METHODS IN MOLECULAR BIOLOGY™

Volume 289

Epidermal Cells

Methods and Protocols

Edited by

Kursad Turksen

HUMANA PRESS

Epidermal Cells

METHODS IN MOLECULAR BIOLOGY™

John M. Walker, SERIES EDITOR

301 **Ubiquitin–Proteasome Protocols,** edited by *Cam Patterson and Douglas M. Cyr, 2005*

300 **Protein Nanotechnology:** *Protocols, Instrumentation, and Applications,* edited by *Tuan Vo-Dinh, 2005*

299 **Amyloid Proteins**: *Methods and Protocols,* edited by *Einar M. Sigurdsson, 2005*

298 **Peptide Synthesis and Application,** edited by *John Howl, 2005*

297 **Forensic DNA Typing Protocols,** edited by *Angel Carracedo, 2005*

296 **Cell Cycle Protocols,** edited by *Tim Humphrey and Gavin Brooks, 2005*

295 **Immunochemical Protocols, Third Edition,** edited by Robert Burns, 2005

294 **Cell Migration:** *Developmental Methods and Protocols,* edited by *Jun-Lin Guan, 2005*

293 **Laser Capture Microdissection:** *Methods and Protocols,* edited by *Graeme I. Murray and Stephanie Curran, 2005*

292 **DNA Viruses:** *Methods and Protocols,* edited by *Paul M. Lieberman, 2005*

291 **Molecular Toxicology Protocols,** edited by *Phouthone Keohavong and Stephen G. Grant, 2005*

290 **Basic Cell Culture, Third Edition,** edited by *Cheryl D. Helgason and Cindy Miller, 2005*

289 **Epidermal Cells,** *Methods and Applications,* edited by *Kursad Turksen, 2005*

288 **Oligonucleotide Synthesis,** *Methods and Applications,* edited by *Piet Herdewijn, 2005*

287 **Epigenetics Protocols,** edited by *Trygve O. Tollefsbol, 2004*

286 **Transgenic Plants:** *Methods and Protocols,* edited by *Leandro Peña, 2005*

285 **Cell Cycle Control and Dysregulation Protocols:** *Cyclins, Cyclin-Dependent Kinases, and Other Factors,* edited by *Antonio Giordano and Gaetano Romano, 2004*

284 **Signal Transduction Protocols, Second Edition,** edited by *Robert C. Dickson and Michael D. Mendenhall, 2004*

283 **Biconjugation Protocols,** edited by *Christof M. Niemeyer, 2004*

282 **Apoptosis Methods and Protocols,** edited by *Hugh J. M. Brady, 2004*

281 **Checkpoint Controls and Cancer, Volume 2:** *Activation and Regulation Protocols,* edited by *Axel H. Schönthal, 2004*

280 **Checkpoint Controls and Cancer, Volume 1:** *Reviews and Model Systems,* edited by *Axel H. Schönthal, 2004*

279 **Nitric Oxide Protocols, Second Edition,** edited by *Aviv Hassid, 2004*

278 **Protein NMR Techniques, Second Edition,** edited by *A. Kristina Downing, 2004*

277 **Trinucleotide Repeat Protocols,** edited by *Yoshinori Kohwi, 2004*

276 **Capillary Electrophoresis of Proteins and Peptides,** edited by *Mark A. Strege and Avinash L. Lagu, 2004*

275 **Chemoinformatics,** edited by *Jürgen Bajorath, 2004*

274 **Photosynthesis Research Protocols,** edited by *Robert Carpentier, 2004*

273 **Platelets and Megakaryocytes, Volume 2:** *Perspectives and Techniques,* edited by *Jonathan M. Gibbins and Martyn P. Mahaut-Smith, 2004*

272 **Platelets and Megakaryocytes, Volume 1:** *Functional Assays,* edited by *Jonathan M. Gibbins and Martyn P. Mahaut-Smith, 2004*

271 **B Cell Protocols,** edited by *Hua Gu and Klaus Rajewsky, 2004*

270 **Parasite Genomics Protocols,** edited by *Sara E. Melville, 2004*

269 **Vaccina Virus and Poxvirology:** *Methods and Protocols,*edited by *Stuart N. Isaacs, 2004*

268 **Public Health Microbiology:** *Methods and Protocols,* edited by *John F. T. Spencer and Alicia L. Ragout de Spencer, 2004*

267 **Recombinant Gene Expression:** *Reviews and Protocols, Second Edition,* edited by *Paulina Balbas and Argelia Johnson, 2004*

266 **Genomics, Proteomics, and Clinical Bacteriology:** *Methods and Reviews,* edited by *Neil Woodford and Alan Johnson, 2004*

265 **RNA Interference, Editing, and Modification:** *Methods and Protocols,* edited by *Jonatha M. Gott, 2004*

264 **Protein Arrays:** *Methods and Protocols,* edited by *Eric Fung, 2004*

263 **Flow Cytometry, Second Edition,** edited by *Teresa S. Hawley and Robert G. Hawley, 2004*

262 **Genetic Recombination Protocols,** edited by *Alan S. Waldman, 2004*

261 **Protein–Protein Interactions:** *Methods and Applications,* edited by *Haian Fu, 2004*

260 **Mobile Genetic Elements:** *Protocols and Genomic Applications,* edited by *Wolfgang J. Miller and Pierre Capy, 2004*

259 **Receptor Signal Transduction Protocols, Second Edition,** edited by *Gary B. Willars and R. A. John Challiss, 2004*

258 **Gene Expression Profiling:** *Methods and Protocols,* edited by *Richard A. Shimkets, 2004*

257 **mRNA Processing and Metabolism:** *Methods and Protocols,* edited by *Daniel R. Schoenberg, 2004*

256 **Bacterial Artifical Chromosomes, Volume 2:** *Functional Studies,* edited by *Shaying Zhao and Marvin Stodolsky, 2004*

255 **Bacterial Artifical Chromosomes, Volume 1:** *Library Construction, Physical Mapping, and Sequencing,* edited by *Shaying Zhao and Marvin Stodolsky, 2004*

254 **Germ Cell Protocols, Volume 2:** *Molecular Embryo Analysis, Live Imaging, Transgenesis, and Cloning,* edited by *Heide Schatten, 2004*

253 **Germ Cell Protocols, Volume 1:** *Sperm and Oocyte Analysis,* edited by *Heide Schatten, 2004*

252 **Ribozymes and siRNA Protocols, Second Edition,** edited by *Mouldy Sioud, 2004*

251 **HPLC of Peptides and Proteins:** *Methods and Protocols,* edited by *Marie-Isabel Aguilar, 2004*

250 **MAP Kinase Signaling Protocols, edited by** *Rony Seger, 2004.*

METHODS IN MOLECULAR BIOLOGY™

Epidermal Cells

Methods and Protocols

Edited by

Kursad Turksen

Ottawa Health Research Institute,
Ottawa, Ontario, Canada

HUMANA PRESS TOTOWA, NEW JERSEY

999 Riverview Drive, Suite 208
Totowa, New Jersey 07512

www.humanapress.com

This publication is printed on acid-free paper. ∞
ANSI Z39.48-1984 (American Standards Institute)
Permanence of Paper for Printed Library Materials.

Production Editor: C. Tirpak
Cover design by Patricia F. Cleary

Color Illustration: From Fig. 2, Chapter 39, "Two-Photon Fluorescence Imaging and Reactive Oxygen Species Detection Within the Epidermis," by Kerry M. Hanson and Robert M. Clegg.

For additional copies, pricing for bulk purchases, and/or information about other Humana titles, contact Humana at the above address or at any of the following numbers: Tel.: 973-256-1699; Fax: 973-256-8341; E-mail: humana@humanapr.com; or visit our Website: humanapress.com

Printed in the United States of America. 10 9 8 7 6 5 4 3 2 1

eISBN 1-59259-830-7

ISSN 1064-3745

Library of Congress Cataloging-in-Publication Data

Epidermal cells : methods and protocols / edited by Kursad Turksen.
p. ; cm. -- (Methods in molecular biology ; v. 289)
Includes bibliographical references and index.
ISBN 1-58829-267-3 (alk. paper)
1. Epidermis--Physiology--Research--Methodology. 2. Epidermis--Cytology--Research--Methodology.
[DNLM: 1. Epidermis--cytology. 2. Cell Culture--methods. 3. Cytological Techniques--methods. 4. Genetic Engineering--methods. 5. Keratinocytes--cytology. 6. Organ Culture--methods. WR 25 E61 2004] I. Turksen, Kursad. II. Series: Methods in molecular biology (Clifton, N.J.) ; v. 289.
QP88.5.E65 2004
616'.0277--dc22

2004010722

Preface

Since Howard Green and colleagues first successfully cultured and maintained epidermal cells in vitro more than two decades ago, our understanding of and ability to manipulate these cells have increased tremendously. Nevertheless, over the years there was, and still is in some circles, an almost mystical notion that epidermal cells are very difficult to work with. Although this may generally be true in comparison to fibroblasts, the field has made exceptional strides in making many methodologies accessible to this cell type. I, therefore, felt that the time was right to collect some of the powerful protocols covering such topics as different methods and models for culturing epidermal cells, enriching for very early epidermal progenitors, and studying epidermal cell commitment and differentiation both in vitro and in vivo. *Epidermal Cells: Methods and Protocols* is not meant to be a comprehensive collection of all possible protocols by which to manipulate epidermal cells, but instead is geared toward protocols that both experienced and novice researchers interested in epidermal biology should find invaluable and easily reproducible in their own labs. If I have achieved this, it is with the willingness of the very committed contributors to share their "hard-won" methodologies. I thank them all.

I would also like to take this opportunity to acknowledge Dr. Jane Aubin for being such a great mentor over many years, but most especially for instilling much enthusiasm and rigor into my own fledgling days of cell culture and differentiation. I similarly thank Dr. Elaine Fuchs for giving me the opportunity to "get down and dirty" with epidermal cells and mouse models to study them. Without their support and the opportunities they gave me, I would not have been able to grow in the scientific directions that I find so exciting.

It is also important to recognize Dr. John Walker, who has been continuously supportive of and helpful in the projects that I have picked. In addition, I would like to acknowledge the enthusiastic support of all at Humana Press who have helped, especially Craig Adams.

I am grateful to N. Urfe for stimulating discussions.

Finally, I would like to thank my great coworker, Tammy Troy. Her endless chipper and enthusiastic support and help have made it a pleasure to complete this book.

Kursad Turksen

Contents

Contributors

Brian J. Aneskievich • *Departments of Nutritional and Pharmaceutical Sciences, School of Pharmacy, University of Connecticut, Storrs, CT*
Claude Backendorf • *Laboratory of Molecular Genetics, Leiden Institute of Chemistry, Leiden, The Netherlands*
Willis Bacon • *Department of Cell Biology, Neurobiology, and Anatomy, University of Cincinnati College of Medicine, Cincinnati, OH*
Jackie R. Bickenbach • *Department of Anatomy and Cell Biology, University of Iowa, Iowa City, IA*
Carolyn Byrne • *Barts and The London Queen Mary School of Medicine and Dentistry, University of London, London, UK*
Alberto Calligaro • *Histology and Embryology Unit, Department of Experimental Medicine, University of Pavia, Pavia, Italy*
Andrea Casasco • *Histology and Embryology Unit, Department of Experimental Medicine, University of Pavia, Pavia, Italy*
Marco Casasco • *Histology and Embryology Unit, Department of Experimental Medicine, University of Pavia, Pavia, Italy*
Pierre Chambon • *Institut Clinique de la Souris (ICS) and IGBMC, Illkirch, France*
Wing Y. Chang • *Department of Physiology and Pharmacology, University of Western Ontario, London, Ontario, Canada*
Robert Man-Kit Cheung • *Ottawa Health Research Institute, Ottawa, Ontario, Canada*
Robert M. Clegg • *Laboratory for Fluorescence Dynamics, Department of Physics, University of Illinois at Urbana-Champaign, Urbana, IL*
Antonia Icaro Cornaglia • *Histology and Embryology Unit, Department of Experimental Medicine, University of Pavia, Pavia, Italy*
Kristine Cuffley • *Laboratoire de Recherche des Grands Brüles/LOEX and Department of Surgery, Laval University, Québec, Québec, Canada*
Lina Dagnino • *Departments of Physiology and Pharmacology and of Pediatrics, University of Western Ontario, London, Ontario, Canada*
Richard L. Davis, Jr. • *NCR Division, Midwest Research Institute, Rockland, MD*
Gys de Jongh • *Department of Dermatology, University Medical Center Nijmegen, Nijmegen, The Netherlands*
Bilge Diker • *Ottawa Health Research Institute, Ottawa, Ontario, Canada*
Thomas P. Dooley • *IntegriDerm Inc., Birmingham, AL*
Rusla M. DuBreuil • *Open Biosystems Inc., Huntsville, AL*
David F. Fischer • *Netherlands Institute for Brain Research, Amsterdam, The Netherlands*
Philip Fleckman • *Department of Medicine (Dermatology), University of Washington, Seattle, WA*
Anthony M. Flores • *Department of Pharmaceutical Sciences, School of Pharmacy, University of Connecticut, Storrs, CT*

Norbert E. Fusenig • *Division of Carcinogenesis and Differentiation, German Cancer Research Center, Heidelberg, Germany*
Jonathan A. Garlick • *Division of Cancer Biology and Tissue Engineering, Department of Oral and Maxillofacial Pathology, School of Dental Medicine and Department of Anatomy and Cellular Biology, School of Medicine, Tufts University, Boston, MA*
Fumitake Gejyo • *Division of Clinical Nephrology and Rheumatology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan*
Lucie Germain • *Laboratoire de Recherche des Grands Brûles/LOEX and Department of Surgery, Laval University, Québec, Québec, Canada*
Marc G. Golightly • *Department of Pathology, State University of New York at Stony Brook, Stony Brook, NY*
Shari Greenberg • *Division of Cancer Biology and Tissue Engineering, Department of Oral and Maxillofacial Pathology, School of Dental Medicine and Department of Anatomy and Cellular Biology, School of Medicine, Tufts University, Boston, MA*
Barbara Hager • *Department of Medicine (Dermatology), University of Washington, Seattle, WA*
Kerry M. Hanson • *Department of Chemistry, University of California, Riverside, CA*
Matthew J. Hardman • *School of Biological Sciences, University of Manchester, Manchester, UK*
Naka Hattori • *Laboratory of Cellular Biochemistry, Animal Resource Sciences/ Veterinary Medical Sciences, University of Tokyo, Tokyo, Japan*
Cindy Hayward • *Laboratoire de Recherche des Grands Brûles/LOEX and Department of Surgery, Laval University, Québec, Québec, Canada*
Robert M. Hoffman • *AntiCancer Inc., San Diego, CA*
Alain Hovnanian • *INSERM CPTP-U563, Service de Génétique Médicale, CHU Purpan, Toulouse, France*
Otmar Huber • *Charité-Medical Universities Berlin, Campus Benjamin Franklin, Institute of Clinical Chemistry and Pathobiochemistry, Berlin, Germany.*
Nam-ho Huh • *Department of Cell Biology, Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan*
Olga Igoucheva • *Department of Dermatology and Cutaneous Biology, Jefferson Institute of Molecular Medicine, Jefferson Medical College, Thomas Jefferson University, Philadelphia, PA*
Wallace Ip • *Department of Cell Biology, Neurobiology, and Anatomy, University of Cincinnati College of Medicine, Cincinnati, OH*
Akemi Ishida-Yamamoto • *Department of Dermatology, Asahikawa Medical College, Asahikawa, Japan*
Mayumi Ito • *Basic Research Laboratory, Kanebo Ltd., Odawara, Japan*
Bastiaan J. H. Jansen • *Department of Tumor Immunology, Nijmegen Center for Molecular Life Sciences, Nijmegen, The Netherlands*
Kim Bak Jensen • *Department of Molecular Biology, University of Aarhus, Aarhus, Denmark*

GYS DE JONGH • *Department of Dermatology, University Medical Center Nijmegen, Nijmegen, The Netherlands*
MONIKA JOST • *SymbioTec GmbH, Saarbrücken, Germany*
TAKASHI KAMEDA • *Department of Biochemistry, Akita University, School of Medicine, Akita, Japan*
MARIKO KASHIWAGI • *Institute of Molecular Oncology, Showa University, Tokyo, Japan*
PRITINDER KAUR • *Epithelial Stem Cell Biology Laboratory, Peter MacCallum Cancer Institute, East Melbourne, Victoria, Australia*
DAVID KIMELMAN • *Department of Biochemistry, University of Washington, Seattle*
KENJI KIZAWA • *Basic Research Laboratory, Kanebo Ltd, Odawara, Japan*
TAKASHI KOBAYASHI • *Department of Dermatology, Chiba University School of Medicine, Chiba, Japan*
LÁSZLÓ G. KÖMÜVES • *Department of Dermatology, VA Medical Center, University of California at San Francisco, San Francisco, CA and Millennium Pharmaceuticals, San Francisco, CA*
PETER KRISTENSEN • *Department of Molecular Biology, University of Aarhus, Aarhus, Denmark*
COREY LARGMAN • *Departments of Dermatology and Medicine, VA Medical Center, University of California at San Francisco, San Francisco, CA*
DANIELLE LAROUCHE • *Laboratoire de Recherche des Grands Brûles/LOEX and Department of Surgery, Laval University, Québec, Québec, Canada*
AMY LI • *Epithelial Stem Cell Biology Laboratory, Peter MacCallum Cancer Institute, East Melbourne, Victoria, Australia*
MEI LI • *Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC) and ICS, Illkirch, France*
NICOLE MAAS-SZABOWSKI • *Division of Carcinogenesis and Differentiation, German Cancer Research Center, Heidelberg, Germany*
ALEXANDER MARGULIS • *Division of Cancer Biology and Tissue Engineering, Department of Oral and Maxillofacial Pathology, School of Dental Medicine and Department of Anatomy and Cellular Biology, School of Medicine, Tufts University, Boston, MA*
HIROKI MARUYAMA • *Division of Clinical Nephrology and Rheumatology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan*
MAJA MATIC • *Departments of Biochemistry and Cell Biology and Pathology, The State University of New York at Stony Brook, Stony Brook, NY*
JIN-JUN MENG • *Department of Cell Biology, Neurobiology, and Anatomy, University of Cincinnati College of Medicine, Cincinnati OH*
DANIEL METZGER • *Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC) and ICS, Illkirch, France*
JUN-ICHI MIYAZAKI • *Division of Stem Cell Regulation Research, Osaka University Medical School, Osaka, Japan*
HITOSHI MIZUTANI • *Department of Dermatology, Faculty of Medicine, Mie University, Mie, Japan*
REBECCA J. MORRIS • *Department of Dermatology, Columbia University College of Physicians and Surgeons, New York, NY*

Jun Ohgane • *Laboratory of Cellular Biochemistry, Animal Resource Sciences/ Veterinary Medical Sciences, University of Tokyo, Tokyo, Japan*

William R. Otto • *Histopathology Unit, London Research Institute, Cancer Research UK, London, UK*

Annalisa Pirrone • *Department of Medicine (Dermatology), University of Washington, Seattle, WA*

Richard B. Presland • *Departments of Oral Biology and Medicine (Dermatology), University of Washington, Seattle, WA*

Christopher Pullis • *Department of Pathology, State University of New York at Stony Brook, Stony Brook, NY*

Ramtin Rahbar • *Ottawa Health Research Institute, Ottawa, Ontario, Canada*

Shanker P. Reddy • *IntegriDerm Inc., Birmingham, AL*

Richard P. Redvers • *Epithelial Stem Cell Biology Laboratory, Peter MacCallum Cancer Institute, East Melbourne, Victoria, Australia*

Federica Riva • *Histology and Embryology Unit, Department of Experimental Medicine, University of Pavia, Pavia, Italy*

Ulrich Rodeck • *Department of Dermatology and Cutaneous Biology, Sidney Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA*

Meghan Rojas • *Department of Cell Biology, Neurobiology, and Anatomy, University of Cincinnati College of Medicine, Cincinnati OH*

Joost Schalkwijk • *Department of Dermatology, University Medical Center Nijmegen, Nijmegen, The Netherlands*

Chun-Shen Shen • *Department of Dermatology, Faculty of Medicine, Mie University, Japan*

Kunio Shiota • *Laboratory of Cellular Biochemistry, Animal Resource Sciences/Veterinary Medical Sciences, University of Tokyo, Tokyo, Japan*

Sanford R. Simon • *Departments of Biochemistry and Cell Biology and Pathology, State University of New York at Stony Brook, Stony Brook, NY*

Hans-Jürgen Stark • *Division of Carcinogenesis and Differentiation, German Cancer Research Center, Heidelberg, Germany*

John T. Stickney • *Department of Cell Biology, Neurobiology, and Anatomy, University of Cincinnati College of Medicine, Cincinnati OH*

Toshihiro Sugiyama • *Department of Biochemistry, Akita University, School of Medicine, Akita, Japan*

Tammy-Claire Troy • *Ottawa Health Research Institute, Ottawa, Ontario, Canada*

Kursad Turksen • *Hormones, Growth, and Development Program, Ottawa Health Research Institute, Ottawa, Ontario, Canada*

Piet E. J. van Erp • *Department of Dermatology, University Medical Center Nijmegen, Nijmegen, The Netherlends*

Fred van Ruissen • *Department of Neurogenetics, Academic Medical Centre, Amsterdam, The Netherlands*

Ashley E. Webb • *Molecular and Cellular Biology Program, Department of Biochemistry, University of Washington, Seattle*

Jörg Weiske • *Charité-Medical Universities Berlin, Campus Benjamin Franklin, Institute of Clinical Chemistry and Pathobiochemistry, Berlin, Germany.*

SARAH H. WILLIAMS • *Pediatric Molecular Genetics, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, University of Oxford, Oxford, UK*

WEI-YANG WU • *Department of Dermatology, Columbia University College of Physicians and Surgeons, New York, NY*

KIYOFUMI YAMANISHI • *Department of Dermatology, Hyogo College of Medicine, Hyogo, Japan*

KYONGGEUN YOON • *Departments of Dermatology and Cutaneous Biology and Biochemistry and Molecular Pharmacology, Jefferson Medical College, Thomas Jefferson University, Philadelphia, PA*

WEITIAN ZHANG • *Division of Cancer Biology and Tissue Engineering, Department of Oral and Maxillofacial Pathology, School of Dental Medicine and Department of Anatomy and Cellular Biology, School of Medicine, Tufts University, Boston, MA*

Color Plates

Color Plates 1–5 appear as an insert following p. 238.

PLATE 1 Confocal immunofluorescence micrographs of HaCat cells double stained with anti-desmoglein-3 antibody and anti-cytokeratin pan antibody. (See full caption on p. 186, Chapter 20.)

PLATE 2 Two-photon fluorescence intensity images before and after UV irradiation of ex vivo human breast tissue incubated with DHR. (See full caption on p. 419, Chapter 39.)

PLATE 3 These images show that similar results as from the ex vivo skin are found with the skin equivalent EpiDerm™ 200. (See full caption on p. 419, Chapter 39.)

PLATE 4 GFP visualization in hair shafts of adenoviral–GFP transduced grafted skin. (See full caption on p. 438, Chapter 42.)

PLATE 5 Hair follicle stem cells in hair growth cycle. (See full caption on p. 442, Chapter 42.)

I

KERATINOCYTE AND ORGAN CULTURES

1

Primary Mouse Keratinocyte Culture

Annalisa Pirrone, Barbara Hager, and Philip Fleckman

Summary

Mouse epidermal keratinocytes have traditionally been difficult to grow in vitro. In this chapter, we present a method for isolating epidermal keratinocytes from a single, newborn mouse pup for long-term culture. The protocols we describe will be especially useful for the isolation and analysis of cells harvested from transgenic or knockout mice. We explain how to use a supplemented fibroblast-conditioned medium, along with mouse collagen IV-coated culture dishes, to establish and subculture these fastidious cells for multiple passages. We describe how to induce expression of markers of the late stages of epidermal differentiation in cultured cells and how to ship whole mouse skins for culture at a site removed from the mice, should it be required. This chapter also contains a method of cryopreservation that ensures high cell viability after periods of storage over liquid nitrogen. The techniques described here in detail should be of interest to investigators currently producing transgenic or null mice with epidermal defects.

Key Words:

Keratinocyte; transgenic; knockout; epidermal differentiation; cell culture; skin.

1. Introduction

The analysis of transgenic or knockout mice with epidermal defects can be enhanced by in vitro studies. The isolation of murine epidermal keratinocytes from a single, transgenic, or knockout mouse pup for long-term culture is an important tool in these investigations. To be successfully maintained and subcultured, mouse keratinocytes require supplemented fibroblast-conditioned medium, a low-calcium environment, and culture dishes coated with mouse collagen IV for attachment. In this chapter, we describe how to make the mouse fibroblast-conditioned medium that is necessary for mouse keratinocyte growth, as well as how to extract and culture cells from individual newborn mouse skins. We include tips for effective subculture with collagen-coated dishes and how to induce expression of markers of the late stages of epidermal differentiation. We also present a method for transporting mouse skins for culture at a site removed from the mice, as well as a protocol for cryogenic preservation of viable cultured keratinocyte stocks. The collection of procedures outlined in this chapter should be of great interest to biologists seeking to augment their investigations of epidermal differentiation through the production of mouse models.

From: *Methods in Molecular Biology, vol. 289: Epidermal Cells: Methods and Protocols*
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

2. Materials

2.1. Chelexed Fetal Bovine Serum (cFBS)

1. Chelex 100 Resin (sodium, 200–400 dry mesh, 75 to 150-µm wet bead; Bio-Rad, Hercules, CA, cat. no. 142-2842); use 200 g Chelex 100 Resin/L of FBS ***(1)***.
2. Whatman Grade No.1 filter paper (Whatman, UK, cat. no. 1093-111) and Buchner funnel (optional, *see* **Note 1**).
3. Endotoxin-free Milli-Q dH_2O (18 megohm-cm resistance, from Milli-Q Plus-UF Water System or similar endotoxin-free water from a type I water filtration system; Millipore, Bedford, MA). When chelexing 1 L of serum, use 1.30 L endotoxin-free Milli-Q dH_2O for each rinse (*see* **Subheading 3.1.1.**).
4. Dulbecco's phosphate-buffered saline (D-PBS; **ref. *2***): 0.2 g of KCl, 0.2 g KH_2PO_4, 8 g NaCl, 2.16 g Na_2HPO_4-$7H_2O$; To make 1 L of 1X D-PBS, add all dry chemicals to 600 mL endotoxin-free Milli-Q dH_2O; adjust to pH 7.4 and bring to 1-L final volume; sterile filter (0.22 µm) into sterile bottles in a laminar flow hood; store at 4°C. When chelexing 1 L serum, use 1.30 L D-PBS for each rinse (*see* **Subheading 3.1.1.**).
5. FBS: serum lots are tested for optimal growth and differentiation of human newborn foreskin keratinocytes and a rat epidermal keratinocyte cell line (current lot is "Premium," nonheat-inactivated; Atlanta Biologicals, Norcross, GA; cat. no. S11150).
6. 500 mL of bottletop filters: 70-mm membrane diameter, pore size 0.45 µm for initial filtering and 0.22 µm for sterile filtering (Corning, Harrodsburg, KY, cat. nos. 430512 and 430521, respectively) of cFBS.

2.2. Primary Fibroblast Conditioned Medium (CM1)

1. Use newborn (d 0–2) mice (*see* **Note 2**).
2. 1 *M* $CaCl_2$ solution: dissolve $CaCl_2$•$2H_2O$ (FW 147.02) in endotoxin-free Milli-Q dH_2O; 0.22 µm sterile-filter.
3. 100 × 15-mm Bacteriological petri dish (BD Falcon Optilux™ or similar style; Becton Dickinson, Franklin Lakes, NJ; cat. no. 351001).
4. Whatman Grade No.1 filter paper (*see* **Subheading 2.1., item 2**): cut to fit 100-mm Petri dish; wrap in aluminum foil and autoclave to sterilize before use.
5. 70% EtOH.
6. D-PBS (*see* **Subheading 2.1., item 4**).
7. D-PBS with added 2% antibiotic/antimycotic (v/v) (antibiotic/antimycotic containing 10,000 U/mL penicillin G sodium, 10,000 µg/mL streptomycin sulfate and 25 µg/mL Amphotericin B; Invitrogen/GIBCO, Carlsbad, CA; cat. no. 15140-022).
8. High-calcium medium (HCM): 100 mL Eagle's minimal essential medium (EMEM) without calcium chloride (with Earles' balanced salt solution, non-essential amino acids and L-glutamine; Cambrex Biosciences, Walkersville, MD; cat. no. 06-174G); 8 mL FBS; 1 mL antibiotic/antimycotic; add 1 *M* $CaCl_2$ solution to bring medium to 0.6 m*M* Ca^{2+} (*see* **Note 3**). Store HCM up to 1 mo at 4°C/darkness.
9. Low-calcium medium (EMEM.06): 100 mL EMEM without calcium chloride; 8 mL cFBS; 1 mL antibiotic/antimycotic; add 1 *M* $CaCl_2$ solution to bring medium to 0.06 m*M* Ca^{2+} (*see* **Note 4**). Store up to 1 mo at 4°C/darkness.
10. Collagenase solution: 0.175 g collagenase (crude, type I, for tissue culture; Invitrogen/GIBCO; cat. no.17100-017); 50 mL Medium 199 (Invitrogen/GIBCO, cat. no. 12340030). Make fresh as needed and warm to 37°C just before use.
11. 0.25% Trypsin (Invitrogen/GIBCO, cat. no. 15050–057): thaw a fresh aliquot the day of use and keep at 4°C until needed.

2.3. Coating Culture Dishes With Mouse Collagen Type IV

1. 0.05 *M* HCl: dilute concentrated HCl (11.6 *M*) with Milli-Q dH_2O (4.3 mL of conc. HCl/L solution) and sterile filter.
2. Mouse collagen IV (Collaborative Biomedical Products; cat. no. 354233): dilute to desired concentration with 0.05 *M* HCl; 1 µg collagen/cm^2 dishes are used for passaging keratinocytes and 5 µg collagen/cm^2 dishes are used for induced differentiation and/or protein extraction (*see* **Note 5**).
3. D-PBS with added 1% antibiotic/antimycotic (*see* **Subheading 2.2., item 7** for description of antibiotic/antimycotic).
4. Tissue culture dishes (Corning, cat. no. 430165 or 430166): 35-mm or 60-mm tissue culture-treated culture dishes (polystyrene, sterile).

2.4. Keratinocyte Isolation and Passage

2.4.1. Primary

1. Use newborn (d 0–2) mice (*see* **Note 2**).
2. 100 × 15-mm Bacteriological Petri dish (*see* **Subheading 2.2., item 3**).
3. Whatman Grade no. 1 filter paper (*see* **Subheading 2.1., item 2**).
4. 70% EtOH.
5. D-PBS with added 1% antibiotic/antimycotic (*see* **Subheading 2.2., item 7** for description of antibiotic/antimycotic).
6. EMEM.06 (*see* **Subheading 2.2., item 9**).
7. CM1 (*see* **Subheading 2.2.**).
8. Humidified incubator at 37°C, set to 4.5% CO_2 (*see* **Note 6**).
9. Collagen IV-coated culture dishes at 1 µg collagen/cm^2 or 5 µg collagen/cm^2 (*see* **Subheadings 2.3.** and **3.3.**).
10. Polystyrene serological pipets (Falcon; BD Biosciences, Franklin Lakes, NJ; cat. nos. 357543, 357551, and 357525).
11. HEPES buffered saline, pH 7.4 (HBS: **ref. 3**): 7.14 g HEPES Powder (30 m*M*), 0.72 g dextrose (D-glucose, anhydrous, 4 m*M*), 0.22 g KCl (3 m*M*), 7.60 g NaCl (130 m*M*), 0.142 g Na_2HPO_4 (1 m*M*); 0.0012 g Phenol Red; ~14 mL 1 *M* NaOH. Add all dry chemicals to 600 mL Milli-Q dH_2O, mix well; add 14 mL 1 *M* NaOH, mix, and adjust to pH 7.4; bring to 1 L final volume; sterile filter.
12. Mouse keratinocyte growth medium (N-medium): Use 1:1 CM1 and EMEM.06 (*see* **Note 7**); N-medium with the following additives is good for 4–5 d at 4°C.
13. 0.25% Trypsin (*see* **Subheading 2.2., item 11**).

2.4.2. Additives

1. Epidermal growth factor (EGF; BD Biosciences, cat. no. 354001): dissolve in sterile solution of 0.1% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO; cat. no. 40001) and HBS; make suggested working solution of 10 µg/mL EGF and add at 1 µL/5 mL (or 2 ng EGF per mL of N-medium); freeze at –20°C in small aliquots; stable for up to 2 yr; avoid freeze–thaw cycles.
2. Aminoguanidine nitrate (AG; MW 137.1; Sigma-Aldrich; cat. no. A5,610-8): dissolve in endotoxin-free dH_2O, sterile-filter; make suggested working solution of 0.75 *M* and add 1 µL/mL (for final concentration of 0.75 m*M* in N-medium); store at 4°C, heat to 37°C, and vortex 10 s to use; make fresh every 6–8 wk.
3. Cholera toxin (CT; *Vibrio cholerae*, Type Inaba 569B; Calbiochem, San Diego, CA; cat. no. 227035): dissolve in HBS; make suggested stock solution of 10^{-6} *M*; from this

make a suggested working solution of 10^{-7} *M* and add 1 µL/mL (for final concentration of 10^{-10} *M* in N-medium); store aqueous solution at 4°C for 6–8 mo.
4. Hydrocortisone (HC; minimum 98%; Sigma-Aldrich; cat. no. H-4001): make suggested working solution of 0.04 mg/mL HC in HBS as follows; dissolve HC in absolute EtOH at the rate of 10 mg HC/1 mL EtOH, then add HBS to bring to volume; sterile filter; add 10 µL/mL of working solution to N-medium; store working solution at 4°C for up to 1 yr.

2.4.3. Subculture

1. 0.25% Trypsin (*see* **Subheading 2.2., item 11**).
2. D-PBS (*see* **Subheading 2.1.**).
3. EMEM.06 (*see* **Subheading 2.2., item 8**).
4. N-medium (*see* **Subheading 2.4.**).
5. Humidified incubator at 37°C, set to 4.5% CO_2.
6. Collagen IV-coated culture dishes at 1 µg collagen/cm^2 or 5 µg collagen/cm^2 (*see* **Subheadings 2.3.** and **3.3.**).
7. Polystyrene serological pipets (*see* **Subheading 2.4., item 7**).

2.5. Shipping and Receiving Mouse Skins

2.5.1. Shipping

1. Washing medium: D-PBS with added 1% antibiotic/antimycotic; 0.1% gentamicin reagent solution (50 mg gentamicin sulfate/mL distilled water; Invitrogen/GIBCO; cat. no. 15750060).
2. Transport medium: Dulbecco's modified Eagle medium containing 1000 mg/L D-glucose, L-glutamine, Pyridoxine HCl, 110 mg/L sodium pyruvate, 3.7 g/L sodium bicarbonate, pH 6.7 (low-glucose DMEM; Invitrogen/GIBCO; cat. no. 31600-034); 20% FBS; 1% HC (of 0.04 mg/mL suggested working solution, or 0.4 µg/mL final volume; *see* **Subheading 2.4.2., item 4**); 1% antibiotic/antimycotic; 0.1% gentamicin reagent solution.
3. 70% EtOH.
4. 15 mL and 50 mL Sterile centrifuge tubes (polyethylene terephthalate; Corning; cat. nos. 430055 and 430304).

2.5.2. Receiving

See **Subheading 2.4.** for keratinocyte isolation and passaging supplies.

2.6. Freezing and Thawing Viable Stocks

2.6.1. Freezing

1. 0.25% Trypsin (*see* **Subheading 2.3., item 11**).
2. Mouse keratinocyte freezing medium (mKFM): EMEM without calcium chloride; 8.33% v/v dimethyl sulfoxide (DMSO) (endotoxin-free sterile-filtered, MW 78.13; Sigma-Aldrich; cat. no. D2650); 20% cFBS. Light-sensitive; prepare the day of use.
3. 95% EtOH at room temperature.
4. 3–4 lbs Dry ice.
5. Vermiculite-insulated beaker and polyurethane-skinned foam ice bucket (*see* **Fig. 1**): Insulated beaker consists of a 600-mL beaker inside a 1000-mL beaker; the space between the two is filled with vermiculite, and the top of the space is sealed with paraffin (*see* **Note 8**).
6. Filter (embedding) bags (approximate size 2.5 × 2.75 in.; made of lens paper; suspended in 95% EtOH with open paper clip or similar wire).
7. Liquid nitrogen freezer, –196°C.

8. Polystyrene serological pipets (*see* **Subheading 2.4.1., item 10**).
9. 1.8 mL NUNC freezing vials, internal thread (Nalge Nunc International; cat. no. 368632): use 1 per 10^6 cells.

2.6.2. Thawing

1. One need the items under **Subheading 2.4.1., items 8–12** plus 70% EtOH).

2.7. Induction of Differentiation Markers

1. D-PBS (*see* **Subheading 2.1., item 4**).
2. 1 *M* $CaCl_2$ solution (*see* **Subheading 2.2., item 2**).
3. EMEM.06 (*see* **Subheading 2.2., item 9**).
4. Keratinocyte growth medium with 0.15 m*M* Ca^{2+} (KGM.15): Keratinocyte Growth Medium (KGM; BulletKit® without Ca^{2+}; Cambrex Bioscience, Walkersville, MD; cat. no. CC-3104; *see* **Note 9**); 1 *M* $CaCl_2$ solution, added 0.15 µL/mL, to bring medium to 0.15 m*M* Ca^{2+}. Store KGM.15 for 1 mo at 4°C/darkness.

3. Methods

Whenever possible, procedures should be conducted in a Type IIA laminar flow hood to minimize the risk of contamination.

3.1. cFBS (1)

*3.1.1. Rinsing Chelex 100 Resin (*see ***Note 1****)*

1. Add 1.3 L Milli-Q dH_2O to flask or beaker.
2. Add 200 g Chelex 100 Resin to Milli-Q dH_2O while stirring.
3. Adjust pH to 7.35-7.4 with HCl (3–6 *M*).
4. Turn off stirrer and let Chelex settle for 30 min.
5. Pour off dH_2O while being careful not to pour off any Chelex.
6. Wash two times with 1.3 L of Milli-Q dH_2O: add dH_2O, stir 5 min, let Chelex settle for 30 min, then pour off dH_2O.
7. Wash two times with 1.3 L of D-PBS as in **step 6**. On the last wash with D-PBS, stabilize pH to 7.35–7.4 before letting Chelex settle.
8. Pour off PBS carefully.

3.1.2. Preparing Chelexed Serum

1. Add 1 L of cold (4°C) FBS.
2. Stir 1 h at 4°C in darkness (wrap with aluminum foil).
3. Filter cFBS once through a 0.45-µm filter to remove remaining resin and again through a 0.22-µm filter to sterilize.
4. Set aside a sample to determine Ca^{2+} concentration (*see* **Note 4**).
5. Label, aliquot into 15-mL or 50-mL sterile containers and store at –20°C/darkness (*see* **Note 10**).

3.2. CM1

The following protocol assumes 30–40 mice will be used, yielding 900–1200 mL CM1. Amount of collagenase solution may need to be modified if using less than 30 mice.

3.2.1. Day 1

1. Obtain live, newborn mouse pups.
2. Decapitate using sterile razor blade, rinse in 70% EtOH, and remove limbs and tail (*see* **Note 11**).
3. Slice skin ventrally from neck to tail and peel off in one piece.
4. Rinse skins in three changes of D-PBS with added 2% antibiotic/antimycotic to prevent contamination. Skins can stay submerged in D-PBS solution at room temperature for up to 1 h.
5. Place precut sterile Whatman Grade no.1 filter paper into 100-mm Petri dish and spread intact skin directly onto dry paper, dermal side down. Make sure edges of the skin are not rolled. Five to six skins can be spread on one 100-mm Petri dish.
6. Add 7–10 mL of cold 0.25% trypsin per dish.
7. Refrigerate 16–18 h at 4°C (*see* **Note 12**).

3.2.2. Day 2

1. Separate epidermis from dermis with fine sterile forceps: Epidermis is shiny, relatively thin, and transparent whereas dermis is red, relatively thick, and opaque (*see* **Note 13**).
2. Place dermis pieces in a 60-mm Petri dish with 5 mL of 37°C collagenase solution (*see* **Note 14**).
3. Mince dermis with sterile scissors and transfer to sterile Erlenmeyer flask.
4. Add 45 mL of 37°C collagenase solution and stir uncovered in a 37°C incubator set to 5.0% CO_2 for 30 min (*see* **Note 15**).
5. Filter through sterile gauze into sterile centrifuge tubes to remove dermis pieces.
6. Centrifuge for 5 min at 200*g* to pellet cells. Carefully aspirate supernatant.
7. Resuspend in HCM. Plate 1 skin/T150 tissue culture flask in 25 mL of HCM (*see* **Note 16**).
8. Culture at 37°C in 5% CO_2 for 1 d (*see* **Note 17**).

3.2.3. Day 3

1. Wash flask two times with D-PBS to remove traces of calcium.
2. Feed with 30 mL of EMEM.06 and culture at 37°C in 5% CO_2 for 2 d.

3.2.4. Day 4

Incubate.

3.2.5. Day 5

1. Filter CM1 from each flask through 0.45-µm 500-mL bottle-top filters (*see* **Note 18**).
2. Aliquot into tubes or bottles for storage at –20°C. Keep frozen until needed, avoiding multiple freezes/thaws (*see* **Note 19**).

3.3. Coating Culture Dishes With Mouse Collagen Type IV

1. Thaw vial of mouse collagen type IV slowly; place vial in container of ice and place container at 4°C (thawing should take approx 24 h). Vortex vigorously for 15 s to fully dissolve.
2. Calculate µg collagen per cm^2 using the µg/mL information provided by the manufacturer. Use 3 mL of collagen solution per 60-mm dish as the standard (*see* **Note 20**). In a laminar flow hood, dilute to the desired concentration with sterile 0.05 *M* HCl.
3. Add working collagen solution to tissue culture dishes at the rate of 1 µg/cm^2 to passage, or 5 µg/cm^2 to induce differentiation and/or to harvest cells for protein extraction. Allow dishes to sit in the laminar flow hood for 1 h.

4. Remove working collagen solution using sterile technique and save for future use (*see* **Note 21**).
5. Rinse each dish two times using D-PBS with added 1% antibiotic/antimycotic to remove the acid. Remove as much D-PBS as possible after the final rinse by tilting the dish and aspirating thoroughly.
6. Use immediately or wrap dishes in plastic wrap—several may be grouped together—and store at 4°C for up to 2 yr.

3.4. Keratinocyte Isolation and Passage

3.4.1. Primary

1. Skin newborn mouse pups and float on 0.25% trypsin overnight at 4°C, as described in **Subheading 3.2.1.** (*see* **Note 22**).
2. Separate epidermis from dermis with sterile forceps.
3. Place epidermis in sterile centrifuge tube with EMEM.06 (EMEM.06 should be at room temperature): 1–4 skins: use a 15-mL tube with 5–10 mL of medium; 5+ skins: use a 50-mL tube with 10–20 mL of medium.
4. Shake tube(s) firmly approx 50 times to separate cells (*see* **Note 23**).
5. Remove large piece(s) of epidermis with a sterile instrument.
6. Count cells in a hemacytometer and centrifuge at 200*g* for 5 min.
7. Aspirate supernatant. Mouse keratinocytes will adhere to glass; using a plastic pipet, resuspend in appropriate volume of N-medium to plate at the following seeding densities on collagen-coated dishes:
   1.0×10^6 cells/60-mm dish in 5 mL of N-medium
   0.45×10^6 cells/35-mm dish in 2.5 mL of N-medium (*see* **Note 24**).
8. Incubate at 37°C, 4.5% CO_2 (*see* **Note 6**). Feed every other day with 5 mL of N-medium/60 mm dish and 2.5 mL of N-medium/35-mm dish, or every third day with 6 mL of N-medium/60-mm dish and 3 mL of N-medium/35-mm dish (e.g., for a 35-mm dish, feed 2.5 mL on Monday, passage on Wednesday, and feed 2.5 mL, then feed 3 mL on Friday). Do not exceed 2 d without feeding.
9. Passage or raise calcium (*see* **Subheading 3.7.**) when cells are completely confluent (every 7–8 d).

3.4.2. Subculture

1. Wash gently with D-PBS.
2. Add 0.25% trypsin as follows: 2 mL/35-mm dish; 3 mL/60-mm dish.
3. Incubate at 37°C for 6–8 min.
4. Using a plastic pipet, triturate with an equal volume of cold (4°C) EMEM.06 to dislodge cells and dilute trypsin. Remove to a cold centrifuge tube (*see* **Note 25**).
5. Count cells in a hemacytometer and centrifuge 3–4 min at 200*g*.
6. Aspirate supernatant and, using a plastic pipet, resuspend in appropriate volume of N-medium to plate at the following seeding densities on collagen-coated dishes: 0.5 m 10^6 cells/60-mm dish in 5 mL of N-medium; 0.2×10^6 cells/35-mm dish in 2.5 mL of N-medium.
7. Incubate at 37°C, 4.5% CO_2. Feed as in the primary protocol (**Subheading 3.4.1., step 8**) described previously.
8. Passage again or raise calcium when cells are completely confluent (every 7–8 d; *see* **Note 26**). Cells can be subcultured indefinitely; however, expression of late epidermal differentiation markers, such as profilaggrin and keratin 1, are lost by the 10th passage ***(4)***.

3.5. Shipping and Receiving Mouse Skins

3.5.1. Shipping

1. Skin newborn mouse pups as described in **Subheading 3.2.1., steps 1–4**.
2. Rinse skins in three changes of washing medium to prevent contamination. Skins can stay submerged in washing medium at room temperature for up to 1 h.
3. Fill one 15-mL centrifuge tube for each skin with transport medium kept at 4°C. Transport medium should fill the tube to the top to ensure coverage of the entire skin. (Leave a small bubble of air to compensate for potential pressure changes en route.)
4. Place each skin in a separate tube of transport medium. Cap tightly and seal with parafilm.
5. Place tubes in styrofoam mailing container with ice packs and added insulation, such as styrofoam peanuts. Ship by overnight express. Notify recipient of impending arrival.

3.5.2. Receiving

1. Carefully open tubes and note any shipping damage (*see* **Note 27**).
2. Remove skins from transport medium with a sterile instrument and rinse in several changes of D-PBS with added 1% antibiotic/antimycotic to remove residual FBS (calcium).
3. Process as described in **Subheading 3.4.**

3.6. Freezing and Thawing Viable Stocks

3.6.1. Freezing (see ***Fig. 1***)

1. Trypsinize cells as described in **Subheading 3.4.2., steps 1–5**.
2. Aspirate supernatant and, using a plastic pipet, resuspend in mKFM at 10^6 cells/mL. DMSO in mKFM may be toxic to cells, especially at room temperature or above, so do not allow cells to linger in freezing medium (no more than 30 min before the cooling procedure is started).
3. Transfer cell suspension into freezing vials at 1 mL/vial (10^6 cells/vial recommended freezing density). Seal cap well, but not too tightly or container may burst during subsequent thawing.
4. Place no more than three vials upright in each 2.5 × 2.75 in embedding bag.
5. Place stir bar in insulated beaker, place beaker in ice bucket; place ice bucket on stirring motor, and fill beaker one half to two thirds full with 95% EtOH and turn on stirring motor.
6. Suspend embedding bags filled with vials by bent paper clips resting on the edge of the freezing beaker. It is best to use no more than four embedding bags, with three vials per bag (or 12 total vials at once). Only submerge the cell suspension, the cap of the vial should be completely above the ethanol level.
7. Fill ice bucket with 95% EtOH to slightly below the ethanol level in the freezing beaker (level will rise with the addition of dry ice pellets).
8. Add dry ice pellets to 95% EtOH in the ice bucket (not in the beaker; the goal is to lower the temperature inside the beaker approx 1°C/min) (*see* **Note 28**). Add pellets sparingly at first to avoid spill-over, but be sure that pellets are visible in the ice bucket throughout the procedure (check periodically).
9. After 1 h, measure the temperature in the freezing beaker. Vials are ready to be transferred to a liquid nitrogen freezer when the temperature of the ethanol in the freezing beaker has reached –40°C or below (takes approx 1.5 h).

3.6.2. Thawing

1. Put 5 mL of N-medium into a 60-mm collagen-coated dish and place in 37°C incubator set to 4.5% CO_2. Allow to equilibrate for 1 h.

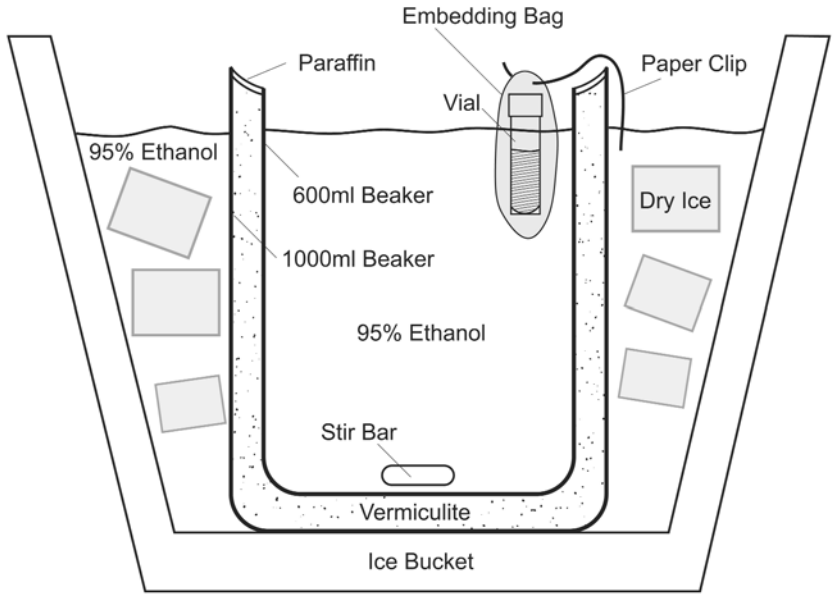


Fig. 1. Ethanol/dry ice freezing method. To ensure high cell viability, use DMSO-containing freezing medium and an insulated container to protect the cells from dehydration and the formation of ice crystals. The figure illustrates a highly effective freezing apparatus that allows the cell suspension to reach –40°C or below at the rate of approx 1°C/min.

2. Remove frozen vial from liquid nitrogen and immediately immerse in a 37°C water bath (*see* **Note 29**).
3. As soon as the solution has thawed, wipe the area around the cap with 70% EtOH or similar antimicrobial reagent, then flick gently to resuspend cells and transfer entire contents of vial to the culture dish with a plastic pipet tip. Again, DMSO may be toxic to cells, especially at higher temperatures, so do not allow cells to linger in freezing medium.
4. Feed after 24 h and resume normal feeding schedule thereafter. Cells should be ready for subculture after approx 7–10 d (when confluent).

3.7. Induction of Differentiation Markers

1. Begin with confluent cells cultured in N-medium, in a dish coated with 5 µg of collagen/cm^2.
2. Wash gently two times with D-PBS.
3. Replace N-medium with EMEM.06 at normal feeding volume to purge the cells of growth factors. Culture in EMEM.06 for 24 h.
4. Refeed with KGM.15. Culture in KGM.15 for 48 h.
5. Wash gently two times with D-PBS; aspirate as much buffer as possible after final rinse.
6. Cells can be harvested for protein extraction or wrapped in aluminum foil and frozen at –80°C for future use (*see* **Note 30**).

4. Notes

1. Quick chelexing method: Rather than letting the Chelex 100 Resin settle, dH_2O or D-PBS can be filtered using a Buchner funnel lined with Whatman Grade no. 1 filter paper. Be sure to scrape the resin from the filter back into the flask between rinses. Do not let the resin become too dry. Quick chelexing method will result in a slightly higher Ca^{2+}

concentration (~0.25 m*M*) because of the calcium in the filter paper, but the results are still acceptable.

2. Species does not seem to matter. Culture of keratinocytes from transgenic mice does not require medium conditioned by fibroblasts from genetically identical mice (i.e., normal mouse fibroblasts can be used to make CM1).
3. For example, FBS with 0.134 mg/mL calcium (concentration provided by manufacturer): because FBS is added at 7.34%, final Ca^{2+} concentration of HCM is 0.25 m*M*. Make a 1 *M* $CaCl_2$ working solution, sterile-filter, and add 0.35 µL/mL to HCM for a final Ca^{2+} concentration of 0.6 m*M*. $CaCl_2$ working solution can be stored at 4°C for 2 yr if sterility is maintained and container is sealed tightly to prevent evaporation.
4. When using cFBS, Ca^{2+} concentration can be determined by atomic absorption spectroscopy; linearity in the University of Washington Medical Center Clinical Laboratory extends to 0.009 m*M*. Please note that the laboratory should be educated about the nature of the specimen to avoid standard dilutions used for determination of serum concentrations. A cFBS Ca^{2+} concentration of approx 0.25 m*M* is normal.
5. Testing multiple lots of collagen is recommended for best results. We test our collagen by ordering two to four distinct lots from the manufacturer, coating dishes, and carrying mouse epidermal keratinocytes in such dishes for two to three passages, taking note of cell attachment. We choose the lot that gives the best cell attachment, determined by visual inspection of Giemsa-stained colonies.
6. Mouse epidermal keratinocytes are best maintained at 4.5% CO_2. We recommend using an external CO2 analyzer, such as a Fyrite® Gas Analyzer (Bacharach, Inc., Pittsburgh, PA; cat. no. 10-5000), to confirm digital CO_2 incubator readings.
7. To save time, a 2X solution of additive (2XE) can be made. 2XE is made with twice the volume of additives (20 µL/mL HC, 2 µL/mL AG, 2 µL/mL CT, 0.4 µL/mL EGF) in EMEM.06 and should be added to CM1 1:1 to make N-medium. Store at 4°C for up to 2 wk.
8. Try to keep the paraffin seal dry because moisture between the layers may cause the beakers to break.
9. We purchase KGM without calcium from Cambrex Biosciences, then add our own calcium chloride. If small amounts of medium are to be prepared, KGM kit supplements provided by the manufacturer should be aliquoted to minimize freezing and thawing.
10. The shelf life of cFBS is unknown; however, our stocks have been successfully used for up to 2 yr when stored at –20°C/darkness and thawed no more than three times.
11. Providone iodine is toxic to keratinocytes and should not be used in this procedure. Antimicrobial preparations aside from those listed in this chapter have not been tested.
12. Placing the dishes on a wire shelf in the refrigerator tends to work better than a flat glass or plastic shelf. Be sure that the refrigeration unit is operating as close to 4°C as possible. The 16- to 18-h refrigeration time is crucial to successful epidermal separation.
13. Epidermis should come off easily, usually in one solid piece. We suggest gripping the dermis with one pair of forceps and using another to peel off the epidermis. Variation of temperature, incubation time, and enzyme used can result in an epidermis that is difficult to remove. If the skins have been in enzyme too long, basal cells are lost and gummy strands of DNA are observed.
14. Dermis pieces from up to 40 mice can be placed in the described amount of collagenase solution. If the epidermis is needed for keratinocyte harvest, dermis pieces can be stored in D-PBS while working with the epidermis first (*see* **Subheading 3.4.1., steps 3–9**). When the epidermal cells are in the incubator, transfer dermal pieces to the collagenase solution and continue the CM1 protocol.

15. We place a stir motor inside a humidified incubator (37°C, 5.0% CO_2) and thread the power cord through an opening in the top of the incubator to plug in. Be sure to use a sterile stir bar (autoclave or submerge in 70% EtOH to sterilize).
16. HCM is used only for plating fibroblasts and ensures that no keratinocytes are left to proliferate in the culture. Calcium concentration should be accurate (0.6 m*M*). You may add one extra flask for every 10–15 skins when plating fibroblasts to ensure the cells are not too confluent when EMEM.06 is added (e.g., 33 flasks for 30 mouse skins).
17. Fibroblasts grow very quickly. The goal is to collect conditioned medium from cells in active growth phase, so be sure to proceed with the d 3 protocol when cells are approx 60% confluent (i.e., check cells early in the morning and do not delay the procedure).
18. Approximately 150 mL of CM1 will go through one filter. Remember to use sterile technique throughout the procedure. Letting the empty flask stand upright for 1 min will allow 1–2 mL of residual medium to pool at the bottom and be pipetted out.
19. CM1 can be stored and used successfully for up to 2 yr. We have not systematically tested CM1 beyond 2 yr. Solution may separate after thawing; shake until color is homogeneous.
20. Example: Corning 60 mm tissue culture dishes have a surface area of 21 cm^2. Approximately 3 mL of collagen solution is sufficient to evenly cover the bottom of such a dish. You will need a working collagen solution of 105 µg/3 mL (35 µg/mL) to coat the dish at 5 µg/cm^2, the suggested concentration for setting up a culture for protein extraction. Cells cultured on 5 µg collagen/cm^2 cannot be passaged successfully; use a 1 µg collagen/cm^2 concentration for cells to be passaged.
21. Solution can be kept at 4°C on ice for up to one week before it will need to be frozen again at –80°C. Thawing more than three times is not recommended.
22. For a transgenic mouse litter, it may be necessary to keep each mouse skin separate, both on trypsin and while harvesting/plating keratinocytes, until the individual mice are genotyped. The amputated tail may be taken at the time of sacrifice to verify genotype.
23. Holding the tube, flick sharply against palm of the hand and repeat approx 50X. Three 15-mL tubes or two 50-mL ones can be shaken at once. A low cell count may be remedied by more vigorous shaking (to adequately dislodge cells from the epidermal sheet) or by confirming enzyme (trypsin) activity. A cell count of 2–3 × 10^6 cells/skin is normal.
24. Large numbers of floating cells are normal, especially in the early passages.
25. This method does not completely inactivate trypsin, and cells should not linger in the solution. Do not use serum on the cells. We have not tested the effect of soybean trypsin inhibitor on mouse keratinocytes.
26. Mouse epidermal keratinocytes should be passaged only when confluent, unlike human epidermal keratinocytes, which should be subconfluent when passaged.
27. If the package was not insulated well enough for the shipping conditions, medium might be partially frozen or warmed to ambient temperature, resulting in cell death. Also, international shipping may cause a day or two of transport delay, which could lead to low cell viability. You may decide to increase seeding density to compensate, or use a Trypan blue viability test when counting cells harvested from skins.
28. Although this freezing method is optimal ***(5)***, other conventional cryofreezing techniques—such as placing freezing vials in a tightly closed polyurethane container at –80°C overnight before transferring to liquid nitrogen storage—have yielded acceptable results. Best results occur when care is taken to lower the temperature of the cell suspension 1°C/min (down to between –40°C and –80°C) before storing vials over liquid nitrogen.
29. To guard against rupture, place vial inside a latex glove or put lid on water bath. Do not completely submerge vial; be sure only the cell suspension is under water.

30. The shift from 0.06 m*M* Ca^{2+} to 0.15 m*M* Ca^{2+} will induce expression of some epidermal differentiation markers. Noninduced control dishes may also be harvested using **steps 5** and **6** ***(3)***.

Acknowledgments

This research was supported by the NIH/NIAMS P01-AR-21557-20 and the Odland Endowed Research Fund. Jackie Bickenbach, PhD, offered invaluable information necessary for the development of many of these protocols ***(3)***. Mary Ann Derr, George M. Martin, and Thomas H. Norwood provided the cryopreservation technique.

References

1 Brennan, J. K., Mansky, J., Roberts, G., and Lichtman, M. A. (1975) Improved methods for reducing calcium and magnesium concentrations in tissue culture medium: application to studies of lymphoblast proliferation in vitro. *In Vitro* **11,** 354–360.

2 Dulbecco, R. and Vogt, M. (1954) Plaque formation and isolation of pure lines with poliomyelitis viruses. *J. Exp. Med.* **99,** 167–182.

3 Tsao, M. C., Whithall, B. J., and Han, R. G. (1982) Clonal growth of normal human epidermal keratinocytes in a defined medium. *J. Cell Physiol.* **110,** 219–229.

4 Hager, B., Bickenbach, J. R., and Fleckman, P. (1999) Long-term culture of murine epidermal keratinocytes. *J. Invest. Dermatol.* **112,** 971–976.

5. Kang, S. J. and Fleckman, P. (1995) Convenient computer colony image analysis using an ordinary flatbed scanner for evaluating keratinocyte cryopreservation methods (abstract). *J. Invest. Dermatol.* **104,** 612.

2

Serial Cultivation of Primary Adult Murine Keratinocytes

Richard P. Redvers and Pritinder Kaur

Summary

In vitro cell culture is a necessary prerequisite in acquiring a thorough understanding of the biology and behavior of the cells of interest and is a critical first step in developing cellular therapies. Somatic stem cell biology is concerned with stem cells of the adult and how they may be utilized in regenerating tissue and ameliorating disease. Moreover, the incidence of disease increases with age, hence the demand for therapeutics is greatest among mature individuals. Therefore, an ability to grow and manipulate primary *adult* epithelial keratinocytes in vitro is of paramount importance in gaining insights into the biology of skin that may have clinical implications. A methodology has been developed that will enable investigators to isolate and serially culture adult basal keratinocytes from the epidermis of the mouse in a supplemented culture medium that selects for epithelial lineages and enhances their proliferation while inhibiting differentiation through many passages.

Key Words:

Adult; murine; mouse; tail; keratinocyte; epidermis; skin; epithelial; culture.

1. Introduction

As the predominant cell lineage of the epidermis, the keratinocyte has been the focus of many seminal studies central to our understanding of skin biology. Early attempts to culture epithelial cells were fraught with difficulties because suboptimal culture conditions required very high seeding densities or failed to maintain keratinocytes in a proliferative state while permitting overgrowth by stromal elements ***(1,2)***. The use of irradiated fibroblast feeders in serum-supplemented media addressed these issues ***(3)***, although these coculture systems made it difficult to elucidate the precise nutritional requirements of epithelial cells and their intrinsic abilities. The development of the MCDB series of media by Ham et al. ***(4–7)*** culminated in the serum-free serial monoculture and frozen storage of human keratinocytes. However, murine keratinocyte culture systems have lagged behind those for human keratinocytes because of their unique nutritional requirements. In recent years, a method has been described that permits the serial passage of neonatal murine keratinocytes in supplemented and conditioned medium on collagen-coated substrates ***(8)***. Subsequently, it was shown that keratinocytes derived from embryonic skins could be passaged 60 times with much less fastidious culture requirements ***(9)***. Recently, a method was described that allowed short-term passage of keratinocytes from 7-wk-old mice in conditioned medium with serum ***(10)***. Alas, the serial culture of adult murine keratinocytes from clonal densities without serum or

From: *Methods in Molecular Biology, vol. 289:*
Epidermal Cells: Methods and Protocols
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

conditioned medium has remained elusive, owing in part to the increased difficulty of culturing adult cells in comparison to their neonatal counterparts ***(11,12)***. The method detailed here describes such a system using the tail of the adult mouse, a convenient and abundant source of interfollicular and hair follicle basal keratinocytes. This should be of interest to investigators pursuing studies in cutaneous biology in the adult mouse.

2. Materials

2.1. Instruments

1. Two fine-point curved forceps.
2. Scalpel holder and blades (no. 22).
3. Scissors.

2.2. Adult Murine Skin

1. Adult (≥8 wk) C57B6/J mouse tails.

2.3. Reagents

All reagents are 0.22-µm filter-sterilized, stored at 4°C, and kept on ice throughout the procedure unless otherwise stated.

2.3.1. Processing of Skin

1. 10% Povidone-iodine solution (equivalent to 1% w/v iodine).
2. PBS-AF (phosphate-buffered saline with anti-fungal and antibiotic agents): add 10 mL of penicillin (600 µg/mL)/gentamycin (8 mg/mL) and 3 mL of Fluconazol (2 mg/mL; Pfizer, France) to 1 L PBS.
3. Dispase solution: neutral dispase II (8 mg/mL, Roche; cat. no. 165 859) in PBS-AF. Prepare and 0.22-µm filter-sterilize just prior to use.

2.3.2. Isolation of Basal Keratinocytes

1. Trypsin-ethylenediamine tetraacetic acid (EDTA) (Thermo Trace Ltd.; cat. no. 21-160-0100V).
2. Trypsin inhibitor (for 0.5 L): DMEM without calcium (*see* **Note 1**); bovine serum albumin (BSA) (tissue culture tested; 0.5 g); and soybean trypsin inhibitor (Sigma; cat. no. T9003; 0.1 g).

2.3.3. Serial Culture of Adult Murine Keratinocytes

1. Collagen (type IV from human placenta, Sigma; cat. no. C5533); make up 1 mg/mL stock in 10 m*M* acetic acid (*see* **Note 2**).
2. Adult mouse keratinocyte growth medium (AM-KGM; for 0.5 L): KBM (Clonetics, San Diego, CA; cat. no. CC-3101; 0.5 L), hydrocortizone (0.5 mg/mL in abs. ethanol, Sigma; cat. no. H0135; 500 µL), epidermal growth factor (100 µg/mL in PBS–AF/1% BSA, Sigma; cat. no. E4127; 50 µL), BPE (35 mg/mL, Hammond Cell Tech, cat. no. 1077, *see* **Note 3**; 1 mL), BSA (tissue culture tested; 1 g), ITT (500X stock solution; 1 mL). For 10 mL: insulin (6.25 mg/mL in 12 m*M* HCl, Sigma; cat. no. I1882; 4 mL), transferrin (25 mg/mL in ddH_2O, Sigma; cat. no. T8158; 1 mL), triiodothyronine (T_3, 4×10^{-10} *M*, Sigma; cat. no. T5516; 5 mL).
   For 10 mL:
   a. Dissolve 13.6 mg T_3 in 50 mL 0.02 *N* NaOH.
   b. Dilute 50 µL T_3 into 5 mL ddH_2O (4×10^{-6} *M*).

c. Dilute 100 µL of 4×10^{-6} *M* T_3 into 9.9 mL ddH_2O (4×10^{-8} *M*).
d. Dilute 100 µL of 4×10^{-8} *M* T_3 into 9.9 mL ddH_2O (4×10^{-10} *M*).
e. EOP (500X stock solution): 1 mL. For 10 mL, add 30 µL of ethanolamine (Sigma; cat. no. E0135) to 10 mL of ddH_2O (50 m*M*). Dissolve 70.55 mg of *o*-phosphorylethanolamine (Sigma; cat. no. P0503) into 10 mL of 50 m*M* ethanolamine.
f. Selenium (2.65×10^{-8} *M* in ddH_2O, Sigma; cat. no. S9133; 1 mL).
g. Adenine (500X stock solution; 1 mL): For 10 mL, dissolve 0.121 g of adenine (Sigma; cat. no. A2786) into 5 mL of 4 *N* NaOH. Make up to 10 mL in KBM (90 m*M*).
h. Progesterone (500X stock solution; 1 mL): For 10 mL, dissolve 1 mg of progesterone (Sigma; cat. no. P8783) into 2 mL ddH_2O. Add 62.9 µL of 0.5 mg/mL progesterone into 10 mL ddH_2O (3.15×10^{-3} mg/mL). Add 1 mL of 3.15×10^{-3} mg/mL progesterone into 9 mL KBM (1×10^{-6} *M*). Also add the following: 50 µL Cholera toxin (1 mg/mL in ddH_2O, Sigma; cat. no. C8052); 100 µL Isoleucine (250 m*M* in 1 *N* HCl, Sigma; cat. no. I7403, *see* **Note 4**); 125 µL Tyrosine (124 m*M* in 1 *N* HCl, Sigma; cat. no. T8566); 200 µL Tryptophan (37.5 m*M* in 1 *N* HCl, Sigma; cat. no. T8941); 500 µL Histidine (230 m*M* in ddH_2O, Sigma; cat. no. H6034); 2 mL Alanine (1.02 *M* in ddH_2O, Sigma; cat. no. A7469); 2 mL Threonine (312.5 m*M* in ddH_2O, Sigma; cat. no. T8441); 1 mL Penicillin (600 µg/mL)/gentamycin (8 mg/mL).
i. 1.5 mL Fluconazol (2 mg/mL solution; Pfizer).

3. KBM and hydrocortizone are stored at 4°C protected from light. EGF and BPE are stored at –80°C for up to a year and all other supplements are stored at –20°C indefinitely. All components are 0.22-µm filter-sterilized and aliquoted for storage. Filter-sterilize medium when all components have been added. Repeated filtration is not recommended.
4. EDTA: 0.05% w/v in PBS-AF.
5. Trypsin–EDTA and trypsin inhibitor as per isolation procedure.
6. Trypan blue solution: 0.4% (Sigma; cat. no. T8154).

3. Method

The reagents and materials described herein are apportioned for harvesting the skin from four adult mouse tails. This number can be varied by scaling the reagents accordingly.

3.1. Processing of Skin

The processing of skin in preparation for keratinocyte isolation is described in this section. Tissue must be handled aseptically as soon as is practicable, and samples must be kept cold (4°C) during processing to preserve cell viability. To maintain asepsis, rinse instruments in 70% ethanol followed by PBS-AF before each handling of the tissue. Prepare in a biohazard laminar flow hood the following: autoclaved instruments immersed in a beaker of 70% ethanol; a beaker of sterile PBS-AF; 10 mL dispase solution in a 60-mm Petri dish; two 100-mm Petri dishes with approx 20 mL of PBS-AF; and tail-washing solutions (10 mL povidone–iodine solution in a sterile 15-mL Falcon tube; 4 × 10 mL PBS-AF in sterile 15-mL Falcon tubes; 10 mL of 70% ethanol in a sterile 15-mL Falcon tube).

1. Sacrifice adult C57B6/J mice with inhalation anesthesia (e.g., isoflurane) followed by cervical dislocation. Collect mice in a box and place in hood.
2. Working on the inverted lid of a sterile 100-mm Petri dish, cut off tails at their proximal end a few millimeters from the body using a scalpel.

3. Place tails in 10 mL of povidone–iodine solution cap and invert tube several times to wash.
4. Transfer tails to a 10 mL PBS-AF cap and invert tube several times to rinse off povidone–iodine. Repeat.
5. Transfer tails to a 10 mL 70% ethanol, cap, and invert as before.
6. Transfer tails to 10 mL of PBS-AF, cap, and invert several times to rinse off ethanol. Repeat.
7. On the inverted lid of a sterile 100-mm Petri dish, hold a tail at the proximal end with forceps, and make a small slit in the skin at the severed end using the scalpel.
8. Tease apart the skin with forceps from the underlying tissue at the incision to create an approx 1-cm flap for gripping.
9. While holding firmly onto the tail cartilage underlying the incision with forceps peel off the skin towards the distal end in one smooth motion with the other forceps using a very firm grip (*see* **Note 5**). Place in 100-mm Petri dish of PBS-AF on ice.
10. Once all tails are peeled, cut each into three equal lengths and then cut the wider proximal piece lengthwise to make four roughly equally-sized pieces. Transfer to fresh PBS-AF in the other 100-mm Petri dish on ice.
11. Place all tail pieces in 10-mL dispase in a 60-mm Petri dish, seal with parafilm, and incubate overnight at 4°C to dissolve dermal–epidermal junctional attachments.

3.2. Isolation of Basal Keratinocytes

This section describes the isolation of basal keratinocytes from adult murine tail skins treated with dispase. Set up the laminar flow hood as before but without dispase or tail washing solutions.

1. Aliquot 15 mL of trypsin inhibitor. Keep on ice.
2. Aliquot 10 mL of trypsin–EDTA into a 100-mL sterile glass jar or beaker. Place sterile magnetic flea into covered vessel and seal with parafilm. Prewarm in 37°C water bath at least 15 min before use.
3. Transfer dispased tails into 100-mm Petri dish of PBS-AF and rinse thoroughly.
4. On the inverted lid of the dish, place skin pieces dermis side down, and while holding the corner with forceps, gently peel the epidermis with the other forceps in the direction of the hair follicles. Transfer the peeled epidermis into fresh PBS-AF and rinse thoroughly. The dispase treatment allows easy peeling of the hairy epidermis with intact follicular structures and minimal loss of hair follicle keratinocytes (*see* **Fig. 1A**,**B**).
5. Gather the peeled skin pieces, lining them up contiguously on the center of the lid, and cut into no more than four or five pieces along their length—do not mince.
6. Press the cut pieces against the side of the lid to squeeze out excess PBS-AF, gather them onto the scalpel blade with forceps, and drop them into the prewarmed trypsin–EDTA. Place immediately on magnetic stirring plate set to 500 rpm and stir for exactly 4 min.
7. Place vessel immediately into ice and quench the reaction with an equal volume of cold trypsin inhibitor solution.
8. Truncate the tip of a plastic 25-mL pipet approx 5 mm with sterile scissors to prevent blockage during aspiration and pass epidermal slurry through a 70-µm cell strainer (Becton Dickenson) into a 50-mL Falcon tube.
9. Filter suspension through a 40-µm cell strainer into a fresh 50-mL Falcon tube and pass an additional 5 mL of trypsin inhibitor through the strainer to collect any residual cells.
10. Centrifuge at 400*g* for 5 min at 4°C. Pour off supernatant and flick tube to disperse pellet. Resuspend in 1 mL of AM-KGM and pipet repeatedly to break up clumps. Bring up to a volume of 15 mL with cold medium.

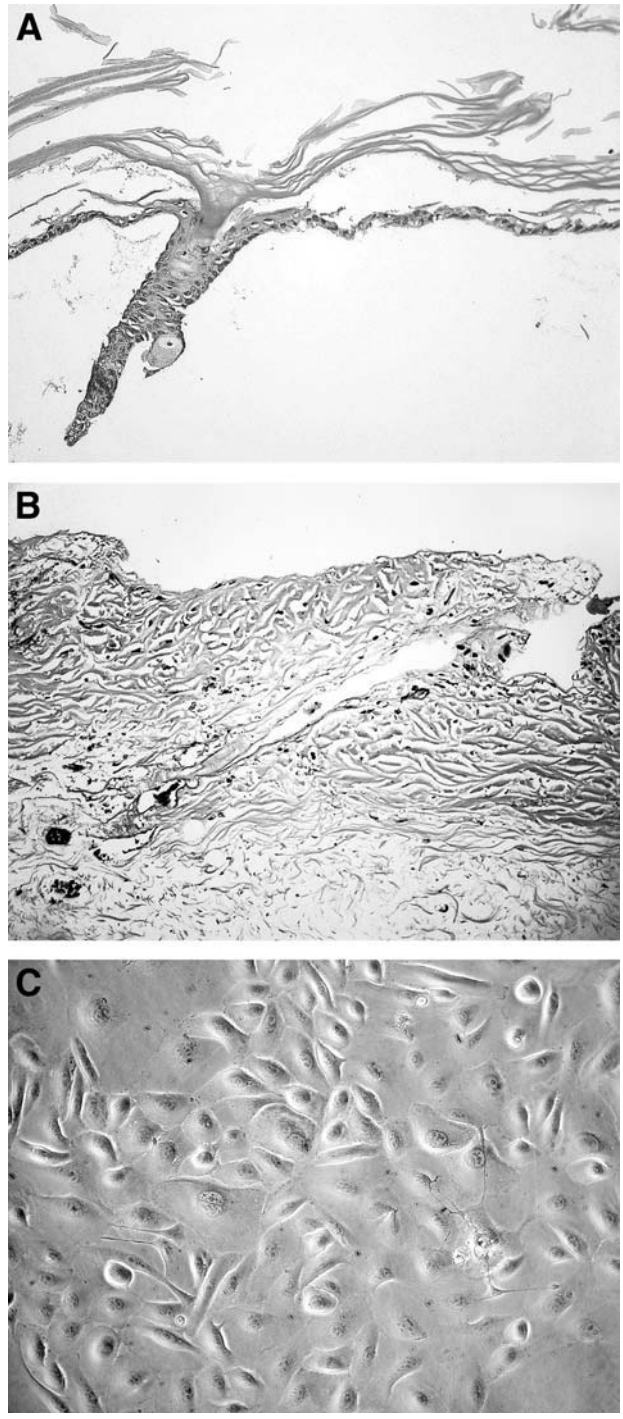


Fig. 1. Adult mouse tail skin. Peeled dispase-treated adult mouse tail epidermis **(A)** retains a continuous basal keratinocyte layer with intact pilosebaceous units that include the bulge region, a known repository for keratinocyte stem cells. Very few epithelial cells remain in the peeled dermis **(B)**. The keratinocytes pictured **(C)** were passaged four times with 1 : 5 splits.

11. Dilute a small aliquot of cell suspension into an equal volume of 0.4% Trypan blue and count viable cells on a hemocytometer. Expect 6–8 × 10^6 basal keratinocytes per tail with 85–90% viability.

3.3. Serial Culture of Adult Murine Keratinocytes

This section describes techniques for serial culture of adult murine keratinocytes from clonal density in serum-free medium without fibroblast conditioning.

3.3.1. Primary Culture

1. Before cell isolation, coat tissue culture flasks or plates with 20 µg/mL collagen IV in PBS-AF (*see* **Note 6**). Ensure volume used covers entire surface. Incubate at 37°C for 3 h or at 4°C overnight. Aspirate collagen solution and wash gently with PBS-AF.
2. Seed cells onto collagen-coated substrate in AM-KGM at desired cell density, that is, 1–10,000 cells/cm^2.
3. Optional: to improve plating efficiency, centrifuge freshly inoculated plates at 200*g* for 5 min at 4°C (*see* **Note 7**).
4. Incubate at 37°C, in 5% CO_2 in a humidified atmosphere.
5. On day 3, rinse culture thoroughly by pipetting medium up and down repeatedly to dislodge dead and differentiating cells. Add fresh medium three times per week thereafter, rinsing cultures as described each time. When nearing confluence, cultures must be fed every day to prevent extensive differentiation.

Keratinocyte colonies will adopt a "cobblestone" morphology of tightly packed cells that may appear slightly rounded (*see* **Fig. 1C**).

3.3.2. Subculture

Keratinocytes should be subcultured before extensive differentiation or growth arrest (~60–80% confluence). If intending immediate subculture, handle cells quickly at ambient temperature. Otherwise, keep cell suspension at 4°C to maintain viability.

1. Rinse cultures thoroughly with PBS-AF twice. Add just enough prewarmed 0.05% EDTA to cover the entire surface and incubate at 37°C for 10–15 min to initiate cell detachment.
2. When intercellular spaces look enlarged, remove EDTA and add prewarmed trypsin–EDTA to cover. Incubate 10–15 min at 37°C.
3. Pipet vigorously to detach cells and transfer to a tube. Wash surface with an equal volume of prewarmed trypsin inhibitor to collect residual cells and pool with trypsinized suspension to quench the reaction.
4. Centrifuge at 400*g* for 5 min.
5. Resuspend pellet with gentle pipetting and plate cells at desired density, for example, 1/3, 1/5, 10^3–10^4/cm^2, in collagen-coated plates or flasks (*see* **Note 8**).
6. Optional: secure lid onto plates by sealing around entire edge with Micropore surgical tape (1.25 cm × 9.1 m, 3 *M*; cat. no. 1530-0) to prevent inadvertent exposure of cultures to the open air.

The culture system described allows cultivation of adult murine keratinocytes from clonal density and serial culture for at least eight passages with 1 : 5 splits over 2 mo. The selectivity of the system for epithelial cells was tested by attempting to grow dermal fibroblasts seeded at 25,000 cells/cm^2 in AM-KGM on collagen IV without success.

4. Notes

1. DMEM may be substituted with an alternative medium provided that Ca^{2+} is omitted, as murine keratinocytes are very sensitive to Ca^{2+} concentration.
2. To make up, add 10 m*M* acetic acid drop wise slowly and swirl. Leave overnight at 4°C to allow solubilization. Collagen stock solution must be kept cold at all times to prevent polymerization.
3. The protein concentration may vary from batch to batch and must be assayed once the crude extract has been processed according to the manufacturer's instructions. Aliquot into vials an amount sufficient to deliver a final protein concentration of 70 µg/mL in 500 mL medium.
4. Some amino acids may require gentle heating and vortexing for solubilization. If a precipitate persists after extended heating and vortexing upon thawing frozen aliquots, discard and use a fresh vial.
5. If unable to grip skins with forceps, they may be peeled by hand using sterile ethanol-sprayed gloves provided skins are given an extra wash to ensure cleanliness.
6. Our cultures consistently perform better on collagen IV-coated substrates than on uncoated tissue culture plastic. However, cells may be plated directly onto tissue culture plastic, though with less efficiency.
7. We have observed better growth with centrifugation of freshly isolated keratinocytes onto their substrate. Indeed, expression of early differentiation markers is initiated in suspension cultures of keratinocytes ***(13)***, hence rapid attachment is desirable.
8. If small numbers of cells are expected and need to be counted, resuspend pellet in 500 µL medium, transfer to a 1.5-mL Eppendorf tube, and recover residual cells with a further 500 µL medium and pool. Centrifuge at 450*g* for 7 min. Aspirate supernatant carefully with a pipettor and resuspend pellet in an appropriate volume of medium. Expect approx 40,000 cells/cm^2 in a culture of 60–80% confluence. For example, a single well of a 24-well plate (~1.8 cm^2) when 80% confluent should yield approx 60,000 cells. For accurate counting, resuspend these at approx 10^6 cells/mL, that is, 60 µL in this example.

Acknowledgments

This work has been supported by the National Health & Medical Research Council (grant no. 145654) and the Anti-Cancer Council of Victoria, Australia.

References

1 Laerum, O. D. and Boyum, A. (1970) The separation and cultivation of basal and differentiating cells from hairless mouse epidermis. A study of the specificity of the separation method by autoradiography, and the viability of the cells during cultivation in diffusion chambers. *J. Invest. Dermatol.* **54,** 279–287.

2 Yuspa, S. H., Morgan, D. L., Walker, R. J., and Bates, R. R. (1970) The growth of fetal mouse skin in cell culture and transplantation to F1 mice. *J. Invest. Dermatol.* **55,** 379–389.

3 Rheinwald, J. G. and Green, H. (1975) Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell* **6,** 331–343.

4 Peehl, D. M. and Ham, R. G. (1980) Clonal growth of human keratinocytes with small amounts of dialyzed serum. *In Vitro* **16,** 526–540.

5 Tsao, M. C., Walthall, B. J., and Ham, R. G. (1982) Clonal growth of normal human epidermal keratinocytes in a defined medium. *J. Cell. Physiol.* **110,** 219–229.

6 Boyce, S. T. and Ham, R. G. (1983) Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal culture and serum-free serial culture. *J. Invest. Dermatol.* **81,** 33s–40s.

7. Boyce, S. T. and Ham, R. G. (1985) Cultivation, frozen storage, and clonal growth of normal human epidermal keratinocytes in serum-free media. *J. Tissue Culture Methods* **9,** 83–93.
8 Hager, B., Bickenbach, J. R., and Fleckman, P. (1999) Long-term culture of murine epidermal keratinocytes. *J. Invest. Dermatol.* **112,** 971–976.
9 Caldelari, R., Suter, M. M., Baumann, D., De Bruin, A., and Muller, E. (2000) Long-term culture of murine epidermal keratinocytes. *J. Invest. Dermatol.* **114,** 1064–1065.
10 Bickenbach, J. R. and Chism, E. (1998) Selection and extended growth of murine epidermal stem cells in culture. *Exp. Cell Res.* **244,** 184–195.
11 Wille, J. J., Jr., Pittelkow, M. R., Shipley, G. D., and Scott, R. E. (1984) Integrated control of growth and differentiation of normal human prokeratinocytes cultured in serum-free medium: clonal analyses, growth kinetics, and cell cycle studies. *J. Cell Physiol.* **121,** 31–44.
12 Tennenbaum, T., Yuspa, S. H., and Kapitulnik, J. (1990) Magnesium and phosphate enrichment of culture medium stimulates the proliferation of epidermal cells from newborn and adult mice. *J. Cell Physiol.* **143,** 431–438.
13. Li, L., Tennenbaum, T., and Yuspa, S. H. (1996) Suspension-induced murine keratinocyte differentiation is mediated by calcium. *J. Invest. Dermatol.* **106,** 254–260.

3

Keratinocyte Culture in the Absence of Substrate Attachment

Monika Jost and Ulrich Rodeck

Summary

This chapter deals with experimental protocols and considerations related to the culture of epithelial cells under anchorage-independent conditions in liquid media. This technique has proven to be a powerful tool in studying the effects of loss of extracellular matrix interaction on crucial aspects of epithelial cell biology. Specifically, examining cells in the absence of substrate attachment, as described in this chapter, will allow the investigator to study the effect of growth factors independently of cell adhesion. Several methods are discussed relating to the preparation of tissue culture plates for suspension cultures and to the choice of a suitable cell culture medium.

Key Words:

Anchorage-independent growth; keratinocytes; adhesion; growth factors; differentiation.

1. Introduction

Adhesion of cells to extracellular matrix components is a crucial part of epithelial cell biology. Matrix attachment controls multiple aspects of cell physiology, including cytoarchitecture ***(1,2)***, cell cycle progression ***(3,4)***, and differentiation ***(5)***. In recent years, it has been realized that substrate attachment is also essential for the survival of epithelial cells, including keratinocytes ***(6–9)***. When removed from substrate, normal or immortalized epithelial cells will undergo apoptosis, a process termed "anoikis" ***(6)***. This aspect of substrate interaction may be rate-limiting to the spread of malignant cells during the metastatic process and has, thus, attracted intense interest. Furthermore, in multilayered epithelia such as the epidermis, detachment of cells from the basement membrane during the stratification process is part of the terminal differentiation program. The question of how terminal differentiation in the upper layers of stratifying epidermis relates to apoptosis incurred after losing contact with the basement membrane is yet to be resolved. However, the examination of molecular events induced by loss of matrix anchorage may help to distinguish matrix-dependent processes that contribute to differentiation and/or apoptosis.

The following brief protocols describe methods to culture either primary human keratinocytes isolated from neonatal foreskin or HaCaT cells, an immortalized keratinocyte cell line ***(10)*** in suspension culture. One of the key elements of these experiments is the medium composition, which is addressed separately. A crucial step in preventing the attachment of epithelial cells to tissue culture plates consists of covering plastic surfaces

From: *Methods in Molecular Biology, vol. 289:*
Epidermal Cells: Methods and Protocols
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

with a substance that prevents cell attachment and the deposition of adhesion-competent extracellular matrix. This is commonly achieved by placing cells on either a poly–HEMA or agarose polymer. Both of these substrates prevent substrate attachment and extracellular matrix (ECM) deposition without interfering with cell-to-cell contact.

2. Materials

2.1. Media Preparation

1. MCDB153 medium (Sigma-Aldrich; St. Louis, MO; cat. no. M7403).
2. Amino acids for media preparation (L-isomers from Sigma-Aldrich [cell culture grade]): histidine–HCl (cat. no. H9511); isoleucine (cat. no. I7383); methionine (cat. no. M2893); phenylalanine (cat. no. P5030); tryptophan (cat. no. T0271); and tyrosine (cat. no. T1145). Amino acids are stored at room temperature, except for tyrosine (stored at 4°C).
3. Sodium bicarbonate (Sigma-Aldrich; cat. no. S5761).
4. Ethanolamine (Sigma-Aldrich; cat. no. E9508).
5. *O*-Phosphoethanolamine (Sigma-Aldrich; cat. no. 0503).
6. Hydrocortisone (Sigma-Aldrich; cat. no. H0888).
7. Bovine serum albumin fraction V, heat shock, fatty acid free (Roche Diagnostics Corporation-Roche Applied Science; Indianapolis, IN; cat. no. 0100062).

2.2. Suspension Culture

1. SeaKem LE agarose (Cambrex; Rockland, ME; cat. no. 50001).
2. Poly(2-hydroxyethyl methacrylate (Poly–HEMA); (Sigma-Aldrich; cat. no. P3932).

3. Methods

3.1. Cell Culture Medium for Anchorage-Independent Growth

The choice of cell culture medium is crucial for the interpretation of phenomena observed in liquid suspension culture. In the following, we will discuss several examples that illustrate how the composition of culture media can affect experimental outcomes. Although it is impossible to determine a "gold standard" medium for experiments in liquid suspension culture, the following considerations may serve as starting point for designing media composition.

3.1.1. Cell Culture Medium for Investigating the Effect of Growth Factors and Cytokines on Anchorage-Independent Growth and/or Apoptosis

To examine the effect of growth factors and cytokines on anchorage-independent cell cycle progression and/or apoptosis, a chemically defined base medium is essential. Preparation of a suitable base medium, in this chapter referred to as MCDB153 base medium ***(11)***, is described in **Subheading 3.1.3.** MCDB153 base medium is buffered with carbonate and contains essential amino acids, hydrocortisone, ethanolamine, and *o*-phosphoethanolamine. This medium will sustain viability albeit not cell cycle progression of keratinocytes for 48–72 h in the attached state. Growth factors and cytokines of interest may be added as required by the experimental design. It is recommended to add fatty-acid free bovine serum albumin as a carrier protein to avoid loss of cytokines as a result of nonspecific binding to the walls of the culture vessel. Any undefined components used for routine culture, including bovine pituitary extract and fetal bovine serum, should be omitted. Commercially available keratinocyte

media similar to MCDB153 base medium may be used as an alternative, as long as they do not contain proprietary or undisclosed components, designed to ensure optimal growth and survival of keratinocytes in adherent culture.

3.1.2. Cell Culture Medium for Investigating Mechanisms of Differentiation and Cell–Cell Adhesion

The MCDB153 base medium is distinguished by a comparatively low Ca^{2+} concentration (29 μ*M*), designed to prevent Ca^{2+}-induced differentiation of keratinocytes under routine culture conditions. An important corollary of low ambient Ca^{2+} in suspension culture is prevention of the formation of cell clumps or spheroids that do form if the Ca^{2+} concentration is higher than 300 μ*M*. This is likely a result of the fact that Ca^{2+} contributes to cadherin-based cell–cell adhesion ***(12,13)***. In effect, the use of low Ca^{2+} media ensures that normal keratinocytes will remain as single cells or loose aggregates during suspension culture; such aggregates can be dispersed by gentle pipetting at least during the first 4 d of suspension culture. Thus, not only cell–matrix but also cell–cell adhesion-mediated signaling events will be prevented in low Ca^{2+} cell culture medium. If cell–cell adhesion is desired, it can be restored by supplementing the base medium with Ca^{2+} at concentrations greater than 500 μ*M*. It should be noted that commercially available media for keratinocyte culture contain Ca^{2+} at a range of concentrations. For example, KGM from GIBCO-Invitrogen (Carlsbad, CA) contains 100 μ*M* of Ca^{2+}, whereas MCDB153 base medium contains only 29 μ*M* of Ca^{2+}. We observed that KGM (GIBCO-Invitrogen) allows the differentiation of keratinocytes in suspension to proceed to a greater extent than MCDB153 base medium as described here (results unpublished). These differences highlight the importance of considering Ca^{2+} concentration as it relates to cellular phenotypes in suspension culture (*see* **Note 1**).

3.1.3. Preparation of MCDB153 Base Medium

1. To 900 mL of dH_2O, add MCDB153 base medium powder (1 bottle for 1 L of final volume) and stir. Rinse the bottle with a little more dH_2O and add to mixture. Stir to dissolve.
2. Add 1.176 g of sodium bicarbonate (Sigma; cat. no. S5761).
3. Ensure that pH is 6.85.
4. Add amino acids as follows:

Amino acid	Final concentration	MW	Add per 1 L of medium
Histidine	$2.4 \times 10e^{-4}$	209.6	50 mg
Isoleucine	$7.5 \times 10e^{-4}$	131.2	98 mg
Methionine	$9.0 \times 10e^{-5}$	149.2	13 mg
Phenyalanine	$9.0 \times 10e^{-5}$	165.2	15 mg
Tryptophan	$4.5 \times 10e^{-5}$	204.2	9 mg
Tyrosine	$7.5 \times 10e^{-5}$	225.2	17 mg

All amino acids are stored at room temperature, except for tyrosine (stored at 4°C).

5. Stir to dissolve (approx 60 min). Adjust volume to 1 L.
6. Adjust pH to 7.6 with 50% w/v NaOH.
7. Filter through 0.2-μm filter; make aliquots of 500 mL and store at –20°C.
8. To 250 mL MCDB-Base medium, add the following:

	Final concentration
250 μL ethanolamine (0.1 *M*)	0.1 m*M*
250 μL *O*-phosphoethanolamine (0.1 *M*)	0.1 m*M*
25 μL hydrocortisone (5 m*M*)	0.5 μ*M*

3.2. Preparation of Culture Plates for Nonadherent Cell Culture

3.2.1. Protocol A: Preparation of Poly–HEMA–Coated Plates

3.2.1.1. Materials

1. 95% Ethanol.
2. Tissue culture medium (*see* **Subheading 2.1.**).
3. Tissue culture-treated plates with various surface area configurations (six-well plates are useful for many applications).
4. Phosphate-buffered saline without Ca^{2+} and Mg^{2+} (sterile).

3.2.1.2. Method

1. Prepare a stock solution of Poly–HEMA by dissolving 10 mg/mL in 95% ethanol while stirring vigorously. To enhance solubility, incubate the solutions in a tightly capped bottle overnight at 37°C. Remove any undissolved material by centrifugation at about 500*g* for 30 min at room temperature (*see* **Note 2**).
2. Coat plates with the solution in a biosafety cabinet. Use approx 0.12 mL/cm^2 surface area (i.e., 1 mL/well of a six-well plate) and let the ethanol evaporate by leaving the plates open in the cabinet.
3. Repeat this process twice to cover the surface completely.
4. Wash the plates several times with phosphate-buffered saline (about 0.25 mL/cm^2). The plates are then ready for use. Unused plates can be sealed with plastic wrap and stored at 4°C.

3.2.2. Protocol B: Preparation of Agarose-Coated Plates

3.2.2.1. Materials

1. Agarose (molecular biology grade).
2. Tissue culture medium (*see* **Subheading 3.1.**).
3. Tissue culture-treated plates of suitable sizes (e.g., six-well plates).
4. Fatty-acid-free bovine serum albumin (optional, *see* **Subheading 3.1.**).

3.2.2.2. Preparation of Agarose-Coated Plates

1. Prepare a 0.9% (w/v) agarose stock solution by weighing 0.9 g agarose into 100 mL of medium (growth-factor free medium, *see* **Subheading 3.2.2.3.**) (*see* **Note 3**).
2. Boil the agarose suspension in a microwave oven to dissolve the agarose and sterilize the solution; alternatively, the solution can be autoclaved in the liquid cycle and should be used before it solidifies.
3. Pipet the solution into plates in a biosafety cabinet. Add approx 0.24 mL per cm^2 surface area (2 mL/well of a six-well plate).
4. Leave the plates open in the biosafety cabinet at room temperature until the agarose has solidified.
5. The plates are ready for use. For storage, add a small amount of medium to each well (about 0.2 mL/cm^2) to prevent drying out, seal with plastic wrap and store at 4°C.

3.2.3. Suspension Culture and Cell Harvest

1. Trypsinize cells and inactivate trypsin using established procedures (either with serum or soybean trypsin inhibitor).
2. Count cells by Trypan blue exclusion assay and resuspend in medium (depending on experimental conditions; *see* **Subheading 3.1.3.**). A recommended starting cell density is $3–5 \times 10^5$ viable cells per milliliter (HaCaT or normal keratinocytes). Use at least 2 mL of medium per well (six-well plate) or more, depending on the number of cells required for

subsequent analysis. When using MCDB153 base medium (*see* above), add 0.2 % (w/v) fatty-acid-free bovine serum albumin.

3. Maintain cells in suspension for desired time intervals up to 72 h. If longer incubation times are required, culture media need to be replaced by centrifugation of the cells, resuspension in fresh medium, and reseeding on agarose.
4. When removing aliquots of cells for analysis, mix the culture by gentle pipetting with a serological pipet, then remove the desired volume and analyze cells.
5. For clonogenic assays, remove 50 to 100 µL cell suspension (~1.5–5 × 10^4 cells) and seed into 24-well plates in optimal growth medium containing all supplements.
6. For molecular or biochemical analyses (e.g., protein or ribonucleic acid extraction), harvest a sufficient number of cells by centrifugation at 400*g* and process as required.

4. Notes

1. HaCaT cells should be cultured for several days in keratinocyte medium before assay. Serum-containing media that are frequently used for routine maintenance of HaCaT cells may exert carryover effects that can affect viability or differentiation in suspension culture, thus making it difficult to distinguish effects of single growth factors
2. It is necessary to repeat coating with Poly–HEMA at least twice to completely cover the surface area. Uncovered areas will allow partial attachment of cells. This can pose a potential source of error if cell numbers are to be determined.
3. It is best to use agarose-coated plates shortly after preparation; longer storage (i.e., for several days or longer) can lead to shrinkage or cracking of the agarose. When this occurs, some cells will manage to slip underneath the agarose while in suspension and grow attached on the plastic, which, as mentioned above, is a potential source of error. For repeated use, the agarose stock solution can be stored at 4°C once it has solidified and may be reheated in a microwave oven whenever more plates are needed. To equilibrate the agarose, a medium similar to the one used in the experiment should be used, with respect to Ca^{2+} concentration. It is not recommended to use growth factor-supplemented medium for the preparation of the agarose solution, since proteins will be denatured by heat (either in the autoclave or in the microwave). Although this is not assumed to negatively affect the experiment, it will waste expensive growth supplements.

References

1 Wang, N., Naruse, K., Stamenovic, D., Fredberg, J. J., Mijailovich, S. M., Tolic-Norrelykke, I. M., et al. (2001) Mechanical behavior in living cells consistent with the tensegrity model. *Proc. Natl. Acad. Sci. USA* **98,** 7765–7770.

2 Ingber, D. E. (2003) Tensegrity II: how structural networks influence cellular information processing networks. *J. Cell Sci.* **116,** 1397–1408.

3 Bottazzi, M. E., Zhu, X., Bohmer, R. M., and Assoian, R. K. (1999) Regulation of p21(cip1) expression by growth factors and the extracellular matrix reveals a role for transient ERK activity in G1 phase. *J. Cell Biol.* **146,** 1255–1264.

4 Roovers, K., Davey, G., Zhu, X., Bottazzi, M. E., and Assoian, R. K. (1999) Alpha5beta1 integrin controls cyclin D1 expression by sustaining mitogen-activated protein kinase activity in growth factor-treated cells. *Mol. Biol. Cell* **10,** 3197–3204.

5 Jones, J., Sugiyama, M., Speight, P. M., and Watt, F. M. (1996) Restoration of alpha v beta 5 integrin expression in neoplastic keratinocytes results in increased capacity for terminal differentiation and suppression of anchorage-independent growth. *Oncogene* **12,** 119–126.

6 Frisch, S. M. and Francis, H. (1994) Disruption of epithelial cell-matrix interactions induces apoptosis. *J. Cell Biol.* **124,** 619–626.

7 Boudreau, N., Werb, Z., and Bissell, M. J. (1996) Suppression of apoptosis by basement membrane requires three-dimensional tissue organization and withdrawal from the cell cycle. *Proc. Natl. Acad. Sci. USA* **93,** 3509–3513.

8 Rodeck, U., Jost, M., DuHadaway, J., Kari, C., Jensen, P. J., Risse, B., and Ewert, D. L. (1997) Regulation of Bcl-xL expression in human keratinocytes by cell-substratum adhesion and the epidermal growth factor receptor. *Proc. Natl. Acad. Sci. USA* **94,** 5067–5072.

9 Frisch, S. M. and Screaton, R. A. (2001) Anoikis mechanisms. *Curr. Opin. Cell Biol.* **13,** 555–562.

10 Boukamp, P., Petrussevska, R. T., Breitkreutz, D., Hornung, J., Markham, A., and Fusenig, N. E. (1988) Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J. Cell Biol.* **106,** 761–771.

11 McNeill, H. and Jensen, P. J. (1990) A high-affinity receptor for urokinase plasminogen activator on human keratinocytes: characterization and potential modulation during migration. *Cell Regul.* **1,** 843–852.

12 Nagafuchi, A., Shirayoshi, Y., Okazaki, K., Yasuda, K., and Takeichi, M. (1987) Transformation of cell adhesion properties by exogenously introduced E-cadherin cDNA. *Nature* **329,** 341–343.

13. Wheelock, M. J. and Jensen, P. J. (1992) Regulation of keratinocyte intercellular junction organization and epidermal morphogenesis by E-cadherin. *J. Cell Biol.* **117,** 415–425.

4

Application of Genetically Modified Feeder Cells for Culture of Keratinocytes

Takashi Kameda and Toshihiro Sugiyama

Summary

In living bodies, there are many cell–cell interactions, including epithelial–mesenchymal interactions, to control cell growth and differentiation, and disturbances to such systems are believed to be a critical reason for many diseases, including cancer. Recently, many growth factors mediating epithelial–mesenchymal interactions have been revealed by molecular biological research. These growth factors are critical tools for both basic research and medical applications. However, obtaining purified forms of such growth factor proteins is difficult. To conveniently analyze the biological effects of such factors on keratinocytes, in this protocol, we describe how to introduce ectopic genes of growth factors of interest (*sonic hedgehog* and *wnt-3* genes as examples) into Swiss-3T3 cells, one of the most widely used feeder cells for epithelial cell culture, and how to coculture the keratinocytes with them.

Key Words:

Keratinocytes; feeder cell; gene transfer; Sonic hedgehog; Wnt-3.

1. Introduction

The most classical and successful method to culture keratinocytes was developed using Swiss-3T3 fibroblast cells as feeder cells ***(1)***. Although it is currently possible to support the growth of keratinocytes using serum-free medium (very low Ca^{2+} concentration containing several expensive growth factors; **ref.** ***2***), using Swiss-3T3 cells as feeders is still frequently applied for the formation of cultured epithelium. However, the key objective to this method is controlling the growth of the fibroblasts.

Although fibroblasts support the growth of keratinocytes, their excess growth conversely suppresses it. To solve this problem, the feeder cells are often unfertilized using γ-irradiation or Mitomycin-C treatment. These unfertilized fibroblasts stop growing and become a good bed for the keratinocytes. Although the nature of factors supplied by fibroblasts is still unclear, some reports have suggested the participation of soluble factors like keratinocyte growth factor (a fibroblast growth factor family protein) or cell membrane-associated mitogens ***(3,4)***. Recently, the more critical factors in keratinocyte growth and differentiation were revealed by molecular biological research on cancer and early developmental processes.

Sonic hedgehog (Shh) is one of the most important factors working in many developmental stages, including hair follicle development ***(5,6)***. Binding of the Shh ligand to the Patched (Ptc) family of transmembrane receptors (Ptc-1 and -2) activates

From: *Methods in Molecular Biology, vol. 289:*
Epidermal Cells: Methods and Protocols
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

Smoothened (Smo), another transmembrane protein suppressed by the members of the Ptc family proteins in the absence of the Shh signal. Derepressed Smo activates the transcription factors of the Gli family (Gli-1, -2, and -3) to regulate the target gene expression *(7)*. Recently, mutations of the *ptc*-1 gene, which encodes the Shh receptor, have been identified in inherited and sporadic forms of human basal cell carcinomas and trichoepitheliomas *(8)*. It is assumed that these skin cancers originate from very immature keratinocytes. These cancers are also known to be induced in transgenic mice by the activation of Shh signaling in the epidermis, suggesting that the Shh signaling has a strong mitogenic effect on immature keratinocytes.

Wnt proteins are also one of the most critical factors in epithelial cell fate control. Wnt proteins bind to receptors of the Frizzled family on the cell surface. Through several cytoplasmic relay components, the signals stabilize the β-catenin protein, which then enters the nucleus and forms a complex with T-cell factor (TCF) to activate the transcription of Wnt target genes *(9)*. Many human epithelial cancers have mutations of Wnt pathway genes, and it was suggested that this pathway is critical for the cell fate determination of keratinocyte stem cells in, for example, hair follicle formation *(10)*.

In addition to the basic interest in the biological effects of these factors on keratinocytes, their application might provide powerful techniques to improve tissue-engineering technology. However, to obtain large amounts of biologically active Shh- or Wnt-purified proteins is difficult at present, and it is troublesome to introduce such oncogenic genes directly into keratinocytes because of the possibility of cancer induction. To solve these problems, we introduced ectopic genes into Swiss-3T3 cells and supplied the proteins sufficiently without inducing genetic changes to the keratinocytes through co-culturing (**Fig. 1**; **ref. *11***).

2. Materials

2.1. Cells

1. Swiss-3T3 (available from most cell banks; *see* **Note 1**).
2. Human epidermal keratinocytes (Cell Applications, Inc.; cat. no. 102-05).

2.2. Antibodies

1. Anti-Sonic hedgehog protein antibodies (Santa Cruz Biotechnology, Inc., cat. no. sc-9024).
2. Anti-HA-antibodies (Santa Cruz Biotechnology, Inc.; cat. no. sc-805).
3. Anti-rabbit IgG HRP linked (Amersham Bioscience; cat. no. NA934).

2.3. Culture Media

2.3.1. Swiss-3T3

1. Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO; cat. no. D 6046), containing 10% fetal calf serum (Sigma; cat. no. F 2442).
2. Trypsin–ethylenediamine tetraacetic acid (EDTA) solution: 0.05% trypsin and 0.02% EDTA in phosphate-buffered saline (PBS).

2.3.2. Keratinocytes

1. For recovery: Defined Keratinocyte-SFM (Gibco; cat. no. 10744-019).
2. For co-culture: DMEM containing 10% fetal bovine serum, 1.8×10^{-4} *M* adenin (Sigma; cat. no. A 9795), 5 µg/mL insulin (Sigma, cat. no. I 9278 [10 mg/mL solution]), 0.5 µg/mL dexamethasone (Sigma; cat. no. D 8893), 1×10^{-10} *M* cholera toxin (Sigma; cat. no. C 8052),

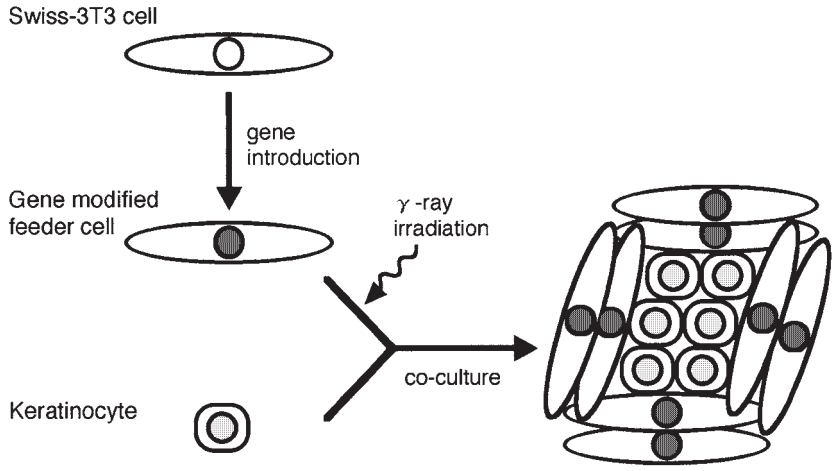


Fig. 1. Schematic diagram of the experiment. To use the growth-stimulating effect of growth factors like Shh or Wnt-3 protein safely and readily, the ectopic gene is introduced into Swiss-3T3 cells, which is commonly used as a feeder for keratinocytes. The established ectopic gene expressing feeder cells are lethally irradiated and used to support the growth of keratinocytes.

10 ng/mL epidermal growth factor (Sigma; cat. no. E 9644), and 2×10^{-9} *M* triiodothyronine (Sigma, cat. no. T 5516).

3. (100X) Adenin: dissolve 0.1 g adenin powder into 30 mL saline, filter, and store at –20°C (stable for several mos.).
4. (1000X) Dexamethasone: dissolve 1 mg dexamethasone powder into 2 mL ethanol, filter and store at –20°C (stable for 1 yr).
5. (100,000X) Cholera toxin: dissolve 0.5 mg cholera toxin into 50 mL saline, filter and store at 4°C (stable for several mos.); *see* **Note 2**).
6. (1000X) EGF: dissolve 200 µg epidermal growth factor into 20 mL sterilized saline and store at –20°C (stable for 1 yr).
7. (10,000X) Triiodothyronine: dissolve 1 mg triiodothyronine into 74.3 mL saline, filter, and store at –20°C in the dark (stable for 1 yr).

2.4. Gene Transfection and G418 Selection

1. Sonic hedgehog expression vector (available from T. Kameda; tkameda@med.akita-u.ac.jp).
2. HA-tagged-Wnt-3 expression vector (Upstate Biotechnology, Lake Placid, NY; cat. no. 21-123).
3. LIPOFECTAMINE PLUS™ Kit (Invitrogen; cat. no. 10964-013).
4. Geneticin (G418, Sigma; cat. no. G 9516).
5. Cloning cylinder with silicone grease (Sigma; cat. no. C 2059).

2.5. Western Blotting

1. Extraction buffer: 10 m*M* Tris-HCl, pH 7.4, 150 m*M* NaCl, 1% NP40, 1% deoxycholate in water.
2. Rubber policeman.
3. Protein assay kit I (Bio-Rad; cat. no. 500-0001JA).
4. 2X sample buffer: 0.2 *M* Tris-HCl, pH 7.4, 4% sodium dodecyl sulfate (SDS), 10% 2-mercaptoethanol, 40% glycerol in water.

5. Electrophoresis gel:
   a. For Shh detection, use a 15% poly-acrylamide gel, such as READY GELS J (15%; Bio-Rad; cat. no. 161-J341).
   b. For Wnt-3 detection: 10% poly-acrylamide gel, such as READY GELS J (10%; Bio-Rad; cat. no. 161-J321).
6. Electrophoresis buffer: dissolve 3.02 g Tris, 14.4 g glycine, and 1 g of SDS into water and adjust the volume to 1 L (adjust pH to 8.3 before adding SDS).
7. Transfer buffer: Remove SDS from the electrophoresis buffer formula.
8. Electroblotting system.
9. Immobilon membrane (Millipore; cat no. IPVH000-10).
10. Whatman 3MM paper (Whatman; cat. no. 3030-917).
11. PBS-T: 0.5% Tween-20 in PBS.
12. Western Blocking Reagent, solution (Roche; cat. no. 1-921-673).
13. ECL Western Blotting Detection System (Amersham; cat. no. RPN2209).

2.6. Staining of Keratinocyte Colonies

1. Fixing solution: 10% formalin in PBS.
2. Rhoda-Nile solution: 1% Rhodamin B (Sigma; cat. no. R6626) and 1% Nile Blue (Sigma; cat. no. N5632) in water. (Caution: Rhodamin B is a weak mutagen).

2.7. Detachment of the Epithelial Sheet

1. Detachment solution: DMEM containing 10% fetal calf serum and 1 U/mL of DISPASE (Invitrogen; cat. no. 17105-041).

3. Methods

3.1. Culture of Swiss-3 T3 Cells

1. Plate 2×10^5 cells per 100-mm culture dish with 10 mL of culture medium.
2. At 80% confluence (about 3 d after inoculation), wash the culture twice with 10 mL of 37°C Trypsin-EDTA solution, incubate at 37°C for 5–10 min, and disperse the cells in 10 mL of culture medium by pipetting. Centrifuge the cells for 5 min at 500*g*, discard the supernatant, and resuspend in 10 mL of culture medium. Count the cell number and plate 2×10^5 cells per 100-mm culture dish with 10 mL culture medium. Repeat **step 2**.

3.2. Transfection of Ectopic Gene into Swiss-3T3 Cells

1. Plate 2×10^5 cells per 35-mm culture dish (or well of six-well plate) with 2 mL culture medium and inoculate to 50–80% confluence (about 18–24 h after inoculation).
2. Discard old culture medium and add 1 mL of new culture medium.
3. Mix the following two tubes. Tube 1: 0.1 mL serum-free DMEM containing 1 µg of plasmid DNA and 6 µL of PLUS Reagent. Tube 2: 0.1 mL serum-free DMEM containing 4 µL Lipofectamine reagent. Wait for 15 min, and add the mixture into the culture.
4. Add 3 mL of new culture medium 3 h after the transfection. Change the medium on the next day.
5. Transfer the culture to new dishes (1 : 10–1 : 20) 2 d after transfection. Change the medium to geneticin (400 µg/mL)-containing medium 3 d after transfection. Change the medium every 3 d.
6. If the transfection succeeds, small vital cell colonies can be detected among dying cells 1–2 wk after the selection. Clone the selected colonies using the cloning cylinder.

3.3. Detection of the Ectopic Protein (Shh or Wnt-3) Expression by Western Blotting

1. Grow transfected and selected cells from **Subheading 3.2.** to confluence in 60-mm culture dishes (*see* **Note 3**).
2. Wash the culture twice with ice-cold PBS. Add 0.1–0.2 mL of the extraction buffer, scrape the cells with the rubber policeman, and mix well. Transfer the extract to a 1.5-mL microtube, centrifuge (10,000 rpm, 5 min), and collect the supernatant.
3. Determine the quantity of protein in the extract using the protein assay kit with protein standard.
4. Make the protein concentration of the samples even with extraction buffer. Add the same volume of sample buffer, mix, and boil for 5 min. Keep the stock sample in the freezer.
5. Electrophorese the samples on a suitable poly-acrylamide gel (15% for Shh and 10% for Wnt-3) with MW standard. Transfer the protein to the Immobilon membrane according to the manuals for each electroblotting machine. We recommend using the transfer buffer described in **Subheading 2.5.**
6. Block the membrane with a suitable blocking solution for 30 min. We do not recommend using crude skim milk. Wash the membrane with PBS-T and transfer it to a suitable hybridization bag. Add primary antibodies (we dilute the anti-Shh antibodies 500 times, and the anti-HA antibodies 100 times with PBS-T) and incubate for 1 h at room temperature (RT).
7. Wash the membrane three times with PBS-T for 10 min each. Transfer the membrane to the hybridization bag and add secondary antibodies (HRP-conjugated) (we dilute them 1000 times with PBS-T). Incubate for 1 h at RT.
8. Wash the membrane three times with PBS-T for 10 min each. Detect the target protein bands using the ECL Western Blotting Detection System according to the manual. Select the cell populations that express the transgene at a high level.

The representative results of Shh and Wnt-3 expressing cells are indicated in **Fig. 2**.

3.4. Preparation of the Feeder Cells for Coculture

1. Grow the feeder cells in 100-mm culture dishes to confluence. Irradiate the cultures with 50–70 Gy of γ-irradiation.
2. Disperse the cells with Trypsin–EDTA solution and inoculate them at 4×10^5/60-mm culture dishes with the culture medium for coculture.

Alternatively, if you do not have a suitable irradiation machine, please use the following protocol: inoculate the feeder cells at $3–4 \times 10^5$/60-mm culture dish. On the next day, treat them with 4 μg/mL mitomycin-C (Sigma; cat. no. 50-07-7) containing culture medium for 4 h (*see* **Note 4**). Wash the culture three times with culture medium, and change the medium to the coculture medium.

The morphological changes in cell shape after the irradiation are indicated in **Fig. 3**.

3.5. Co-Culture of Human Keratinocytes With the Feeder Cells

1. Recover the human epidermal keratinocytes following the manufacturer's protocol. Grow them for 2–3 d in the recovery medium.
2. Prepare the feeder cells according to method in **Subheading 3.4.**
3. Wash the keratinocyte culture with trypsin-EDTA solution, incubate for 5 min at RT, and collect the cells with a rubber policeman. Wash the cells twice with ice-cold PBS, count the cell number, suspend in the medium for coculture, and inoculate a suitable number (*see* **Note 5**; we used 5000–40,000 cells for the inoculation) onto the prepared feeder cells. Change the medium twice a week.

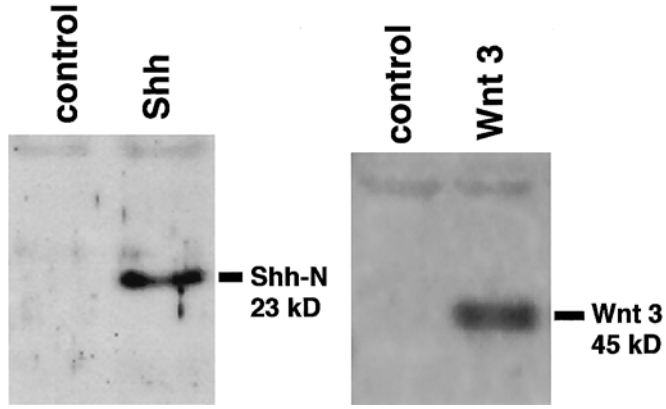


Fig. 2. Ectopic expression of the Shh and Wnt-3 in the Swiss-3T3 cell.

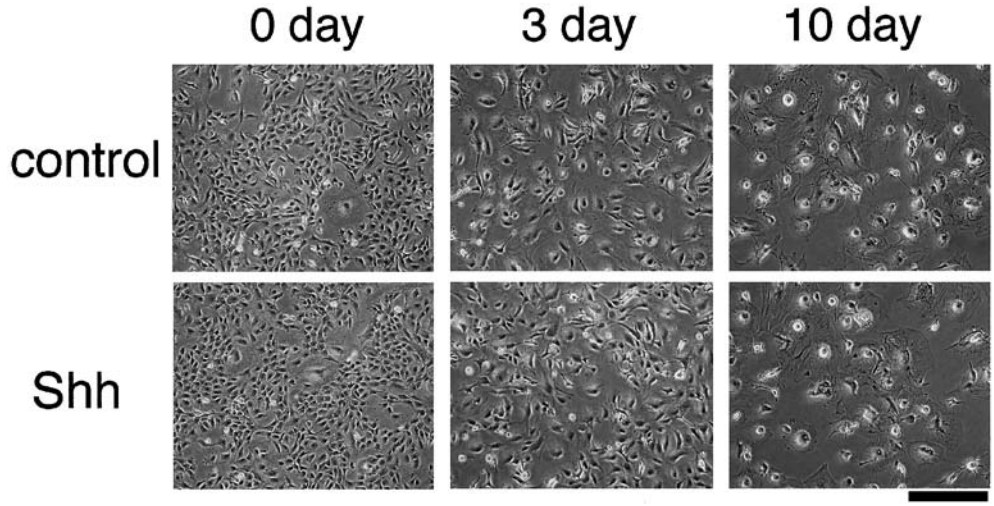


Fig. 3. Morphological change in the control and the Shh-expressing Swiss-3T3 cells after γ-ray irradiation (70 Gy). The images of d 0 show the cell shapes before the irradiation. The irradiated cells gradually hypertrophied and peeled off. Bar = 150 μm.

3.6. Staining of the Keratinocyte Colonies

1. Wash the culture twice with PBS and fix it with fixing solution for 30 min at RT.
2. Discard the fixing solution, add 2 mL Rhoda-Nile solution, and gently shake the dishes for 10 min at RT. Wash the plate with running water in a bucket for several minutes. Stop washing after confirmation of the keratinocyte colony staining. Drain the water, and dry the dishes.

The staining results of a comparison of the feeding effect of the normal and Shh expressing feeder cells are indicated in **Fig. 4**.

3.7. Detachment of the Keratinocyte Sheet From the Culture Dish

Change the medium to the detachment solution and incubate the culture for 30 min in a CO_2 incubator. Peel the cell sheet off with two pairs of tweezers. The vertical section of the detached cell sheet, fed with Shh expressing feeder cells, is indicated in **Fig. 5**.

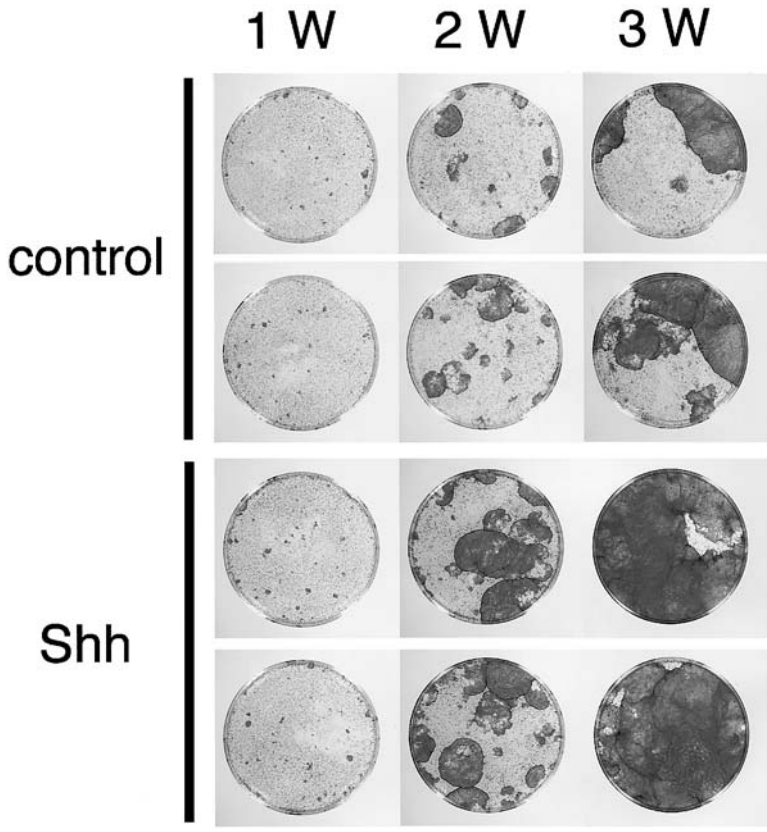


Fig. 4. Comparison of the feeding effect of the lethally irradiated control and Shh-expressing Swiss-3T3 cells. Cultures were fixed and stained after 1 wk (1 W), 2 wk (2W), and 3 wk (3 W) after keratinocyte inoculation.

Fig. 5. The vertical section of the cultured epidermis that was fed by Shh-expressing feeder cells. Twenty-seven days after keratinocyte inoculation, the cell layer was detached from the culture dish by dispase treatment, fixed, embedded in paraffin, and cut at 10 μ. The sections were stained with hematoxylin-eosin. Bar = 100 μm.

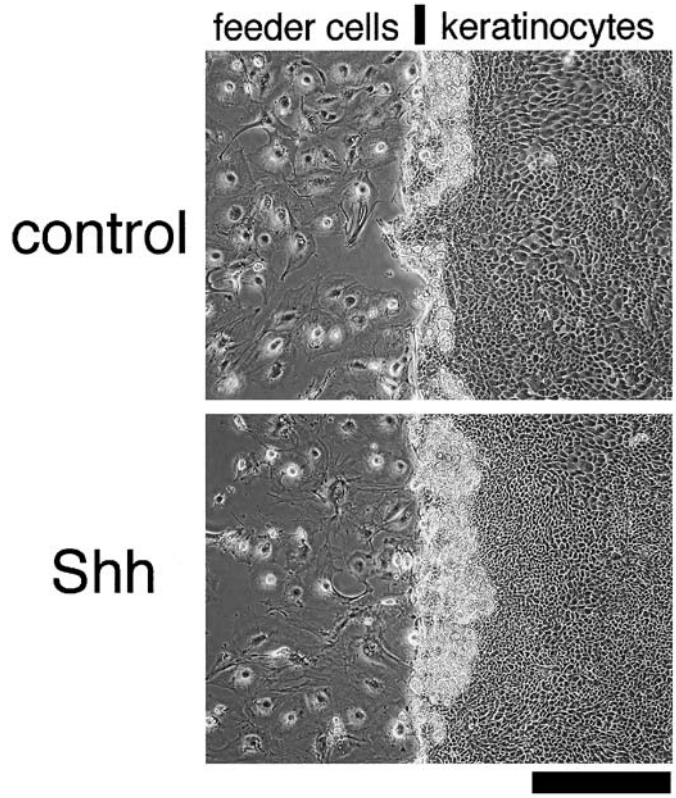


Fig. 6. Microscopic observation of the keratinocyte colonies that grew well fed by control (top) or Shh-expressing feeder cells (bottom). Two weeks after inoculation of keratinocytes, the representative well-growing colonies were selected, and their peripheral regions were photographed. Bar = 500 μm.

3.8. Appendix: Effect of the Shh- or Wnt-3-Expressing Feeder Cells on the Keratinocytes

1. As shown in **Fig. 4**, using the Shh expressing feeder cells is effective in accelerating the growth of keratinocytes. Microscopic observation revealed that the keratinocyte colonies, consisting of small sized cells, are rich in coculture with Shh expressing feeders (**Fig. 6**).
2. We observed extraordinary shaped keratinocyte colonies (loss of the tight cell-cell adhesion as shown in **Fig. 7**) specifically in the co-culture with Wnt-3 expressing feeders, whereas the effect on the growth of keratinocytes was not obvious (data not shown). These changes might relate to the epithelial–mesenchymal transition, including the loss of cell polarity, and acquisition of elongated cell shape and invasive potential ***(12)***.

We speculated that both Shh and Wnt-3 signal-responsive keratinocytes might represent a minority in the population and are very immature. This must be proved by further analyses.

4. Notes

1. Swiss-3T3 is not a cloned cell line. We must establish good clones as feeder cells if we want to make the genetic background equal for comparative analysis.

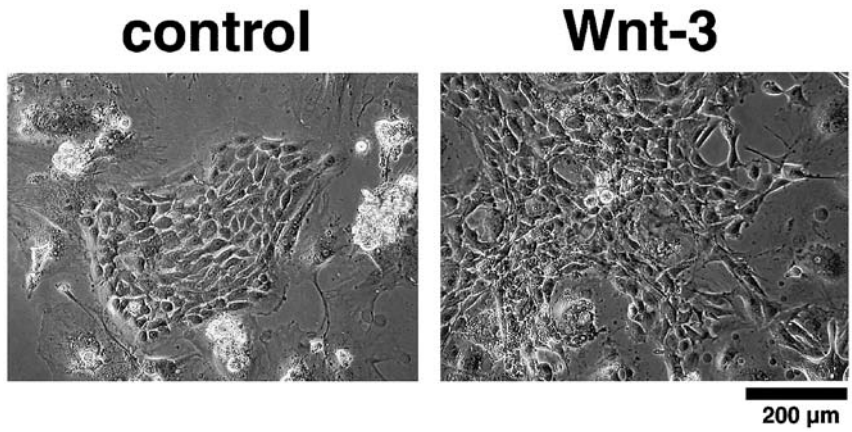


Fig. 7. Effect of the Wnt-3 expressing feeder cells on the shape of keratinocyte colonies (7 d after the inoculation). Loss of the tight cell–cell adhesion was observed in a restricted population only when cocultured with Wnt-3 expressing feeders. Bar = 200 µm.

2. In our experience, too strong a growth stimulation causes cellular senescence or apoptosis paradoxically. We believe that the addition of cholera toxin is useful in attenuating and controlling the strong effect of Shh by inducing PKA activation, which is inhibitory to Shh signaling.
3. Remember to keep a replica culture or freeze a stock!
4. Please coordinate suitable times for the treatment. Too long a treatment causes rapid cell death and too short a treatment is unsuitable to stop the cell growth.
5. The quality of purchased keratinocytes is not stable (including cell viability and differentiation status). This means that we must check the quality and set up proper experimental conditions for each lot.

References

1 Rheinwald, J. G. and Green, H. (1975) Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell* **6,** 331–343.
2 Boyce, S. T. and Ham, R. G. (1983) Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal culture and serum-free serial culture. *J. Invest. Dermatol.* **81,** 33s–40s.
3 Werner, S. (1998) Keratinocyte growth factor: a unique player in epithelial repair processes. *Cytokine Growth Factor Rev.* **9,** 153–165.
4 Yaeger, P. C., Stiles, C. D., and Rollins, B. J. (1991) Human keratinocyte growth-promoting activity on the surface of fibroblasts. *J. Cell Physiol.* **149,** 110–116.
5 Oro, A. E. and Scott, M. P. (1998) Splitting hairs: dissecting roles of signaling systems in epidermal development. *Cell* **95,** 575–578.
6 Callahan, C. A. and Oro, A. E. (2001) Monstrous attempts at adnexogenesis: regulating hair follicle progenitors through Sonic hedgehog signaling. *Curr. Opin. Genet. Dev.* **11,** 541–546.
7 McMahon, A. P. (2000) More surprises in the Hedgehog signaling pathway. *Cell* **100,** 185–188.
8 Bale, A. E. and Yu, K.-P. (2001) The hedgehog pathway and basal cell carcinomas. *Hum. Mol. Genet.* **10,** 757–762.
9 Moon, R. T., Bowerman, B., Boutros, M., and Perrimon, N. (2002) The promise and perils of Wnt signaling through beta-catenin. *Science* **296,** 1644–1646.

10 Fuchs, E., Merrill, B. J., Jamora, C., and DasGupta, R. (2001) At the roots of a never-ending cycle. *Dev. Cell.* **1,** 13–25.

11 Kameda, T., Hatakeyama, S., Terada, K., and Sugiyama, T. (2001) Acceleration of the formation of cultured epithelium using the Sonic hedgehog expressing feeder cells. *Tissue Eng.* **7,** 545–555.

12. Savagner, P. (2001) Leaving the neighborhood: molecular mechanisms involved during epithelial-mesenchymal transition. *Bioessays* **23,** 912–923.

5

Organ Culture of Developing Mouse Skin and Its Application for Molecular Mechanistic Studies of Morphogenesis

Mariko Kashiwagi and Nam-ho Huh

Summary

In this chapter, we describe an organ culture method that allows skin tissues isolated from 12.5- or 13.5-d postcoitus mouse embryos to develop in a manner histologically and temporally similar to the process in vivo. In this culture system, epidermal growth factor specifically and completely inhibited the hair follicle formation with marginal effects on interfollicular epidermis. Using an adenovirus vector, one can transduce a gene into the epidermal and dermal cell layers without appreciable toxicity.

Key Words:

Organ culture; skin; development; adenovirus.

1. Introduction

Studies to elucidate molecular mechanisms of morphogenesis in mammalian systems are hampered by the limited accessibility to intrauterine embryos. One of the most practical and fertile approaches to overcome this difficulty is organ culture. Various organs, including lung ***(1)***, teeth ***(2)***, kidney ***(3)***, and mammary gland ***(4)***, have been shown to develop in various organ culture systems in a similar way as in vivo.

In mouse embryos, the epidermis is composed of simple layers of epithelial cells and periderm at 13.5 dpc (days postcoitus). On 14.5 dpc, hair buds, rudiments of hair follicles, appear as local thickenings of the epidermis surrounded by condensed mesenchymal cells ***(5)***. Once formed, hair buds continuously elongate and penetrate into the dermal tissue. The interfollicular epidermis becomes stratified and progressively differentiates to form the keratinized layers, eventually sloughing off from the surface by 16.5 dpc. Thus, development of embryonic mouse skin from 13.5 to 16.5 dpc includes the principal morphogenic events, that is, pattern formation, determination of cell fate, control of growth, differentiation, and apoptosis of a defined subpopulation of cells, as well as tissue remodeling.

Here we describe how to cultivate embryonic mouse skin at 12.5 or 13.5 dpc while permitting development in a similar way as in vivo for approx 3–4 d ***(6)***. In this system, it is possible to manipulate the morphogenic processes, thus enabling molecular mechanistic studies. Transduction of genes to the cultured skin is also described.

From: *Methods in Molecular Biology, vol. 289:*
Epidermal Cells: Methods and Protocols
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

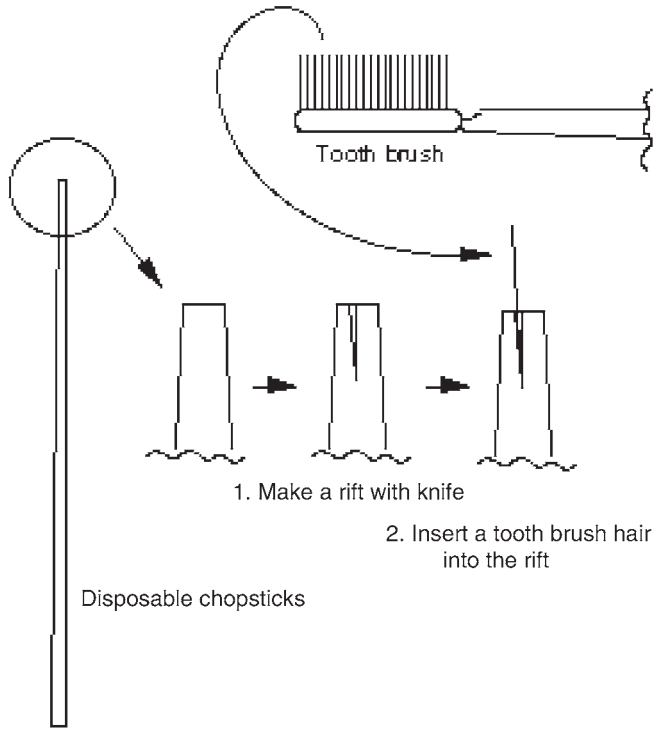


Fig. 1. Preparation of hair-sticks from chopsticks and a toothbrush.

2. Materials

2.1. Dissection and Handling of Embryonic Mouse Skin

1. Pregnant mice with defined gestational days (12.5 or 13.5 dpc).
2. A dissecting microscope (Carl-Zeiss, Stemi 2000-C; or equivalents) and a dissecting plate.
3. Dissecting instruments: fine forceps (can be purchased from your local suppliers); scissors (one of normal size and one of a very small size that is designed for operating on the cornea with a blade length of approx 5 mm; can be purchased from your local suppliers); and "hair-sticks:" Hand-made tissue-handling instruments (*see* **Fig. 1** and **Note 1**).
4. Phosphate-buffered saline (PBS) (Ca^{2+}- and Mg^{2+}-free): 8.00 g NaCl; 1.15 g Na_2HPO_4; 0.20 g KCl; and 0.20 g KH_2PO_4. Dissolve in water and adjust up to 1000 mL.
5. Dulbecco's MEM (DMEM; Nissui Pharmaceutical Co., Tokyo, 05919) with 1% fetal bovine serum (FBS). Prepare DMEM according to the manufacturer's instructions and mix 99 vol of DMEM with 1 vol of FBS.
6. Plastic dishes (100 mm, 35 mm; Corning or any equivalent companies).

2.2. Culture

1. Culture medium: DMEM with 10% FBS and 50 µg/mL kanamycin (*see* **Note 2**).
2. Nuclepore polycarbonate filters (pore size, 8 µm; Structure Probe Inc., F8013-MB).
3. Collagen (type IC, 3.0 mg/mL, pH 3.0; Nitta Gelatin, Osaka, Japan; *see* **Note 3**).
4. Plastic dishes (35 mm, 60 mm).
5. A CO_2 incubator (5% CO_2, 37°C).

2.3. Materials Needed for Other Purposes

1. 4% paraformaldehyde in PBS: 8.0 g paraformaldehyde and 100 m L water. Dissolve by warming up to 65°C and by adding a few drops of 1 *N* NaOH. Cool down and add an equal volume of 2× PBS.
2. Ethanol (75%, 95%, 100%).
3. Xylene.
4. Paraffin (Histprep 568, Wako, Osaka, Japan, cat. no. 415-25791).
5. Instruments for making tissue sections, for example, a microtome with a blade; slide glasses; and a tissue section spreader.
6. Hematoxilin and eosin solutions. Hematoxilin solution can be obtained from Merck (Darmstadt, Germany; cat. no. 1.09249.0500). Eosin solution: 1 g eosin Y (Merck; cat. no. 1.15935.0025) and 100 mL water. Dissolve and add 200 µL of acetic acid.
7. 10 m*M* ethylene diamine tetraacetate (EDTA) in PBS: 0.372 g EDTA/2 Na/2 H_2O (mw 372.24); 100 mL PBS. Dissolve by adjusting the pH at about 8.0 with 1 *N* NaOH.
8. An incubator with a shaking platform at 37°C (Eyela, Tokyo, MHS-2000).
9. Cell Proliferation Kit (Amersham Biosciences, RPN20).
10. An adenovirus vector construct (e.g., adenovirus vector carrying lacZ under the CAG promoter; *see* **ref. *7***).

3. Methods

3.1. Organ Culture of Embryonic Mouse Skin

3.1.1. Coating the Filters With Collagen

1. Autoclave Nuclepore filters in advance.
2. Put the filters with the shiny side up in a plastic dish without overlap under a sterile condition.
3. Apply the collagen solution (3.0 mg/mL) to cover the filters and suck off the solution immediately.
4. Air-dry until no liquid remains on the filters.
5. The coated filters can be stored at room temperature for at least a few days.

3.1.2. Removing Embryos From Pregnant Mice

1. Sacrifice pregnant mice at 12.5 or 13.5 dpc by cervical dislocation under ether anesthesia.
2. Open the abdomen, hold the uterine stem with forceps, and cut it. Cut the connecting membranous tissues while lifting up the uterus and transfer it into a dish containing PBS.
3. Cut the muscle layer of uterus and release embryos keeping the chorioamniotic membrane intact.
4. Under a dissecting microscope, open the chorioaminiotic membrane, cut the umbilical cord, and transfer the embryos into DMEM with 1% FBS on ice. Don't pinch the embryos with forceps but scoop them to avoid damaging tissues (*see* **Note 4**).

3.1.3. Cutting Out Skin Tissues Under a Dissecting Microscope

1. Transfer an embryo into a 35-mm dish with DMEM with 1% FBS. (The amount of medium should be at a level narrowly covering the embryo so that the body does not move freely.)
2. Cut the back skin with the small scissors along a line shown in **Fig. 2**.
3. Carefully tear the skin tissue away from the muscle layer by gentle scraping with the blade of a small scissors. Avoid breaking the skin tissue (*see* **Note 5**).
4. Keep the skin tissues in the medium on ice.

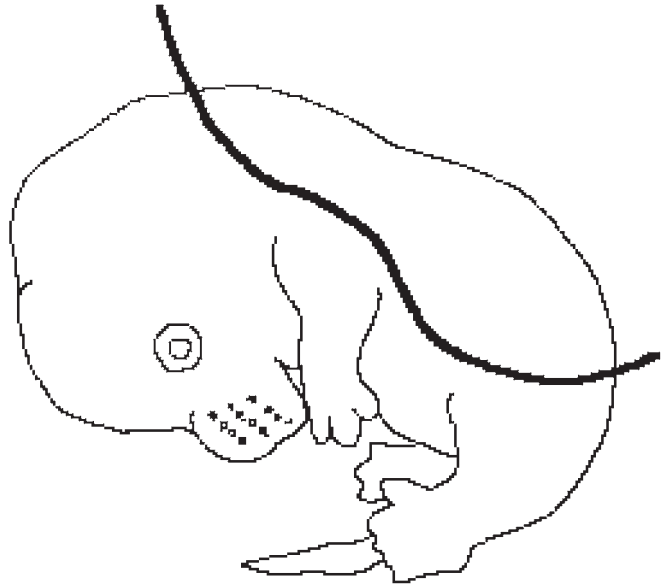


Fig. 2. Cutting line in a mouse embryo.

3.1.4. Spreading the Skin Tissues and Cultivation

1. Transfer the skin tissue onto the collagen-coated dried filter with a small amount of DMEM with 10% FBS (~100 µL).
2. Put the skin tissue with dermal-side up **(Fig. 3)** and spread with the hair-sticks.
3. Turn the skin tissues to epidermal-side up and spread with the hair-sticks.
4. During the spreading procedure, aspirate the medium little by little to facilitate the spreading. Avoid the tissues being dried up. Finally, leave the amount of medium narrowly covering the skin tissue.
5. Transfer the filter with skin tissue onto 2 mL DMEM with 10% FBS in a 35-mm dish **(Fig. 4)**.
6. Incubate the dish allowing the filter to freely float on the medium to facilitate gas- and medium-exchange (*see* **Note 6**).

3.2. Morphological Analysis of the Cultured Skin Tissues

3.2.1. Preparation of Tissue Sections and Staining With Hematoxilin and Eosin

1. Wash the skin tissues cultured on filter once with PBS.
2. Fix the tissues in 4% paraformaldehyde in PBS for 2 h at 4°C. Cut the tissues with filter into two pieces.
3. Dehydrate the tissues via the following:
   a. Dip the tissues in increasing concentration of ethanol (70%, 80%, 90%, 95%, 100% [twice] each for >1 h).
   b. Dip the tissues in 100% ethanol overnight.
   c. Dip the tissues twice in xylene for >2 h.
   d. Dip the tissues in xylene overnight.
   e. Dip the tissues twice in paraffin at for >2 h.
   f. Dip the tissues in paraffin overnight.
4. Embed the tissues in a vertical position in paraffin.

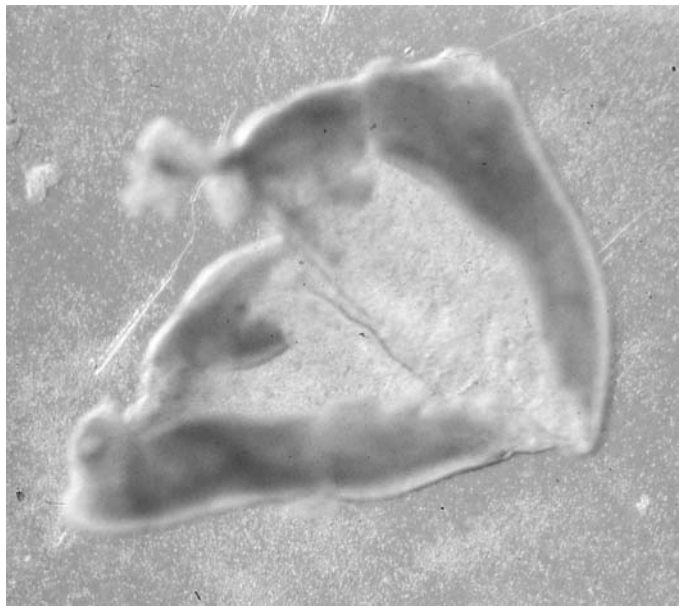


Fig. 3. A skin tissue with the dermal side up, cut out from a mouse embryo at 13.5 dpc before spreading.

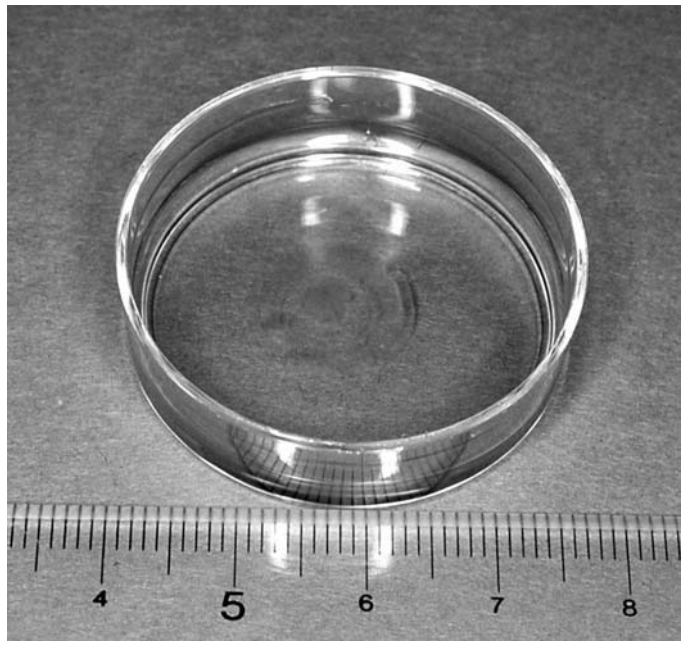


Fig. 4. Organ culture of embryonic mouse skin on a floating Nuclepore filter.

5. Cut the tissues into with 4-µm thick sections.
6. Deparaffinize the sections by 1) dipping the sections in two series of xylene each for approx 3–5 min; 2) dipping the sections in two series of 100% ethanol each for approx 3–5 min; and 3) dipping the sections in 75% ethanol for approx 3–5 min.

7. Stain the sections with hematoxilin and eosin under the conventional conditions:
   a. Wash the sections with tap water for approx 2–4 min.
   b. Dip the sections in the Mayer's hematoxilin solution for approx 3–5 min.
   c. Wash the sections with tap water for approx 10–20 min.
   d. Dip the sections in the eosin solution for approx 2–4 min.
   e. Wash the sections with tap water for a few seconds.
   f. Dehydrate the sections by passing serially through 75% ethanol, 95% ethanol, 100% ethanol twice, and xylene twice.
   g. Mount the tissue sections in Entellan (Merck; cat. no. 940389088).

3.2.2. Separation of Epidermis From Dermis

1. Incubate the skin tissues in 10 m*M* EDTA in PBS at 37°C for approx 10–30 min with gentle shaking (*see* **Note 7**).
2. Separate epidermis from dermis using hair-stick under a dissecting microscopy.
3. Observe the epidermis after spreading or process the individual tissues for biochemical analysis.

3.3. Miscellaneous Techniques

3.3.1. Monitoring Growth of Cells (see ***Note 8***)

1. Cultivate mouse embryonic skin tissues as described in **Subheading 3.1.**
2. Replace the medium containing 10 n*M* 5-bromo-2′-deoxyuridine (BrdU) and 1 n*M* 5-fluoro-2′-deoxyuridine 1 h prior to harvesting tissues.
3. Process the skin tissues as described in **Subheading 3.2.1.**
4. Detect BrdU incorporated into DNA using Cell Proliferation Kit (Amersham).

3.3.2. Specific Inhibition of Hair Germ Formation by Epidermal Growth Factor (EGF; see ***Note 9***)

1. Prepare and spread mouse embryonic skin tissues as described in **Subheading 3.1.**
2. Replace the medium covering the skin tissues with that containing 30 ng/mL EGF.
3. Put the skin tissues with the filter onto the culture medium containing 30 ng/mL EGF.
4. Observe the morphology after making tissue sections as described in **Subheading 3.2.1.** or after separating epidermis as described in **Subheading 3.2.2.** (*see* **ref. *6***).

3.3.3. Transduction of Genes into Skin Tissues by an Adenovirus Vector ***(8)***

1. Prepare, partially purify, and titrate an Adenovirus vector construct containing desired cDNA according to Kanegae et al. ***(9)***.
2. Incubate the skin tissues freshly isolated and spread on Nuclepore filter floating on 1 mL of the culture medium containing 8×10^8 pfu of the Adnenovirus vector. Exchange the medium covering skin tissues with the bulk medium once an hour. (Incubation in the solution containing lower amount of Adenovirus results in remarkable lower infection efficiency.)
3. Transfer the skin tissues into fresh culture medium 6 h after the onset of incubation. (Incubation for approx 1–3 h and for 12–24 h results in lower infection efficiency and appreciable toxicity, respectively; *see* **Note 10**).

4. Notes

1. Hair-sticks: toothbrushes with different stiffness of hair are commercially available. We recommend to prepare hair-sticks with different degrees of stiffness. Hair-sticks with softer hair are suitable for handling finer tissues. The hair-sticks are very helpful to handle any delicate tissues without damaging tissues.

2. When handling mouse embryos and the skin tissues, it is important not to pinch them with forceps but scoop them to avoid damaging tissues. Damage to the skin tissues retards the growth and development of the part surrounding the wound.
3. Difficulty in tearing skin tissues from the muscle layer depends on the development of loose subcutaneous tissues. The tearing is difficult at early 12.5 dpc but becomes easy at late 12.5 dpc.
4. The collagen solution, type IC (Nitta gelatin), is prepared from porcine skin and suitable for coating because of its low viscosity. Any equivalent products can be used for coating the membranes.
5. We usually use DMEM with 10% FBS, but one may reduce the concentration of FBS to 1% without any significant effect.
6. The skin tissues follow the developmental process similar to that in vivo for approx 3–4 d. The longer cultivation results in gradual decomposition of the tissues.
7. During the cultivation, growing cells are found only in the basal layer of epidermis and hair germs.
8. By incubating with 30 ng/mL EGF, formation of hair germs is completely inhibited whereas development of the epidermis is marginally affected ***(6)***.
9. The time necessary for dissociating epidermis from dermis with EDTA depends on tissue origins. Cultured tissues and tissues of younger embryos are easy to dissociate whereas tissues from embryos at 16.5 or 17.5 dpc need longer treatment.
10. Efficiency of gene transduction by Adenovirus vector is practically 100% under the present conditions. When epidermis is stratified and keratinized, the efficiency drops dramatically.

References

1 Schuger, L., Varani, J., Mitra, R., and Gilbride, K. (1993) Retinoic acid stimulates mouse lung development by a mechanism involving epithelial-mesenchymal interaction and regulation of epidermal growth factor receptors. *Dev. Biol.* **159,** 462–473.
2 Chai, Y., Mah, A., Crohin, C., Groff, S., Bringas, P., Le, T., et al. (1994) Specific transforming growth factor-beta subtypes regulate embryonic mouse Meckel's cartilage and tooth development. *Dev. Biol.* **162,** 85–103.
3 Weller, A., Sorokin, L., Illgen, E. M., and Ekblom, P. (1991) Development and growth of mouse embryonic kidney in organ culture and modulation of development by soluble growth factor. *Dev. Biol.* **144,** 248–261.
4 Coleman, S. and Daniel, C. W. (1990) Inhibition of mouse mammary ductal morphogenesis and down-regulation of the EGF receptor by epidermal growth factor. *Dev. Biol.* **137,** 425–433.
5. Sengel, P. (1976) *Morphogenesis of Skin.* Cambridge University Press, Cambridge.
6 Kashiwagi, M., Kuroki, T., and Huh, N. (1997) Specific inhibition of hair follicle formation by epidermal growth factor in an organ culture of developing mouse skin. *Dev. Biol.* **189,** 22–32.
7. Kanegae, Y., Lee, G., Sato, Y., Tanaka, M., Nakai, M., Sakaki, T., et al. (1995) Efficient gene activation in mammalian cells by using recombinant adenovirus expressing site-specific Cre recombinase. *Nucl. Acid Res.* **23,** 3816–3821.
8. Kuroki,T., Kashiwagi,M., Ishino, K., Huh, N., and Ohba, M. (1999) Adenovirus-mediated gene transfer to keratinocytes-review. *J. Invest. Dermatol. Symp. Proc.* **4,** 153–157.
9. Kanegae, Y., Makimura, M., and Saito, I. (1994) A simple and efficient method for purification of infectious recombinant adenovirus. *Jpn. J. Med. Sci. Biol.* **47,** 157–166.

6

Experimental Models to Analyze Differentiation Functions of Cultured Keratinocytes In Vitro and In Vivo

Nicole Maas-Szabowski, Norbert E. Fusenig, and Hans-Jürgen Stark

Summary

In this chapter, we present technical details for the generation of in vitro skin equivalents consisting of collagen gels with incorporated fibroblasts covered by proliferating and differentiating keratinocytes. Epithelial–mesenchymal interactions are clearly manifest in these skin equivalents. Therefore, they have proven to be suitable experimental tools for a broad range of applications, e.g., for studies on the the paracrine regulation of keratinocyte differentiation and proliferation. On the other hand, in vivo assays cannot be abandoned totally, in particular, when such properties as malignant growth potential, disturbed differentiation control in carcinogenesis, and impact on angiogenesis are concerned. For that reason, we additionally describe xenotransplantation techniques to graft human keratinocytes and skin equivalents, respectively, onto the dorsal muscle fascia of thymus-aplastic mice.

Key Words:

Organotypic cocultures; skin equivalents; keratinocyte differentiation; epithelial–mesenchymal interaction; surface transplantation assay; tumorigenicity test.

1. Introduction

The development of an orderly structured and well-organized squamous epithelium such as skin epidermis strictly depends on epithelial cell interactions with its connective tissue. The formation and maintenance of the mature epidermis consisting of basal, spinous, granular, and cornified strata, which develops in a continuous process involving keratinocyte proliferation and terminal differentiation, is regulated by mesenchymal influences (reviewed in **ref.** ***1***). Cell cultures are simplified experimental tools to study regulating factors and mechanisms controling the biology of cells. However, because of the reduced complexity of cell cultures, most cell types, including keratinocytes, merely show truncated versions of their intrinsic differentiation potentials in vitro. For example, keratinocytes cultivated in monolayers never achieve the state of terminal differentiation as they do in vivo, even if cocultured with fibroblasts.

Nevertheless, in coculture with mesenchymal cells, isolated and cultured epithelial cells are responsive to appropriate extracellular regulatory stimuli, thus realizing their intrinsic potential for tissue specific differentiation ***(2–4)***. Thus, in vitro model systems are appropriate tools to study epithelial–mesenchymal interactions and to identify regulating factors.

From: *Methods in Molecular Biology, vol. 289:*
Epidermal Cells: Methods and Protocols
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

Since the pioneering work of Rheinwald and Green ***(5)***, the normal procedure to expand primary keratinocytes is the feeder-layer-coculture method, whereby keratinocytes grow in coculture with postmitotic fibroblasts (feeder cells) that are submerged in culture medium, yielding a highly proliferative epithelial cell layer, however, without normal stratification and differentiation.

In contrast, three-dimensional in vitro systems or in vivo transplantation assays are required to enable keratinocytes to develop well-ordered epithelia that allow the analysis of the differentiation process and its regulation. As subsequently outlined, these organotypic cocultures can be composed of normal epithelial and mesenchymal cells, neoplastically transformed epithelial cells, immortalized cell lines, combinations of both, or even combinations of cells from different species (human/mouse). The use of such organotypic cocultures remarkably expands the options to study the influence of locally and systemically acting factors, to characterize the altered growth and differentiation features of transformed cells, and to understand the characteristic behavior of malignant cells, such as loss of histotypic architecture, tumor formation, and invasion. Actually, many aspects of epidermal biology depending on regular keratinocyte differentiation have already been investigated in this setting, for example, the re-epithelialization during wound healing, the regulation of human papilloma viruses, and the development of the stratum corneum barrier ***(6–8)***.

Despite growing legal constraints and decreasing public acceptance, investigations exploiting living organisms can not be abandoned totally, for example, in cancer research. Although epithelial–mesenchymal interactions are operative in in vitro model systems, the biological significance and in vivo relevance of the effects observed in vitro should always be confirmed under more physiologic, that is, in vivo conditions. Beside the examination of altered gene function in knockout mice or transgenic mice (*see* **ref. *9***), the surface transplantation assay is considered the most reliable test to identify normal epithelial physiology ***(10,11)*** as well as alterations in growth and differentiation associated with malignant transformation ***(9,12)***.

2. Materials

2.1. Preparation of Organotypic Cultures

2.1.1. Collagen Preparations

1. Type I collagen, in house isolated from rat tail tendons (*see* **Note 1**). Below, some alternative commercial sources of native type I-collagen are listed:
2. Vitrogen-100, bovine dermal type I-collagen (Collagen Corp., Palo Alto, CA; cat. no. FXP-019, *see* **refs. *13,14***).
3. Type I collagen, rat tail tendons (Sigma, St. Louis, MO; cat. no. C-7661).
4. Type I collagen, calf skin (IBFB Pharma GmbH, Leipzig, Germany; cat. designation ‘collagen type I’, *see* **ref. *15***).

2.1.2. Membrane Filter Holders and Labware for Organotypic Cultures

1. Falcon cell culture inserts: cat. no. 3102 (1.0-µm pores) or cat. no. 3091 (3.0-µm pores) (BD Biosciences, Bedford, MA).
2. BioCoat sixfold Deep Well Plate (BD Biosciences; cat. no. 5467). Alternative cell culture filter inserts are listed under **steps 3–5**.
3. Millicell filter holder inserts (30 mm) with mixed cellulose ester membranes (0.4-µm pores; Millipore, Billerica, MA; cat. no. PICM 030 50, *see* **ref. *13***).

4. Six-well-Transwell permeable supports with polycarbonate membranes (pore size: 0.4 µm, cat. no. 3412, or 3.0 µm, cat. no. 3414, Corning-Costar, Acton, MA).
5. Nunc cell culture filter inserts, 25 mm, with 3.0-µm pores polycarbonate membranes (Nalge Nunc International, Rochester, NY; cat. no. 137435).
6. Glass rings, 20-mm external diameter, 18-mm internal diameter, 12-mm height.
7. Fine curved forceps (Dumont type, no. 5).

2.1.3. Solutions and Culture Media

1. 10X Hank's salt solution with 8 mg/mL phenol red (Biochrom, Berlin, Germany; cat. no. L 2025).
2. Fetal bovine serum (FBS), tested for mycoplasma (Biochrom, cat. no. S 0115).
3. Collagen gel mixture: for 20 mL, combine 16 mL of 4 mg/mL collagen stock solution, 2 mL 10X Hank's salt solution containing phenol red, and 2 mL FBS with or without fibroblasts (*see* **Subheading 3.1.1.**).
4. Culture medium (for organotypic cocultures with normal human keratinocytes): Mixture of three parts Dulbecco's modification of Eagle's medium and one part Ham's F12 (Biochrom; cat. nos. FG 0435 and FG 4815) supplemented with 10% FBS, penicillin/streptomycin (100 U/mL/100 µg/mL, Biochrom: cat. no. A 2213), 0.5 m*M* Na–pyruvate (Biochrom; cat. no. L 0473), 10^{-10} *M* cholera toxin (Sigma; cat. no. C 3012), 0.4 mg/mL hydrocortizone (Sigma; cat. no. H 0135), and 50 µg/mL L-ascorbic acid (Sigma; cat. no. A 4034, *see* **Note 2**).

2.2. Transplantation Assays

1. Thymus-aplastic Swiss-nude mice (nu/nu), females, not younger than 5–6 wk, with a body weight of 22 to 26 g, to be kept under clean-room conditions (*see* **Note 3**).
2. Transplantation chamber made of silicone with a dome-shaped central part and a broad thin brim (Renner KG, Dannstadt, Germany, type F2U; cat. no. 30268; *see* **Note 4**).
3. Combi Ring Dish (CRD) culture chamber consisting of pairs of concentric Teflon rings of different diameters for mounting extracellular matrices between the tightly fitting Teflon rings (Renner KG, inner ring: type b; cat. no. 30907, outer ring: type c; cat. no. 30910, *see* **Note 4**).
4. Polypropylene film (20-µm thickness, Renner KG; cat. no. 30274 A, *see* **Note 4**).
5. 'Stanzen' Petri dishes, 35-mm tissue culture Petri dishes with four (8-mm diameter) rings with a height of 1 mm on the culture surface of the dish (Renner KG; cat. no. 30272, *see* **Note 4**).
6. Sterile disposable cannulae, gauge sizes 2 and 3/4.
7. To anaesthetize mice: Ketavet (Ketamin hydrochloride, Parke-Davis, Berlin, Germany, and Rompun (Xylazin hydrochloride, Bayer-Vital, Leverkusen, Germany).
8. Surgical wound clips, 14 mm (Diener GmbH, Tuttlingen, Germany; cat. no. 845.14).

2.3. Analytical Reagents

1. For histological fixation: 37% formaldehyde diluted 1 : 10 in phosphate-buffered saline (PBS), pH 7.4.
2. For embedding of cryospecimens: Tissue Tek OCT-compound (Lab Tec. Prods., Naperville, IL; cat. no. 4583).
3. Bromodeoxyuridine and bromodeoxycytidine (ICN Biomedicals Inc., Irvine, CA; cat. nos. 100171 and 100166, respectively).
4. Anti-BrdU-antibodies: Clone Bu5.1 (working dilution 1:20 in PBS, Progen, Heidelberg, Germany; cat. no. 61015) and polyclonal sheep BrdU-antiserum (working dilution 1 : 200 in PBS, Exalpha Biologicals, Boston, MA; cat. no. A 205 P).

5. Fluorescent DNA stains: DAPI (diamidino phenylindole, working concentration 0.5 µg/mL, Molecular Probes, Eugene, OR; cat. no. D-1306) and Hoechst 33258 (bis-benzimide, working concentration 2.5 µg/mL, Molecular Probes; cat. no. H-1398).
6. Monoclonal antibody against Ki-67 nuclear antigen (clone MIB-1, DAKO A/S, Glenstrup, Denmark; cat. no. M 7240).

3. Methods

3.1. Organotypic Cocultures of Epithelial and Mesenchymal Cells

To study the molecular mechanism of epithelial–mesenchymal interactions suitable in vivo like organotypic culture models have been developed *(16,17)*. In such organotypic cultures, human epidermal keratinocytes grow air-exposed in special filter inserts on a matrix of collagen type I, (e.g., isolated from rat or bovine tendons, skin or placenta; **Fig. 1**). Alternatively, dermal equivalents were reconstituted from collagen type IV, matrigel, soft agar, or mixtures of collagen and glycosaminoglycan *(18–21)*. To become functional dermal equivalents, the collagen gels contain dermal fibroblasts, which may proliferate in the collagen gel and eventually reorganize this matrix by producing extracellular matrix components comparable to the wound situation *(3,4,22,23)*. In these organotypic models, the contact of the cultures with medium is restricted to the bottom of the gels so that fibroblasts and keratinocytes are nourished by diffusion from below.

However, while contracting the gels to a densely structured lattice with oriented collagen fibrils, the fibroblasts reduce their proliferative activity, alter their protein synthesis and acquire a resting state comparable to dermis *(22)*. By increasing the collagen concentration (to about 4 mg/mL) fibroblast contraction can be prevented without reduction of epithelial support function. To standardize further the fibroblast compartment in the organotypic culture system, fibroblasts were rendered permanently postmitotic by high-dose X-irradiation because those were shown to be still functionally active, as far as cellular integrity and longevity, expression and secretion of growth factors and cytokines, as well as the response to specific inducers are concerned *(3,23–27)*.

When plated on the upper surface of collagen gels containing embedded fibroblasts epithelial cells rapidly attach and form confluent layers within 1–2 d. Subsequently, keratinocytes proliferate, reconstituting an epithelial tissue architecture resembling the epidermis and expressing characteristic epidermal differentiation markers *(1,2,28)*. In the absence of fibroblasts, only thin epithelia arise with rapid loss of proliferation and incomplete differentiation *(1,4,23)*.

In these organotypic cocultures mimicking the in vivo situation of mesenchymal and epithelial cells, the function of diffusible factors mediating epithelial–mesenchymal interactions and the cooperation of keratinocytes and fibroblasts in basement membrane formation has been demonstrated *(4,29)*.

3.1.1. Production of Dermal Equivalents

1. To prepare 50 mL of a collagen stock solution with 4 mg/mL, 200 mg lyophilized type I-collagen is resolubilized in 50 mL of 0.1% acetic acid under gentle stirring in the cold and stored at 4°C.
2. Before preparing the gel mixture, all components are chilled on ice. Eight parts of collagen solution are mixed with one part 10X Hanks buffer containing phenol red and adjusted to pH 7.4 by adding dropwise 2 *N* NaOH while continuously but slowly stirring on ice. An

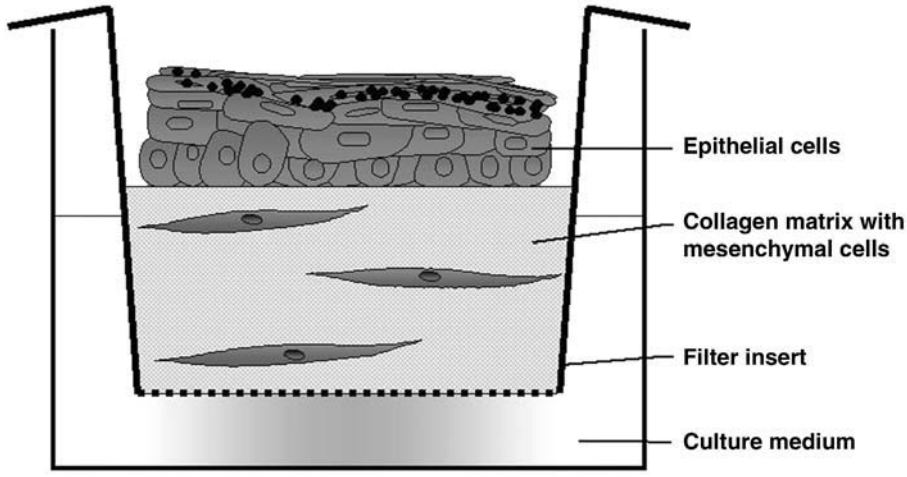


Fig. 1. Schematic illustration of skin equivalents in the organotypic culture system. Keratinocytes grow exposed to air on a fibroblast containing collagen gel nourished by medium from underneath.

orange–red color indicates that the gel solution has been titrated to pH 7.4, what usually requires addition of about 80 µL of 2 *N* NaOH per 12 mL of collagen solution.

3. The fibroblast number necessary for the desired cell density in the gel (1×10^5–1×10^6 per mL) is resuspended in the FBS portion (one part of total volume) and mixed with the gel solution on ice under cautious stirring. In the case of cell free gels, just FBS (10% of total volume) is added.
4. With cooled pipets, 2.5-mL aliquots of the complete collagen gel mixture solution (final collagen concentration about 3.2 mg/mL, *see* **Subheading 2.1.3., step 3**) are dispensed into Falcon filter inserts (six-well filters; cat. no. 3091; *see* **Subheading 2.1.2., step 1**; **Fig. 1**).
5. For gelation, these filter inserts are placed within a BD-BioCoat sixfold deep well plate (*see* **Subheading 2.1.2., step 2**) and incubated for 1 h at 37°C in a humidified incubator.
6. Glass rings corresponding to the diameter of the filter inserts (*see* **Subheading 2.1.2., step 6**) are placed on the gel and gently pushed down by mild pressure with forceps (*see* **Subheading 2.1.2., step 7**) to compress the meniscus of the gel and to delineate a horizontal area for seeding the keratinocytes.
7. Gels with the glass rings are placed for 1 h at 37°C in a humidified incubator.
8. The excess liquid on top of the gels is gently aspirated without touching the gel or the glass ring.
9. Thereafter, the gels are equilibrated while completely submerged in culture medium for 24 h.

3.1.2. Application of the Epithelial Cells

1. Epithelial cells, for example, human skin keratinocytes, (1×10^6 in 1 mL of medium for 2.5-cm inserts) are plated inside the inner ring. Freshly isolated keratinocytes are usually taken in secondary cultures as later passages are less suited. They attach within 12–24 h and form a nearly confluent layer on top of the collagen gel.
2. 24–30 h after seeding, the keratinocyte cell layer is carefully rinsed with 1 mL of medium, and the glass rings are removed, cautiously avoiding any mechanical distortion of the epithelial cell sheet (*see* **Note 5**).

3. Culture medium is changed, and with a reduced medium volume (10–11 mL each in deep-well plates) the liquid level is lowered to the base of the collagen gels, thus raising the cultures to the air-liquid interphase and restricting nourishment to diffusion from below.

3.1.3. Analysis

In such polarized cultures, the regulation of directional processes such as secretory functions or apical differentiation mechanisms are studied. Furthermore, it is possible to investigate the effects of agents applied either topically (to the upper surface of the epithelial cultures) or systemically (added to the culture medium, contacting first the fibroblasts in the gel and then the basal-layer epithelial cells).

Morphology as the most important qualitative parameter to monitor epidermal tissue reconstitution is evaluated by light and electron microscopy after adequate processing. The spatial expression of differentiation products is detected by immunohistochemistry.

For histological analysis or immunohistochemistry, the organotypic cocultures are fixed in formaldehyde (3.7%), embedded in agar (2%) to prevent dislodgement of the epidermal and dermal compartment during further preparation, and then processed *en bloc* in paraffin or prepared as specimens for cryosectioning by embedding in Tissue Tek-OTC-compound and subsequent snap freezing in liquid nitrogen ***(30)***.

Proliferating cells and mitotic index can be determined by labelling the cultures with the thymidine-analogue 5-bromodeoxyuridine (BrdU) and subsequent detection of its incorporation on paraffin or frozen sections by specific anti-BrdU antibodies (**ref. *31***, *see* **Note 6**). Positive nuclear reaction appears in cells that had been in S-phase during the BrdU exposure time and can be recorded in relation to the total number of nuclei counterstained by a DNA dye, such as DAPI or Hoechst 33258. Alternatively, immunohistochemical monitoring of proliferation without any prelabeling is possible with anti-Ki-67 antibodies specific for nuclear antigens in cycling cells ***(32)***.

By simple mechanical separation of the epithelial and dermal portions of organotypic cocultures with fine forceps both compartments can be analyzed individually without a serious risk of material loss and cross contamination. Protein expression patterns are analyzed on their ribonucleic acid and protein levels according to appropriate molecular methods ***(4,23,30)***.

Conditioned medium can be collected from the organotypic cultures (stored at –80°C) for determination of secreted proteins (extracellular matrix components, growth factors, proteases) by ELISA and Western blot analysis, respectively (*see* **refs. *4,33,34***).

Usually, the formation of a regular tissue architecture is paralleled by normal expression patterns of differentiation products. Therefore, histochemical analysis of frozen or fixed tissue sections is important to demonstrate specific differentiation products and their distribution in the tissue. For this purpose, several useful antibodies to tissue specific differentiation proteins, cell surface structures, desmosomal or junctional proteins or basement membrane components and various others are available (*see* **refs. *2,29***).

3.2. Modifications of Organotypic Cocultures

Depending on the researchers' requirements, the organotypic coculture model allows a broad range of modifications. In this context, different cell types, epithelial as well as mesenchymal ones, can be introduced and combined, variable medium formulations and culture conditions can be exploited, and the matrix composition of the dermal equivalents altered.

Both isologous and heterologous combinations of epithelial and mesenchymal cells from different species have been used. Successful organotypic cocultivation has been reported for a variety of epithelial-derived cells and cell lines, such as mouse skin keratinocytes ***(35,36)***; normal human keratinocytes from interfollicular epidermis ***(10,30)*** and foreskin ***(37)***; hair follicle keratinocytes from outer root sheath ***(38)*** and hair matrix ***(39)***; as well as sqamous cell carcinoma keratinocytes ***(40)***, conjunctival epithelial cells ***(41)***, human ovarian epithelial cells ***(42)***, mucosal keratinocytes ***(43)***, and the spontaneously immortalized keratinocyte line HaCaT ***(11,44,45)*** and some of its different genetically modified variants ***(46,47)***.

Further attractive perspectives for the exploitation of organotypic systems arise from the inclusion of additional cell types such as melanocytes and Langerhans cells into the epithelium ***(48,49)*** and/or incorporation of endothelial cells into the dermal equivalents ***(50)***.

Depending on the experimental purpose, different dermal cell types have been incorporated into the matrix such as mouse 3T3 fibroblasts ***(37,51,52)***, human dermal fibroblasts ***(2,4,10,11,38,53)***, scleroderma fibroblasts ***(54)***, or even tumor-derived fibroblasts from squamous cell carcinoma (SCC) ***(55)***.

Although the majority of cell culture experiments are performed in conventional culture media (e.g., Dulbecco's modified Eagle's medium or other formulations) containing serum and additional supplements (such as insulin, epidermal growth factor, cholera toxin), now more defined culture conditions have become available by the development of serum-free culture media ***(2)***. This represents an important step towards standardized in vitro skin models and their application in pharmacotoxicology.

3.3. Transplantation Models for Surface Epithelia

Still, the optimal approach to evaluate properly the capacity of cultured epithelial cells to differentiate and reorganize into structured epithelia is to transfer them into the organism. For human keratinocytes, the method of choice is the orthotopic transplantation onto the back muscle fascia of nude mice ***(1,10)***. Additionally, this surface transplantation assay allows to check for tumorigenic potential and invasive growth behavior of transformed keratinocytes ***(12,30,56)***.

The particular feature of the technique presented here is the use of dome-shaped silicone transplantation chambers (*see* **Subheading 2.**) to protect the graft from desiccation and isolate it from contiguous host epidermis. In general, two modes of applying keratinocytes into the transplantation chamber are feasible: 1) as single-cell suspension directly onto the host dorsal muscle fascia (**Fig. 2**, left) and 2) as preformed epithelial sheets on an intermediate matrix separating epithelium and mesenchyme (**Fig. 2**, right). Preferably, the latter version provides more controlled conditions of cell attachment and growth causing improved reproducibility of the graft take.

The first step in producing these grafts is to precultivate keratinocytes (typically 2×10^5/graft) on a collagen type I gel layer (1–2 mm thick) prepared as described for organotypic cultures above either with or without incorporated fibroblasts. Two concentric Teflon rings into which the collagen gels are mounted provide higher stability and better handling. This CRD described by Noser and Limat ***(38)*** fits exactly into the silicone transplantation chambers. Keratinocytes are seeded onto the collagen surface and incubated submerged for 24 to 48 h. These organotypic CRD cultures are then

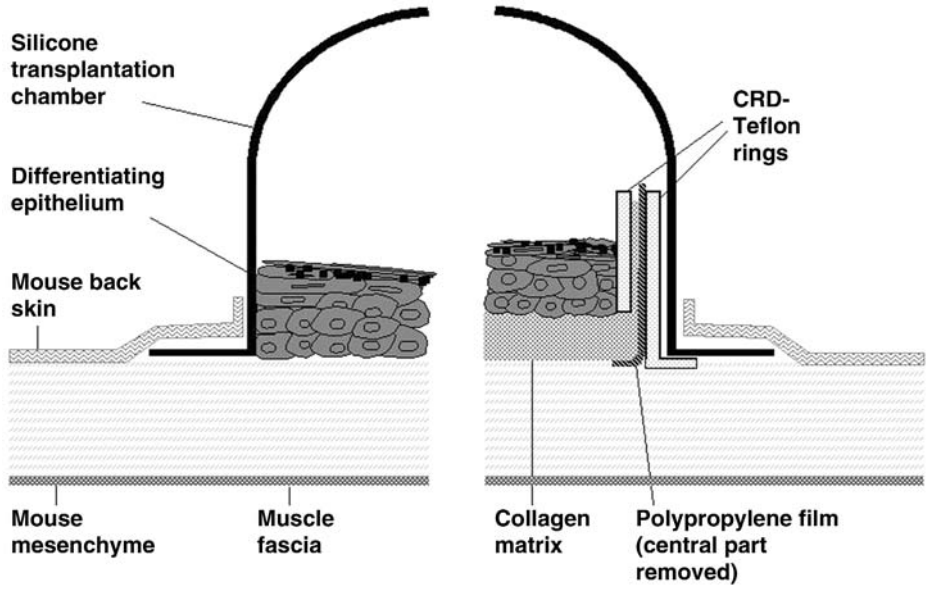


Fig. 2. The surface transplantation system for keratinocytes in schematic cross section. Left, keratinocytes directly inoculated as single cells onto the host mesenchyme. Right, implant of an intact organotypic keratinocyte culture on a collagen gel (mounted in CRD) that separates epithelium and host mesenchyme, illustrating the configuration of all components involved.

covered with the silicone chamber and subsequently implanted onto the dorsal muscle fascia of nude mice (for details, *see* **Subheading 3.3.2.**).

The advantages of this grafting assay for cultured cells are as follows:

1. A low cell number is required ($1–2 \times 10^5$ per graft) for a high take rate (95–100%).
2. Cell losses from preformed keratinocyte sheets are minimized in comparison to injected cell suspensions.
3. Despite histocompatibility barriers, allogenic transplants remain vital for at least 4 wk. Obviously, the low number of allogenic cells and the virtual absence of a larger number of dead cells that might act as immunogens avoid induction of early rejection ***(10,56,57)***.
4. Because of the protective containment, host keratinocytes are prevented from overgrowing the graft that, in turn, can be easily recognized and dissected *en bloc*.
5. As important advantage, early mesenchymal reactions (e.g., granulation tissue formation, angiogenesis) can be studied in detail because of the interposed collagen gel.
6. Lastly, the stable geometry typical for a surface epithelium is maintained in these surface transplants. With an identical configuration as established in organotypic cocultures in vitro, epithelial–mesenchymal interactions can be analyzed and directly compared.

3.3.1. Transplantation of Cell Suspensions

1. The silicon transplantation chamber (without CRD) is implanted onto the mouse back muscle fascia under aseptic conditions. Cells are inoculated directly into the chamber. Usually 1×10^5 to 2×10^6 cells in 100-µL medium are applied by syringe with a needle (gauge size 2) pricked through the roof of the silicon chamber, while a second needle is inserted to avoid overpressure (**Fig. 2**, left).

2. After gently pulling out both needles, the mice have to be kept anaesthetized and carefully immobilized for about half an hour so that the chambers can stay horizontally for cell attachment to the muscle fascia and to prevent cell outflow under the rim of the chambers. This could lead to irregular cell distribution or failure of take.

3.3.2. Transplantation of Epithelial Cells on a Collagen Matrix

Steps 1–7 of the following procedure require sterile conditions and should be performed in a laminar air cabinet.

1. To prepare gels of appropriate size to fit into the CRD assembly (16 mm in diameter), collagen gel solution, prepared according to **Subheading 3.1.1.**, is poured into 24-well plates, 500 µL per well. Gelation requires 1 h at 37°C in a humidified incubator. Subsequently, the gels are covered with culture medium for equilibration.
2. Gels are taken out of the wells and placed on a piece of polypropylene film (3 × 3 cm, sterilized in 80% ethanol) with a central circular perforation corresponding to the internal diameter of the inner Teflon ring. This important auxiliary tool for mounting secures the gel from being ruptured while being mounted in between the concentric Teflon rings of the CRD. Gel and polypropylene film are carefully centered above the outer ring of the CRD.
3. Next, the inner ring is placed centrally on top of the collagen gel and pushed gently together with the gel on the film into the outer ring, so that the gel now stretches over the lower open end of the inner ring of the CRD-assembly and its margin is clamped between the rings (*see* **Fig. 2**, right).
4. After the central part of the polypropylene foil has been peeled off (important!) and its overhanging parts protruding from the upper end of the CRD have been clipped off with scissors, the assembled CRDs are transferred into Stanzen Petri dishes providing free medium access from below (*see* **Subheading 2.2., step 5**). The gels mounted in CRDs are then equilibrated with culture medium and incubated at 37°C. At this stage an incubation period of 16–24 h is strongly recommended to check for microbial contamination.
5. Epithelial cells (2×10^5 in 100 µL of medium) are plated onto the collagen gel in the inner ring of the CRD and allowed to attach for 12–24 h.
6. The cells in the CRDs are rinsed, and the medium is aspirated before the CRD is covered with the silicon transplantation chamber fitting tightly the outer Teflon ring. To prevent the risk of damaging the collagen matrix by overpressure inside the chamber and mechanical distortion, a cannula is inserted into the dome-shaped roof of the transplantation chamber which finally is gently pushed down with forceps.
7. For transplantation, nude mice are anaesthetized by intraperitoneal injection of Ketavet (0.1 mg per g/body weight) and Rompun (0.015 mg per g/body weight) before saggital incisions in their back skin are made. The complete CRD transplantation chamber unit is transferred onto the muscle fascia and kept in place by closing the skin above the brim of the silicone chamber and tightly fixing the wound margins with wound clips.
8. At desired time points, transplants are excised *en bloc* and further processed for histology and cryotome sectioning. For BrdU labeling, refer to **Note 6**.

Both methods are suited for rapid reconstitution of epithelial tissue by grafted cells displaying advanced differentiation features ***(35,58)***. Furthermore, this transplantation assay represents a reliable method to discriminate benign (noninvasive) and malignant phenotypes of epithelial cells. Therefore, it is an indispensable tool, primarily in cancer research, to evaluate altered cell regulation in the context of a physiologic environment that, so far, can not be provided in in vitro models (for review, *see* **ref. *56***).

4. Notes

1. Tendons are highly enriched in type I collagen and easily available from rat tails. Therefore, native type I collagen is isolated from this convenient source by cold (4°C) extraction in 0.1% acetic acid. After clarification by centrifugation, the resulting solution is stable for six months when stored at 4°C and for years when frozen or even lyophilized. Because of the high homology, the species origin of a collagen preparation is less critical for its effects on cells; more important is the kind and concentration of the extracting acid, optimally 0.1% acetic acid. The optimal collagen concentration of stock solutions is 4 mg/mL; above 6 mg/mL, they are very viscous and difficult to handle.
2. According to its instability at physiologic pH, L-ascorbic acid has to be added freshly to the cell culture medium.
3. To avoid graft rejections of human cells xenotransplanted onto mice it is essential to use animals that are deficient in their cellular immunity such as athymic nude mice.
4. The exclusive supplier for the surface transplantation equipment described here (silicone chambers, Stanzen Petri dishes, CRD components, polypropylene foil, and the punches to make circular perforations in them) is Renner KG, Riedstr. 6, D-67125 Dannstadt, Germany (http://www.renner-laborbedarf.de).
5. Occasionally, the margins of the keratinocyte layers seeded onto collagen gels stick to the glass rings and have to be detached from them with fine forceps before taking them off.
6. The nucleotide analog BrdU is an excellent tool to demarcate replicating cells. However, like thymidine, BrdU is a negative effector of ribonucleotide-reductase, thus leading to a cellular depletion of deoxycytidine and a blockade of DNA synthesis ***(59)***. This can be prevented by providing the cells additionally with equimolar amounts of deoxycytidine. Moreover, the use of 5-bromodeoxycytidine (BrdC) enhances the sensitivity of the test by further increasing the incorporation of 5-bromodeoxynucleotides into the deoxyribonucleic acid (DNA). A 1000X stock solution of a BrdU/BrdC mixture (65 m*M* each) is prepared by dissolving 500 mg each of BrdU and BrdC in 25 mL of 0.9% NaCl solution at 37°C protected from light. This stock is stored in aliquots frozen at –80°C. Cells in culture are labeled by adding 1 µL of this BrdU/BrdC stock per milliliter of culture medium and incubating for a period of 4–16 h. However, for BrdU-labeling of cells grafted onto mice, the animals are injected intraveneously with 0.1 mL of the BrdU/BrdC-stock 2 h before being sacrificed. For the immunohistochemical detection of incorporated BrdU/BrdC, cell cultures or cryosections of tissues are fixed in 80% methanol at 4°C for 5 min and subsequently in absolute acetone at –20°C for 2 min before the DNA is denatured by incubation in 2 *M* hydrochloric acid at 20°C for 8 min. After thorough washing in PBS pH 7.4 (three changes), the anti BrdU-antibody dilution is applied. Immunofluorescence detection offers the opportunity to easily counterstain nuclear DNA with DAPI or Hoechst 33258. For that, these dyes are included at the concentrations indicated (*see* **Subheading 2.3.5.**) in the incubation with the secondary antibodies.

References

1. Fusenig, N. E. (1994) Epithelial-mesenchymal interactions regulate keratinocyte growth and differentiation in vitro, in *The Keratinocyte Handbook* (Leigh, I., Lane, B., and Watt, F., eds.), Cambridge University Press, Cambridge, UK, pp. 71–94.
2 Stark, H. J., Baur, M., Breitkreutz, D., Mirancea, N., and Fusenig, N. E. (1999) Organotypic keratinocyte cocultures in defined medium with regular epidermal morphogenesis and differentiation. *J. Invest. Dermatol.* **112,** 681–691.
3 Maas-Szabowski, N. and Fusenig, N. E. (1996) Interleukin 1 induced growth factor expression in postmitotic and resting fibroblasts. *J. Invest. Dermatol.* **107,** 849–855.

4 Maas-Szabowski, N., Stark, H. J., and Fusenig, N. E. (2000) Keratinocyte growth regulation in defined organotypic cultures through IL-1-induced KGF expression in resting fibroblasts. *J. Invest. Dermatol.* **114,** 1075–1084.

5 Rheinwald, J. G. and Green, H. (1975) Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell* **6,** 331–344.

6 Garlick, J. A. and Taichman, L. B. (1994) Fate of human keratinocytes during reepithelialization in an organotypic culture model. *Lab. Invest.* **70(6),** 916–924.

7. Nuss Parker, J., Zhao, W., Askins, K. J., Broker, T. R., and Chow, L. T. (1997) Mutational analyses of differentiation-dependent human papillomavirus type 18 enhance elements in epithelial raft cultures of neonatal foreskin keratinocytes. *Cell Growth Differ.* **8,** 751–762.

8 Ponec, M., Gibbs, S., Weerheim, A., Kempenaar, J., Mulder, A., and Mommaas, A. M. (1997) Epithelial growth factor and temperature regulate keratinocyte differentiation. *Arch. Dermatol. Res.* **289,** 317–326.

9 Bajou, K., Noel, A., Gerard, R. D., Masson, V., Brunner, N., Holst-Hansen, C., et al. (1998) Absence of host plasminogen activator inhibitor 1 prevents cancer invasion and vascularization. *Nat. Med.* **4(8),** 923–928.

10 Fusenig, N. E., Breitkreutz, D., Dzarlieva, R. I., Boukamp, P., Bohnert, A., and Tilgen, W. (1983) Growth and differentiation of transformed keratinocytes from mouse and human skin in vitro and in vivo. *J. Invest. Dermatol.* **81,** 168–175.

11 Breitkreutz, D., Schoop, V. M., Mirancea, N., Baur, M., Stark, H. J., and Fusenig, N. E. (1998) Epidermal differentiation and basement membrane formation by HaCaT cells in surface transplants. *Eur. J. Cell Biol.* **75,** 273–286.

12 Skobe, M., Rockwell, P., Goldstein, N., Vosseler, S., and Fusenig, N. E. (1997) Halting angiogenesis suppresses carcinoma cell invasion. *Nat. Med.* **3,** 1222–1227.

13 Madison, K. C., Swartzendruber, C. D., Wertz, P. W., and Downing, D. T. (1998) Lamellar granule extrusion and stratum corneum intercellular lamellae in murine keratinocyte cultures. *J. Invest. Dermatol.* **90,** 110–116.

14 Grinnell, F., Toda, K. I., and Lamke-Seymour, C. (1987) Reconstitution of human epidermis in vitro is accompanied by transient activation. *Exp. Cell Res.* **172,** 439–449.

15 Herzhoff, K., Sollberg, S., Huerkamp, C., Krieg, T., and Eckes, B. (1999) Fibroblast expression of collagen integrin receptors alpha1beta1 and alpha2beta1 is not changed in systemic scleroderma. *Br. J. Dermatol.* **141,** 218–223.

16 Bell, E., Ehrlich, H. P., Buttle, D. J., and Nakatsuji, T. (1981) Living tissue formed in vivo and accepted as skin-equivalent tissue of full thickness. *Science* **211,** 1052–1054.

17 Mackenzie, I. C. and Fusenig, N. E. (1983) Regeneration of organized epithelial structure. *J. Invest. Dermatol.* **81,** 189–194.

18 Dawson, R. A., Goberdhan, N. J., Freedlander, E., and MacNeil, S. (1996) Influence of extracellular matrix proteins on human keratinocyte attachment, proliferation and transfer to a dermal wound model. *Burns* **22,** 93–100.

19 Supp, D. M., Supp, A. P., Bell, S. M., and Boyce, S. T. (2000) Enhanced vascularization of cultured skin substitutes genetically modified to overexpress vascular endothelial growth factor 1. *J. Invest. Dermatol.* **114,** 5–13.

20 Takahashi, Y. and Nogawa, H. (1991) Branching morphogenesis of mouse salivary epithelium in basement membrane-like substratum separated from mesenchyme by the membrane filter. *Development* **111,** 327–335.

21 Zheng, J. and Vaheri, A. (1995) Human skin fibroblasts induce anchorage-independent growth of HPV-16-DNA-immortalized cervical epithelial cells. *Int. J. Cancer* **61,** 658–665.

22 Coulomb, B., Dubertret, L., Merrill, C., Touraine, R., and Bell, E. (1984) The collagen lattice: A model for studying epidermalization in vitro. *Br. J. Dermatol.* **114,** 91–101.

23 Smola, H., Thiekötter, G., and Fusenig, N. E. (1993) Mutual induction of growth factor gene expression by epidermal-dermal cell interaction. *J. Cell Biol.* **122,** 417–429.

24 Limat, A., Hunziker, T., Boillat, C., Bayreuther, K., and Noser, F. (1989) Post-mitotic human dermal fibroblasts efficiently support the growth of human follicular keratinocytes. *J. Invest. Dermatol.* **92,** 758–762.

25 Waelti, E. R., Inaebnit, S. P., Rast, H. P., Hunziker, T., Limat, A., Braathen, L., and Wiesmann, U. (1992) Coculture of human keratinocytes on post-mitotic human dermal fibroblast feeder cells: production of large amounts of interleukin 6. *J. Invest. Dermatol.* **98,** 805–808.

26 Smola, H., Thiekötter, G., Baur, M., Stark, H. J., Breitkreutz, D., and Fusenig, N. E. (1994) Organotypic and epidermal-dermal co-cultures of normal human keratinocytes and dermal cells: regulation of transforming growth factor α, β1 and β2, mRNA levels. *Toxicol. In Vitro* **8,** 641–650.

27. Bumann, J., Santo-Holtje, L., Loffler, H., Bamberg, M., and Rodemann, H. P. (1995) Radiation-induced alterations of the proliferation dynamics of human skin fibroblasts after repeated irradiation in the subtherapeutic dose range. *Strahlenther. Oncol.* **171,** 35–41.

28 Parenteau, N. L., Nolte, C. M., Bilbo, P., Rosenberg, M., Wilkins, L. M., Johnson E. W., et al. (1991) Epidermis generated in vitro: practical considerations and applications. *J. Cell Biochem.* **45,** 245–251.

29 Smola, H., Stark, H. J., Thiekötter, G., Mirancea, N., Krieg, T., and Fusenig, N. E. (1998) Dynamics of basement membrane formation by keratinocyte-fibroblast interactions in organotypic skin culture. *Exp. Cell Res.* **239,** 399–410.

30. Maas-Szabowski, N., Stark, H. J., and Fusenig, N. E. (2002) Cell interaction and epithelial differentiation, in Culture of Epithelial Cells, 2nd ed. (Freshney, I. R., and Freshney, M. G., eds.), Wiley-Liss, Inc., New York, pp. 31–63.

31 Gratzner, H. G. (1982) Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: a new reagent for detection of DNA replication. *Science* **218,** 474–475.

32 Cattoretti, G., Becker, M. H., Key, G., Duchrow, M., Schluter, C., Galle, J., and Gerdes, J. (1992) Monoclonal antibodies against recombinant parts of the Ki-67 antigen (MIB 1 and MIB 3) detect proliferating cells in microwave-processed formalin-fixed paraffin sections. *J. Pathol.* **168,** 357–363.

33 Maas-Szabowski, N., Shimotoyodome, A., and Fusenig, N. E. (1999) Keratinocyte growth regulation in fibroblast cocultures via a double paracrine mechanism. *J. Cell Sci.* **112,** 1843–1853.

34 Szabowski, A., Maas-Szabowski, N., Andrecht, S., Kolbus, A., Schorpp-Kistner, M., Fusenig, N. E., et al. (2000) c-Jun and JunB antagonistically control cytokine-regulated mesenchymal-epidermal interaction in skin. *Cell* **103,** 745–755.

35 Breitkreutz, D., Bohnert, A., Herzmann, E., Bowden, P. E., Boukamp, P., and Fusenig, N. E. (1984) Differentiation specific functions in cultured and transplanted mouse keratinocytes: Environmental influences on ultrastructure and keratin expression. *Differentiation* **26,** 154–169.

36. Mackenzie, I., Rittman, G., Bohnert, A., Breitkreutz, D., and Fusenig, N. E. (1993) Influence of connective tissues on the in vitro growth and differentiation of murine epidermis. *Epithelial Cell Biol.* **2,** 107–119.

37 Kaur, P., and Carter, W. G. (1992) Integrin expression and differentiation in transformed human epidermal cells is regulated by fibroblasts. *J. Cell Sci.* **103,** 755–763.

38. Noser, F. K. and Limat, A. (1987) Organotypic culture of outer root sheath cells from human hair follicles using a new culture device. *In Vitro Cell Devel. Biol.* **23,** 541–545.

39 Detmar, M., Schaart, F. M., Blume, U., and Orfanos, C. E. (1993) Culture of hair matrix and follicular keratinocytes. *J. Invest. Dermatol.* **101,** 130–134.

40 Kopan, R. and Fuchs, E. (1989) The use of retinoic acid to probe the relation between hyperproliferation-associated keratins and cell proliferation in normal and malignant epidermal cells. *J. Cell Biol.* **109(1),** 295–307.

41 Chen, Y. Q., Mauviel, A., Ryynanen, J., Sollberg, S., and Uitto, J. (1994) Type VII collagen gene expression by human skin fibroblasts and keratinocytes in culture: influence of donor age and cytokine responses. *J. Invest. Dermatol.* **102,** 205–209.

42 Gregoire, L., Munkareh, A., Rabah, R., Morris, R. T., and Lancaster, W. D. (1998) Organotypic culture of human ovarian surface epithelial cells: a potential model for ovarian carcinogenesis. *In Vitro Cell Dev. Biol. Anim.* **34,** 636–639.

43 Tomakidi, P., Breitkreutz, D., Fusenig, N. E., Zoller, J., Kohl, A., and Komposch, G. (1998) Establishment of oral mucosa phenotype in vitro in correlation to epithelial anchorage. *Cell Tissue Res.* **298,** 355–366.

44 Schoop, V. M., Mirancea, N., and Fusenig, N. E. (1999) Epidermal organization and differentiation of HaCaT keratinocytes in organotypic cucultures with human dermal fibroblasts. *J. Invest. Dermatol.* **112,** 343–353.

45 Maas-Szabowski, N., Starker, A., and Fusenig, N. E. (2003) Epidermal tissue regeneration and stromal interaction in HaCaT cells is initiated by TGF-α. *J. Cell Sci.* **116,** 2937–2948.

46 Vaccariello, M., Javaherian, A., Wang, Y., Fusenig, N. E., and Garlick, J. A. (1999) Cell interactions control the fate of malignant keratinocytes in an organotypic model of early neoplasia. *J. Invest. Dermatol.* **113,** 384–391.

47 Cerezo, A., Stark, H. J., Moshir, S., and Boukamp, P. (2003) Constitutive overexpression of human telomerase reverse transcriptase but not c-myc blocks terminal differentiation in human HaCaT skin keratinocytes. *J. Invest. Dermatol.* **121,** 110–119.

48 Regnier, M., Staquet, M. J., Schmitt, D., and Schmidt, R. (1997) Integration of Langerhans cells into a pigmented reconstructed human epidermis. *J. Invest. Dermatol.* **109,** 510–512.

49 Laning, J. C., DeLuca, J. E., and Hardin-Young, J. (1999) Effects of immunoregulatory cytokines on the immunogenic potential of the cellular components of a bilayered living skin equivalent. *Tissue Eng.* **5,** 171–181.

50. Black, A. F., Berthold, F., L'Heureux, N., Germain, L., and Auger, F. A. (1998) In vitro reconstruction of a capillary-like network in a tissue-engineered skin equivalent. *FASEB J.* **12,** 1311–1340.

51 Kopan, R., Traska, G., and Fuchs, E. (1987) Retinoids as important regulators of terminal differentiation: examining keratin expression in individual epidermal cells at various stages of keratinization. *J. Cell Biol.* **105,** 427–440.

52 Chen, C. S. J., Lavker, R. M., Rodeck, U., Risse, B., and Jensen, P. (1995) Use of a serum-free epidermal culture model to show deleterious effects of epidermal growth factor on morphogenesis and differentiation. *J. Invest. Dermatol.* **104,** 107–112.

53 Asselineau, D. and Prunieras, M. (1984) Reconstruction of simplified skin: control of fabrication. *Br. J. Dermatol.* **111,** 219–222.

54 Mauch, C., Kozlowska, E., Eckes, B., and Krieg, T. (1992) Altered regulation of collagen metabolism in scleroderma fibroblasts growth within three-dimensional collagen gels. *Exp. Dermatol.* **1,** 185–190.

55 Atula, S., Grenman, R., and Syrjänen, S. (1997) Fibroblasts can modulate the phenotype of malignant epithelial cells in vitro. *Exp. Cell Res.* **235,** 180–187.

56. Fusenig, N. E., Skobe, M., Vosseler, S., Hansen, M., Lederle, W., Airola, K., et al. (2002) Tissue models to study tumor-stroma interactions, in *Proteases and Their Inhibitors in Cancer Metastasis* (Muschel, R. J. and Foidard, J. M., eds.), Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 205–223.

57. Fusenig, N. E., Breitkreutz, D., Lueder, M., Boukamp, P., and Worst, P. K. M. (1981) Keratinization and structural organization in epidermal cell cultures, in *International*

Cell Biology 1980-1981 (Schwaiger, H. G., ed.), Springer Verlag, Berlin, Germany, pp. 1004–1014.

58 Breitkreutz, D., Stark, H. J., Mirancea, N., Tomakidi, P., Steinbauer, H., and Fusenig, N. E. (1997) Integrin and basement membrane normalization in mouse grafts of human keratinocytes—implications for epithelial homeostasis. *Differentiation* **61,** 195–209.

59. Meuth, M. and Green, H. (1974) Induction of a deoxycytidineless state in cultured mammalian cells by bromodeoxyuridine. *Cell* **2,** 109–112.

7

In Vitro Fabrication of Engineered Human Skin

Alexander Margulis, Weitian Zhang, and Jonathan A. Garlick

Summary

In vitro fabrication of human epidermal tissues that mimic the biochemical and morphologic properties of human skin, known as skin-equivalent (organotypic) cultures, has opened new avenues in the study of skin biology. In this chapter, methods for the generation of these tissues from their component parts are described. Conditions for culture of human keratinocytes and fibroblasts that allow optimal growth in skin equivalent cultures are delineated. These cell types are then sequentially combined so that keratinocytes are grown at an air-liquid interface on a contracted collagen gel containing dermal fibroblasts. The methods described enable the generation of human epidermal tissues that show in vivo-like tissue architecture and phenotype.

Key Words:

Skin equivalents; epidermis; human keratinocytes; fibroblasts; organotypic culture.

1. Introduction

The development of three-dimensional human tissue models to further our understanding of the biology of epidermal cells requires the ability to engineer tissues that faithfully mimic their in vivo counterparts. Biologically meaningful signaling pathways that mediate the linkage between growth and differentiation function optimally when cells are spatially organized to display the architectural features seen in vivo but are uncoupled and lost in two-dimensional culture systems ***(1,2)***. Over the last decade, the development of tissue-engineered models that mimic human skin, known as skin equivalents or organotypic cultures, have provided novel experimental systems to study epidermal biology. The organotypic culture is an in vitro tissue that consists of a stratified squamous epithelium grown at an air–liquid interface on a collagen matrix populated with dermal fibroblasts. This generates three-dimensional tissues that demonstrate in vivo-like epithelial differentiation and morphology, as well as rates of cell division similar to those found in human skin. This chapter will describe how to generate organotypic cultures from their component parts. This will be accomplished by first describing methods for preparation of keratinocytes and fibroblasts in submerged, monolayer culture, and will then be followed by methods that describe incorporation of these constituent cells into organotypic cultures. The protocols described will allow the generation of human tissues that mimic the morphology, differentiation, and growth of human epidermis.

From: *Methods in Molecular Biology, vol. 289:*
Epidermal Cells: Methods and Protocols
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

Table 1
Media Formulations for 1 L of Total Volume[a]

	Epidermalization I	Epidermalization II	Cornification (at airlift)
DME	725	725	474
F12	240	240	474
L-glutamine	20	20	20
Hydrocortizone	2	2	2
ITES	2	2	2
O-phos	2	2	2
$CaCl_2$	2	2	2
Triiodothyranine	2	2	2
Progesterone	2	2	–
Adenine	2	2	2
Serum	1 (FBJ)	1 (FBJ)	20 (FBJ)

[a]All volumes are in milliliters.

Table 2
Components for the Collagen Matrix With Incorporated Dermal Fibroblasts

	Acellular matrix for 6 mL (1 mL/insert)	Cellular matrix for 18 mL (3 mL/insert)
10X DMEM	0.60 mL	1.65 mL
L-glutamine	0.05 mL	0.15 mL
Fetal bovine serum	0.70 mL	1.85 mL
Sodium bicarbonate	0.17 mL	0.52 mL
Collagen	4.60 mL	14.00 mL
Fibroblasts	–	4.5×10^5 cells in 1.5 mL of fibroblast media

2. Materials

2.1. Submerged Cultures

2.1.1. Submerged Keratinocyte Culture Media

1. Source of human keratinocytes: newborn foreskins (average size: 1–2 cm^2; *see* **Note 1**).
2. Keratinocyte tissue culture medium:
   a. 3:1 Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 medium (Gibco, BRL); DMEM (Invitrogen, Carlsbad, CA; cat. no. 31600-083); and Ham's F12 (Invitrogen; cat. no. 21700-108).
   b. Fetal bovine serum 5% (Hyclone Laboratories, Logan, UT; cat. no. SH30071.03).
   c. Penicillin–streptomycin 100 IU/mL (100 µg/mL): penicillin (Sigma, St. Louis, MO; cat. no. P-3032) and streptomycin (Sigma; cat. no. S-6501).
   d. HEPES (Sigma; cat. no. H-4034): Dissolve 47.24 g in 250 mL of 2X dH_2O to make a 100X stock. Filter sterilize, aliquot, and store at –20°C.
   e. Adenine (ICN, Aurora, OH; cat. no. 100190): Dissolve 0.972 g in 2.4 mL of 4 *N* NaOH; bring volume to 400 mL with 2X dH_2O to make a 100X stock (18 m*M*); store at –20°C.
   f. Cholera toxin (ICN; cat. no. 190329): Dissolve 9 ng/mL to make a 1000X stock and store at –20°C.

g. Epidermal growth factor (Austral Biological, San Ramon, CA; cat. no. GF-010-9): Dissolve 10 µg/mL in 0.1% bovine serum albumin to make a 1000X stock and store at –20°C.
h. Hydrocortisone (Sigma; cat. no. H-4881): Dissolve 0.0538 g in 200 mL of 2X dH_2O to make a 500X stock (0.269 mg/mL) and store at –20°C.
i. Insulin (EMD Biosciences, San Diego, CA; cat. no. 40769): Dissolve 50 mg in 10 mL 0.005 *N* HCl to make a 1000X stock (5 mg/mL) and store at –20°C.

Note: all the components can be stored at –20°C for up to 1 yr.

2.1.2. Fibroblasts for Submerged Keratinocyte Culture (Feeder Layers)

1. Irradiated 3T3-J2 cells are used as feeder cells ***(3)***.
2. 3T3 media:
   a. DMEM (*see* **Subheading 2.1.1.**).
   b. Bovine calf serum 10% (Hyclone; cat. no. SH30072.03).
   c. Penicillin–streptomycin 100 IU/mL (100 µg/mL; *see* **Subheading 2.1.1.**).
   d. HEPES (*see* **Subheading 2.1.1.**).

2.2. Organotypic Cultures

2.2.1. Fibroblasts for Organotypic Culture

1. Source of human fibroblasts: newborn foreskins (average size: 1–2 cm^2).
2. Fibroblast tissue culture medium:
   a. DMEM (*see* **Subheading 2.1.1.**).
   b. Fetal bovine serum 10% (*see* **Subheading 2.1.1.**).
   c. Penicillin-streptomycin 100 IU/mL-100 µg/mL (*see* **Subheading 2.1.1.**).
   d. HEPES (*see* **Subheading 2.1.1.**).

2.2.2. Materials for Collagen Matrix

1. A confluent culture of human foreskin fibroblasts.
2. Six-well deep tissue culture tray containing culture inserts with a 3-µm porous polycarbonate membrane (Organogenesis, Canton, MA; cat no. MS-10-305).
3. Bovine tendon Type I collagen (Organogenesis; cat. no. 200-201).
4. 10X Minimum essential medium with Earle's salts (DMEM) (Cambrex, Walkersville, MD; cat. no. 12-684F).
5. Fetal bovine serum (Hyclone; cat. no. SH30071.01).
6. L-glutamine (200 m*M*; Cambrex; cat. no. 17-605E).
7. Sodium bicarbonate (71.2 mg/mL; Cambrex; cat. no. 17-613E).
8. Fibroblast tissue culture medium (*see* **Subheading 2.2.1.**).
9. Green's trypsin (2 L):
   a. 2 g Trypsin 1-300 (ICN; cat. no. 103140).
   b. 2 g Glucose (Sigma; cat no. G-7528).
   c. 200 mL 10X phosphate-buffered saline (PBS).
   d. 0.2 g Streptomycin (*see* **Subheading 2.1.1.**).
   e. 0.2 g Penicillin (*see* **Subheading 2.1.1.**).

Mix ingredients on ice, adjust the volume to 2 L with double-distilled water, and adjust the pH to 7.25–7.40 with a few drops of 2 *N* NaOH. Extract trypsin by filtering it through the filter paper. Repeat the procedure again with Nalgene filter (Fisher, Pittsburgh, PA; cat. no. 09-740-32). Aliquot and store at –20°C for up to a year.

2.2.3. Organotypic Culture Media

1. DME Base Modified: this DME is a special formulation from JRH Inc., Lenexa, KS (cat. #56430-10L) that needs to be ordered in bulk quantities. 0.0086 g of DME powder is dissolved in 1 mL ddH_2O together with 0.0001 g of $MgSO_4$ and 0.0037 g of $NaHCO_3$.
2. Ham's F12 (*see* **Subheading 2.1.1.**).
3. L-glutamine (*see* **Subheading 2.2.2.**) 200 m*M* is a 500X stock.
4. Hydrocortizone (*see* **Subheading 2.1.1.**).
5. ITES (Cambrex; cat. no. 17-839Z: this contains insulin (5 µg/mL), transferrin (5 µg/mL), ethanolamine (5 µg/mL), and selenium (5 µg/mL).
6. Triiodothyronine (Sigma; cat. no. T-5516): dissolve 1 mg in 1 mL 1 *N* NaOH and add 19 mL DMEM (50 µg/mL) then dilute 4 µL in 31 mL of DMEM to make a 500X stock (10^9 *M*).
7. *O*-phosphorylethanolamine (O-phos) (Sigma; cat. no. P-0503): dissolve 705 mg in 100 mL DME to make the 500X stock (50 m*M*).
8. Adenine (Sigma; cat. no. A-9795): dissolve 1.55 g in 100 mL of acidified water warmed in a 37°C water bath to make a 500X stock (90 m*M*).
9. Calcium chloride: dissolve 3.55 g in 20 mL ddH_2O to make a 500X stock.
10. Progesterone (Sigma; cat. no. P-8783): dissolve 1 mg in 1 mL absolute ethanol and add 14.7 mL of ddH_2O, then dilute 1 mL in 100 mL of DMEM to make a 500X stock (2×10^9 *M*).
11. Serum:
    a. Chelated fetal bovine serum (cFBS). Chelate serum (*see* **Subheading 2.2.2.**) by mixing 10 g CHELEX 100 (Sigma; cat. no. C-7901) with 100 mL of serum and stirring in cold room for 3 h. Serum can then be filtered through Whatman filter paper and then through sterile filter (*see* **Note 8**).
    b. FBS.

2.2.4. Connective Tissue Substrates Placed on the Contracted Collagen Gel (see ***Note 3***)

1. De-epidermalized human dermis (Alloderm™, LifeCell Inc., Branchburg, NJ; cat. no. 102-009).
2. Inserts with individual basement membrane components (all inserts are from Becton-Dickinson Inc.): Collagen I (cat. no. 354442), Laminin I (cat. no. 354446), Fibrillar Collagen I (cat. no. 354472), Fibronectin/Collagen I (cat. no. 354633), Fibronectin (cat. no. 354440), Collagen IV (cat. no. 354544).
3. 1.4-cm stainless-steel dermatologic punch (Delasco, Inc, Council Bluffs, IA; cat. no. KP-14).

3. Methods

3.1. Method for Incorporation of Fibroblasts Into Organotypic Cultures

3.1.1. Preparation of Submerged Cultures of Fibroblasts To Be Incorporated into Collagen Matrix

Culture human foreskin fibroblasts so that they are confluent one day before the collagen matrix is to be cast. Passage the cells at high density (9 : 10 split) so that cultures will be confluent again and used the following day (*see* **Note 4**).

3.1.2. Construction of the Collagen Matrix ***(Fig. 1)***

Mix the following components in a 100-mL sterile media bottle, placed on ice, to generate acellular and cellular layers for the contracted collagen gel. The goal is to create

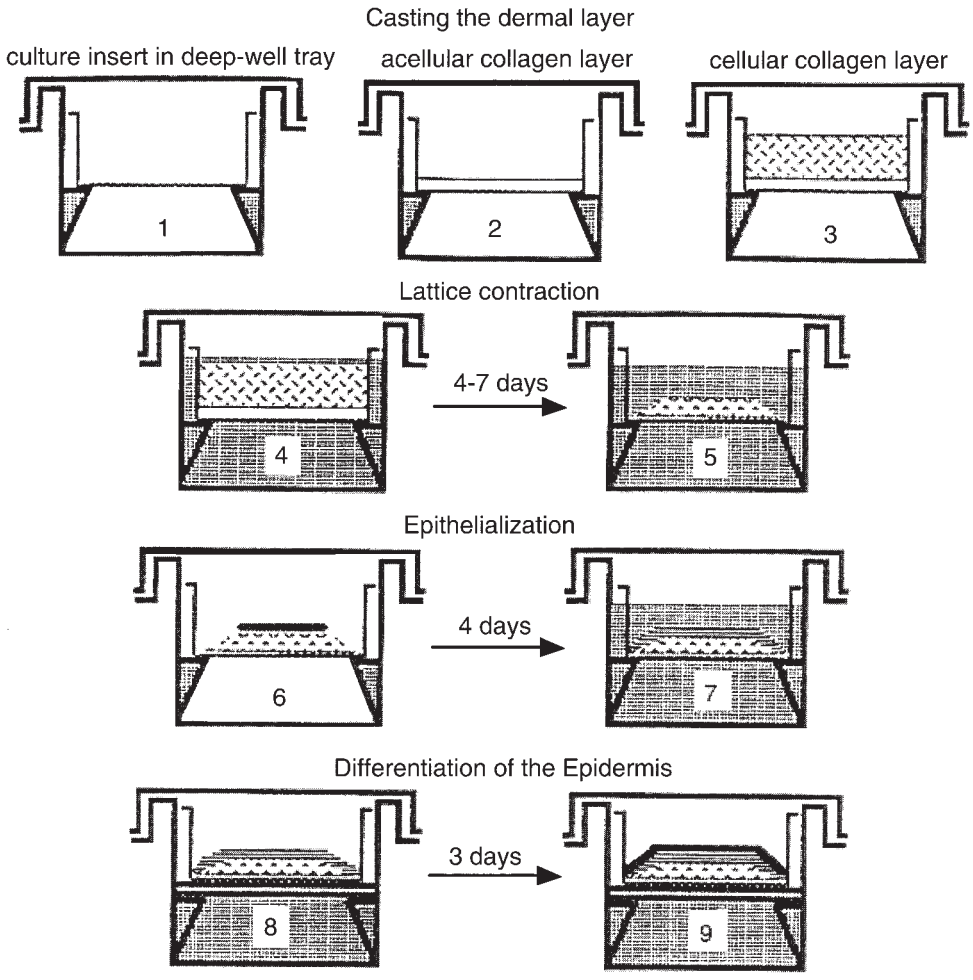


Fig. 1. Construction of organotypic cultures grown directly on contracted collagen gel. 1). Culture insert in deep well tray. 2) A 1-mL layer of acellular collagen is allowed to gel over the polycarbonate membrane of the insert. 3) Cellular collagen (3 mL) is allowed to gel over the acellular collagen. 4,5) The collagen gel contracts from the sides of the collagen insert to form a contracted collagen gel with a central, raised area. Addition of keratinocytes for epithelialization of cultures. 6) A 50-µL suspension of ke atinocytes (500,000 cells/insert) is seeded onto the raised, central part of the insert. 7) Cultures are maintained submerged for 7 d to allow complete coverage and early stratification. 8,9) The developing cultures are raised to the air–liquid interface.

a thin layer of acellular collagen that will act as a substrate for the cellular collagen. This will prevent the cellular collagen from contracting completely from the insert membrane (*see* **Note 5**).

1. Keeping all components on ice, mix the acellular matrix components in the order listed in **Fig. 1** and add 1 mL per insert **(Fig. 1A,2)**. The color of the solution should be straw-yellow to light pink. Any deviations from this color suggests an alteration in pH and the collagen may not gel. If the final solution is bright yellow, slowly titrate in more sodium bicarbon-

ate until the straw-yellow color is achieved. Make sure the mixture coats the entire bottom of the insert. Once the gel has been poured, allow it to set at room temperature. Do not move the tray until the collagen gel has fully polymerized (10–15 min). As the gel polymerizes, the color of the matrix will change to a pink color (*see* **Note 5**).

2. While the acellular matrix layer is polymerizing, trypsinize the fibroblasts, count, and resuspend these cells in fibroblast media to a final concentration of 3.0×10^5 cells/mL. Resuspend these cells fully with trituration since they must be incorporated into the collagen gel as single cells.
3. For the cellular matrix, again keep all components on ice and mix in the order indicated above. Again, if the color needs to be adjusted after mixing, slowly add sodium bicarbonate. Fibroblasts should be added last as they need to be added to the mix after it has been neutralized by the addition of collagen so that the cells are not damaged by an alkaline pH. Gently triturate in order to obtain an even suspension of cells in the collagen, add 3 mL of the mix into the inserts and allow mixture to gel at room temperature **(Fig. 1A3)**. This process is usually complete within 30 min, when gels are firm and have a pink color. At this point, the gels are covered with 12 mL fibroblast media by adding 10 mL to the bottom of the well and 2 mL to the top of the insert **(Fig. 1A4)**. Gels are then incubated for 5–7 d until gel contraction is complete (*see* **Note 6**) and the gel no longer contracts **(Fig. 1A5)**.

3.2. Method for Growth of Keratinocytes in Organotypic Cultures

3.2.1. Adding Keratinocytes to the Collagen Matrix

1. It is first necessary to decide whether cells will be grown directly on the contracted gel or on an intervening interface of de-epidermalized dermis or coated, polycarbonate insert (*see* **Note 7**). If de-epidermalized dermis is to be used, it should be cut out to fit the diameter of the contracted collagen gel with a dermatologic punch and rehydrated in PBS for 1 h at 37°C. Coated inserts are cut away from their plastic insert by using a scalpel at their periphery and should then be rehydrated in serum-free DMEM for 1 h at 37°C. Alternatively, keratinocytes can be seeded and grown directly on the contracted collagen gel **(Fig. 1B)**.
2. Using the feeder layer system, cultures should be trypsinized when keratinocytes are no more than 50% confluent, which will minimize the number of differentiated cells seeded (*see* **Note 2**). Remove the 3T3 feeder cells from the culture by incubating plates in PBS/EDTA for 2 min at 37°C. By gently triturating, 3T3 will be displaced from the plate and the keratinocytes will remain attached. Be careful not to allow the cultures to incubate for an excessive time in PBS/EDTA, as this may cause keratinocytes to lift off the plate, as well. As soon as 3T3 have begun to detach, immediately replace PBS/EDTA with PBS and gently rinse the plate several times until the 3T3s have been completely removed and only small keratinocyte colonies remain.
3. Trypsinize keratinocyte colonies with Green's trypsin/EDTA for 5 min at 37°C to obtain a single cell suspension. Once cells have detached, gently resuspend them in keratinocyte media, count cells and add the appropriate number of cells to a 15-mL tube so that there will be enough cells (500,000 cells/insert) to seed onto the desired number of inserts.
4. Centrifuge at 2000*g* for 5 min.
5. Remove all fibroblast media from the matrices. At this time, the de-epidermalized dermis or coated insert can be layered directly onto the collagen matrix. Alternatively, cells can be seeded directly onto the contracted collagen gels.
6. Resuspend the keratinocytes so that they are in the small volume needed for plating onto the matrix. It is important to resuspend cells in a small volume as 500,000 cells will be seeded onto the small surface area of the connective tissue (1.4-cm diameter). This is performed by using a sterile, plastic 1-mL pipet to add 50 µL of epidermalization I media

to the 15-mL tube per 500,000 keratinocytes present. Gently dislodge the cell pellet and transfer it to a 1.5-mL sterile Eppendorf tube with a 1-mL plastic pipet. Gently resuspend the cell pellet in the eppendorf tube with a 200-µL pipetman until it is cloudy and well-suspended and transfer 50 µL of the cell suspension to the center of the contracted collagen gel. Do not touch the tray for 10 min to allow cells to begin to attach. Incubate at 37°C for 30–60 min without any media to allow the keratinocytes to fully adhere.

7. At this point, add 12 mL of epidermalization I media by adding 10 mL to the bottom of the well and 2 mL gently into the insert top of the keratinocytes and incubate cultures at 37°C (**Fig. 1B6**; *see* **Note 8**).
8. Cultures are fed every 2 d in the following manner:
   a. Epidermalization I media: 12 mL for first 2 d.
   b. Epidermalization II media: 12 mL for next 2 d **(Fig. 1B7)**.
   c. Cornification media: cultures are raised to the air/liquid interface by adding only 6.5 mL to the bottom of the well so that the bottom of the insert just contacts the interface with the media. Additional feedings with 6.5 mL cornification media are performed every 2 d (**Fig. 1B8,9**; *see* **Note 9**).

*3.2.2. Processing of Tissues for Morphologic and Immunohistochemical Analysis (*see ***Note 10****)*

1. Gently rinse organotypic cultures twice in PBS.
2. Using a scalpel, cut away the insert membrane from the plastic insert.
3. Bisect the cultures and place one half of the culture in a cassette and immediately immerse in 10% formalin. The tissues are very thin and only require fixation for several hours before paraffin processing. If tissues will not be processed within 24 h, formalin should be replaced with 50% ethanol.
4. The other half of the tissue can be placed into a 2 *M* sucrose solution (*see* **Note 10**) that was prepared in water. Tissues should be soaked with sucrose at 4°C for at least 1 h and not more than 24 h.
5. To embed sucrose-soaked tissue for frozen preservation, make a small mold with aluminum foil using a small bottle cap (2 cm in diameter) which is then three-quarters filled with embedding media.
6. Gently remove the tissue from the sucrose using narrow-tipped forceps making sure to keep it on its nylon membrane. Gently touch the membrane side of the tissue to a kimwipe to remove some of the excess sucrose.
7. Using a thin spatula, lift the tissue on its membrane side and place it into the embedding media. Remove the spatula by gently holding the forceps on top of the tissue.
8. Allow the tissue to soak in embedding media for 20–30 min at room temperature. This will prevent the tissue from binding to the embedding media during sectioning.
9. Place a metal test-tube rack into a styrofoam box and fill it with liquid nitrogen so that it is just below the height of the rack. Place the aluminum foil mold on top of the rack and adjust the tissue to an upright position by gently holding the tissue on top. Gently hold the tissue in this position with the forceps for less than a minute. After releasing the specimen, check to ensure that it remains upright as it slowly freezes in the liquid nitrogen vapor.
10. The tissue will be completely frozen in 5 min and can be stored in a pill box at –80°C.

4. Notes

1. Although the methods described are for the fabrication of organtypic cultures using skin keratinocytes, they can be adapted to grow tissues from other stratified epithelia such as the oral and cervical mucosa, esophageal lining, conjunctiva and laryngeal epithelium.

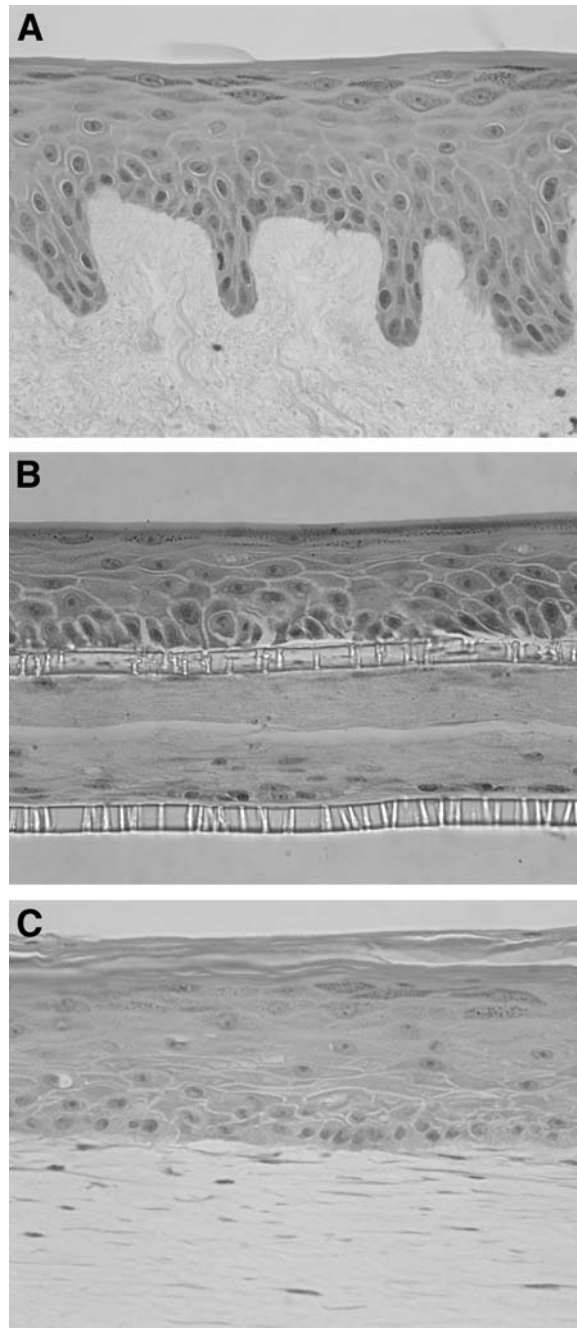


Fig. 2. Morphologic appearance of human stratified squamous epithelium grown on different connective tissue substrates. When keratinocytes were grown on the acellular, de-epidermalized dermis (AlloDerm), the epithelium demonstrated in vivo-like tissue architecture and organization, as seen by the presence of cuboidal basal cells nested in undulating rete pegs and well-defined spinous, granular layers and an orthokeratinized stratum corneum **(A)**. When grown on Type IV collagen-coated polycarbonate membranes, tissues were well-stratified with polarized basal cells and a well-formed spinous layer **(B)**. A slightly altered pattern of morphologic differentiation was seen when cells were grown directly on a contracted Type I collagen gel as the transition between strata was not as distinct as was seen in AlloDerm cultures and no polarization of the basal layer was seen **(C)**.

It is suggested that efforts be made to optimize the growth of these cells in monolayer culture before incorporating them into organotpyic cultures.

2. The rationale for growing keratinocytes initially in low calcium media containing chelated calf serum is to allow growth optimization. This step is useful in obtaining complete coverage of the connective tissue surface after seeding keratinocytes in organotypic culture.
3. The protocols described provide techniques that allow growth of epithelial tissues on a variety of connective tissue substrates. Each of these systems presents specific applications that can be tailored to answer specific experimental questions. Tissues grown on the de-epidermalized dermis (AlloDerm) serve as an interface on which the rapid assembly of structured basement membrane occurs and tissue morphology is optimized ***(4)***. AlloDerm is commercially available from LifeCell, Inc. (Branchburg, NJ) and is used in a variety of clinical applications to treat burns, periodontal disease and surgical defects ***(7)***. Tissues grown directly on contracted collagen gels do not assemble intact basement membrane, but provide excellent support for keratinocyte growth and differentiation ***(4)***. Cultures grown on polycarbonate membranes coated with specific extracellular matrix proteins can be used to directly study the effect of these proteins on tissue architecture and phenotype of these tissues (*see* **Subheading 2.2.4.**). Examples of tissues grown on these different substrates are seen in **Fig. 2**.
4. We have found some variability in the degree to which fibroblast strains support keratinocyte growth after their incorporation into the collagen gel. We have seen that fiboblast-mediated support of keratinocyte growth is directly correlated to the degree to which fibroblasts are able to contract the collagen matrix. Fibroblast strains demonstrating more shrinkage of the collagen gel before adding keratinocytes are better able to support keratinocyte growth. This parameter can be used to screen fibroblast strains for optimal growth-support in organotypic culture.
5. Fabrication of the collagen gel requires that all components be kept on ice until the gel mixture is placed into the insert. This will ensure that collagen will not prematurely precipitate from these solutions. Plastic pipets used for collagen should be chilled before use.
6. To generate tissues with normalized tissue architecture, it is critical that keratinocytes seeded into organotypic culture have an elevated growth potential. Nearly all keratinocytes seeded in organotypic cultures will adhere to their connective tissue substrate but only replicating cells will grow after plating. Keratinocytes that have undergone terminal differentiation while in submerged culture will also attach to their substrate but will not generate actively growing, well-stratified organotypic cultures. It is therefore important to grow keratinocytes so that a high growth fraction is present in the monolayer cultures at the time of passage to organotypic culture. This can be accomplished by growing keratinocytes as small colonies at high clonal density in submerged cultures on 3T3 feeder layers so that terminal differentiation will be minimized and the fraction of replicating cells will be maximized. Alternatively, this can also be accomplished by growing keratinocytes in a low calcium media before passage to organotypic culture using defined media conditions without 3T3 fibroblasts. We have found that growth and morphology of organotypic cultures can vary when different keratinocyte and fibroblast strains are incorporated. Keratinocyte strains can therefore be tested in organotypic culture to determine those that will provide the best growth and morphologic differentiation. This may also be accomplished by testing the clonogenic growth of keratinocyte strains in monolayer, feeder layer cultures in order to determine the growth potential of these cells.
7. It should be kept in mind that although keratinocytes grown in organotypic cultures share many morphologic and biochemical features in common with human skin keratinocytes, they do differ somewhat in phenotype. For example, integrin receptors not normally expressed in skin are constituitively expressed in keratinocytes grown in organotypic

culture. In addition, these cultures are somewhat deficient in barrier function. A good way to think about keratinocytes grown in organotypic cultures is to compare them to newly re-epithelialized, healed wounds in vivo, wherein morphologic differentiation is complete but cells are still in a somewhat "activated" state.

8. An advantage to studying keratinocyte phenotype in organotypic culture lies in the ability to control and modify the cellular milleu in which these tissues are grown. For example, it is possible to add soluble factors directly to these cultures to determine the phenotypic response to such environmental conditions ***(5)***. The environment can be modified by growing cultures in the absence of fibroblasts that can be eliminated from cultures after contraction of the collagen gel ***(4)***. In addition, control of the milleu facilitates analysis of these tissues as it is possible to directly determine proliferation indices by adding a pulse of 5-bromodeoxyuridine directly to media.
9. Several points regarding some subtleties of keratinocyte behavior in organotypic cultures should be mentioned. The first concerns the length of time which cultures can be maintained at the air–liquid interface. In our experience, cultures can be kept at this interface for up to 10 to 14 d. At this time, the surface layer of the epithelium becomes very thick due to a failure to desquamate. As a result, lower layers of the epithelium become compressed and the longevity of cultures is limited. A second and related issue concerns the growth potential of keratinocytes in organotypic culture. While organotypic cultures demonstrate a basal level of proliferation that is greater than human skin ***(4)***, it has been shown that these cultures have a tremendous potential for cell growth and are very responsive to external growth stimuli. For example, disruption of the organotypic cultures upon wounding results in a 10-fold increase in basal cell proliferation ***(5,6)***.
10. As described previously, it is critical to preserve tissue architecture during processing of tissues after culture is complete. Because collagen gels are greatly hydrated, they can undergo significant artifactual damage during tissue processing. For this reason, tissues are soaked in 2 *M* sucrose to replace water in the tissue and prevent freezing damage (*see* **Subheading 3.2.2.**). Furthermore, tissues need to be gradually frozen in liquid nitrogen vapor to prevent destruction of tissue architecture. Cultures should never be snap-frozen by immersing tissues in liquid nitrogen.

References

1 Hagios, C., Lochter, A., and Bissell, M. J. (1998) Tissue architecture: the ultimate regulator of epithelial function? *Phil. Trans. R. Soc. Lond. B Biol. Sci.* **353,** 857–870.

2. O'Brien, L. E., Zegers, M. M., and Mostov, K. E. (2002) Opinion: building epithelial architecture: insights from three-dimensional culture models. *Nat. Rev. Mol. Cell Biol.* **3,** 531–537.

3 Rheinwald, J. G. and Green, H. (1975) Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell* **6,** 331–343.

4 Andriani, F., Margulis, A., Lin, N., Griffey, S., and Garlick, J. A. (2003) Analysis of microenvironmental factors contributing to basement membrane assembly and normalized epidermal phenotype. *J. Invest. Dermatol.* **120,** 923–931.

5 Garlick, J. A. and Taichman, L. B. (1994) Effect of TGF-β1 on re-epithelialization of human keratinocytes in vitro: an organotypic model. *J. Invest. Dermatol.* **103,** 554–559.

6 Garlick, J. A. and Taichman, L. B. (1993) The fate of genetically marked human oral keratinocytes in vitro. *Arch. Oral Biol.* **38,** 903–910.

7. Wainwright, D. J. (1995) Use of an acellular allograft dermal matrix (AlloDerm) in the management of full-thickness burns. *Burns* **21,** 243–248.

II

Epidermal Stem Cells

8

In Vivo Labeling and Analysis of Epidermal Stem Cells

Wei-Yang Wu and Rebecca J. Morris

Summary

To identify slowly cycling (label-retaining) keratinocytes in the cutaneous epithelium of mice, newborn female pups were injected with [^{3}H]-thymidine twice daily for 3 d beginning the third day after birth. At 8–10 wk of age, skin from the dorsal side of the mice was removed and fixed. The skin was processed for paraffin embedding, and serial sections were prepared. Dewaxed and ethanol-treated slides were dried and dipped into autoradiographic emulsion and allowed to dry in light tight box for 4 wk at 4°C. After fixation with photographic fixer, the slides were washed, lightly stained, and viewed with a light microscope to observe and quantify cells that retained the labeling. Label-retaining cells were identified by the black silver grains over their nuclei.

Key Words:

Hair follicle; radiolabeling; autoradiography; mice; skin.

1. Introduction

Proliferative subpopulations of keratinocytes from the cutaneous epithelium can be defined by various characteristics—location within the tissue, responses to chemical treatments, mitotic behavior in vitro or in vivo, morphology, and expression of marker proteins. Each of these parameters helps define a subpopulation that may contain stem cells, but none of them, taken individually, provides an accepted operational definition of the keratinocyte stem cells. However, take together, these parameters show great promise for identification and isolation of the keratinocytes stem cells.

This chapter focuses on one of the first techniques used for identification of keratinocyte stem cells from the cutaneous epithelium of mice: identification of slowly cycling (label-retaining) keratinocytes by light microscopic autoradiography. Since their first identification ***(1)***, a number of laboratories, including ours, using variations of continuous labeling with [^{3}H]thymidine followed by a chase, have identified several subpopulations of keratinocytes, notably the central cells in the epidermal proliferative units and the cells in the hair follicle bulge ***(2)***. The underlying premise for all of these variations is labeling of deoxyribonucleic acid (DNA) at a time when all epidermal cells are undergoing DNA synthesis followed by a chase during which label is diluted and lost by the more rapidly proliferating cells, leaving the slowly cycling cells identified in autoradiographs. The following procedure is the one used by our laboratory for the identification of label-retaining cells.

From: *Methods in Molecular Biology, vol. 289:*
Epidermal Cells: Methods and Protocols
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

2. Materials

2.1. Mice

1. 13-d-old pregnant mice. Mice can be ordered from Charles Rivers and requested to be at 13 d of gestation upon arrival.

2.2. Injection Supplies

1. 30-gauge 0.5-inch needles (BD Biosciences, Franklin Lakes, NJ; cat. no. 305106).
2. 1-mL syringe (BD Biosciences, Franklin Lakes, NJ.; cat. no. 309602).
3. Sterile gauze pads (Johnson & Johnson, Arlington, TX; cat. no. 2315).
4. 26-gauge 5/8-inch needles (BD Biosciences, Franklin Lakes, NJ; cat. no. 305115).
5. 5-mL syringe (BD Biosciences, Franklin Lakes, NJ; cat. no. 9603).
6. Disposable plastic cages (Ancare, Bellmore, NY; cat. no. N10PS).
7. [*methyl*-^{3}H] Thymidine 1 mCi, 40–60 Ci/mmol (Amersham Biosciences, Amersham Place Little Chalfont, Buckinghamshire HP 7 9NA, UK; cat. no. TRK418).
8. Dulbecco's phosphate-buffered saline, Ca^{2+} and Mg^{2+} free, sterile (Cambrex Biosciences, Walkersville, MD; cat. no. 17-515Q).

2.3. Section Preparation Supplies

1. One pair of scissors (Biomedical Research Instruments, Rockville, MD; cat. no. 25-1050).
2. One pair of full-curve eye dressing forceps (Miltex, Bethpage, NY; cat. no. 18-784).
3. Index cards.
4. Tissue cassette (Fisher, Suwanee, GA; cat. no. 15-182-500F).
5. 10% formalin (Fisher, Suwanee, GA; cat. no. SF98-4).
6. 70% ethanol in water.
7. 95% ethanol in water.
8. 100% ethanol.
9. Commercial hair remover (local drug store).

2.4. Autoradiography Supplies

1. Humidifier.
2. Humidity gauge.
3. Thermometer.
4. Glass stirring rod.
5. Plastic spatula.
6. Plastic-coated test-tube rack with 1-inch openings.
7. Kodak NTB-2 nuclear track emulsion (Eastman Kodak, Rochester, NY; cat. no. 165-4433).
8. Black light-tight drying box.
9. Freshly made and filtered Kodak Dektol developer diluted 1:1 with distilled water (Eastman Kodak, Rochester, NY, cat. no. 146-4700).
10. Kodak fixer (Eastman Kodak, Rochester, NY; cat. no. 197-1746).
11. Distilled water.
12. 100% ethanol.
13. Xylene.
14. Permount (Fisher, Fisher, Suwanee, GA; cat. no. SP15-100).
15. Acid alcohol: 1 L 70% ethanol and 10 mL concentrated HCl.
16. Phosphate-buffered saline (PBS; to neutralize acid alcohol; Cambrex, Walkersville, MD; cat. no. 17-515Q).

3. Methods

3.1. Labeling Mice

1. Before delivery, provide nesting materials from animal husbandry for the mice. This will increase survivability of the pups. Once the pups are born, avoid contact until the day of injections.
2. Prepare ^{3}H-thymidine injection solution using a 26-gauge needle. With a 5-mL syringe, inject 4 mL sterile PBS into the bottle of ^{3}H-thymidine, bringing the final concentration to 10 µCi/50 µL.
3. At 3 d of age, separate and euthanize all male pups with CO_2 inhalation. Before handling the pups to be injected, use fresh gloves and rub the gloves with some of the bedding to avoid stressing the mice. Place female pup on sterile gauze pad. Using 30-gauge needle with the 1-mL syringe, inject 50 µL of ^{3}H-thymidine/PBS solution subcutaneously under the scruff of the neck (*see* **Note 1**). Injections are made at 9 AM and 5 PM each day for three consecutive days. Each mouse will receive a total of 60 µCi. To ensure survivability of pups, change gloves after each litter.
4. After injections, transfer all labeled pups with mother to disposable cages for one week. After 1 wk, the mice can be kept in conventional cages.
5. The pups are weaned at 3–4 wk of age. The mothers and any remaining male pups are euthanized via CO_2 inhalation at that time.

3.2. Section Preparation

1. At 8 wk of age, the mice are euthanized with CO_2 followed by cervical dislocation. Clip the dorsal fur with electric clipper. Spread a generous portion of hair remover on the clipped portion. Do not brush in the paste, which will destroy the epidermal layer. Allow the hair remover to remain on mice for 2 min and wash off with cold running water.
2. Using forceps and scissors, remove the smooth portion of skin denuded of hair. Cut a rectangle of the skin (2 cm × 1.5 cm), making sure that the long axis of the rectangle of the skin runs parallel to the length of the mouse. Stretch the skin out on a piece of index card, place index card in a numbered tissue cassette, and place tissue cassette in 10% formalin.
3. Allow tissue to fix in 10% formalin for at least overnight.
4. Pour 10% formalin into formalin waste container and dehydrate tissue with series of 30-min washes of 70%, 95%, and 100% ethanol.
5. Remove tissue from index card and trim tissue into a rectangular strip (1.5 cm × 1 cm). In trimming the tissue, retain the long axis of the tissue.

3.3. Autoradiography With Liquid Emulsion (Fig. 1)

1. Before proceeding procedure, check room temperature and humidity. If humidity is below 50%, use humidifier (*see* **Note 2**).
2. Deparaffinize the slides with the series of washes (*see* **Note 3**):
   a. Xylene I for 3–5 min
   b. Xylene II for 3–5 min
   c. Xylene III for 3–5 min
   d. 100% Ethanol for 3 min
   e. 100% Ethanol for 3 min
   f. 100% Ethanol for 3 min
3. Warm beaker of water to 41°C in a water bath. Fill dipping vessel half full with distilled water.
4. Open emulsion and break up with spatula. Add emulsion carefully to plastic vial up to half an inch from the top.

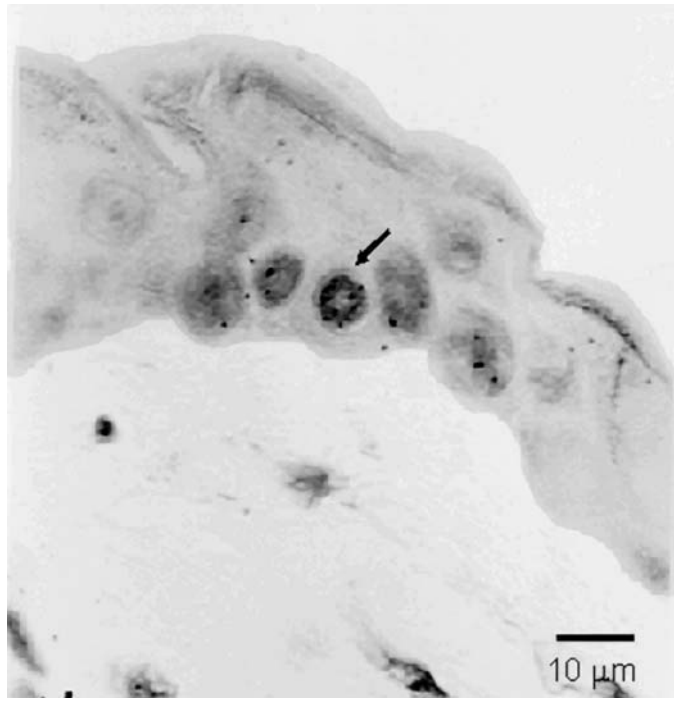


Fig. 1. Autoradiography photomicrograph of label retaining mouse skin. Slightly darker region is the epidermis, which stains darker from the keratin fibers. The arrow denotes a silver grain exposed from the ^{3}[H]thymidine retaining nucleus of the keratinocyte.

5. Put emulsion and water vial in beaker in the water bath gently stirring emulsion/water mixture constantly for 10 min.
6. Dip 12 blank slides into emulsion until no bubbles are on the slides. Dip experimental slide into vial slowly. Tap excess emulsion on dampened paper towels.
7. Lean slide on a test tube rack and continue to dip remaining slides.
8. Place test tube rack into light tight box for 1–3 h to allow drying of the slides.
9. Take dried slides and place in a black box or light tight box. To make certain that no light can penetrate the box, seal it with black photographic tape and then wrap the box in aluminum foil. Place box at 4°C for 28 d.
10. Remove boxes from 4°C and allow them to come to room temperature. Put developer, distilled water, and fixer solutions into a large tray of chipped ice. Allow the solutions to cool to 18°C for about 30 min.
11. Under safelight illumination, remove slides and place in glass slide rack. Put slide rack into developer for 3 min. Tap slide rack once to remove any air bubbles on slides but do not agitate. Remove slide rack from developer and place into distilled water for 10 s. Put slide rack into fixer for 3 min and then 3 min more with agitation. Place slide rack into distilled water for 3 min.
12. Place slides into staining racks into fresh jars of water and rinse for at least 1 h in cold running RO water. Alternatively use several changes of distilled water (*see* **Note 4**).
13. Stain slides with the series of stains and washes:
    a. Ehrlich's hematoxylin (10 min).
    b. Gently running deionized water (10 min).
    c. 50% acid alcohol with five to six fast.

Table 1
Autoradiograph Problems and Probable Causes

High background	• Light leak • Low humidity (below 50%) • Developer and fixer were warm • Chemical dust present
Bubbles	• Emulsion was stirred too fast • Not enough blank slides were dipped.
Uneven staining	• Emulsion not mixed well enough
Black spots on side edges of slides	• Test-tube rack was not clean or was not plastic coated.
Black spots on bottom of slides	• Slides were not drained well enough before drying • Forgot to dampen paper towels in test tube racks.
Exposure (black areas) on some or all slides	• Cracked black box • Black box not closed tightly • Tape not sealing well
Streaks of dark emulsion across slide	• Bubble in the elmusion sliding down the slide left a trail

d. Gently running water (10 min).
e. Place slides into a large staining jar with PBS (3 min),
f. Gently running water (10 min); 7) 70% ethanol (10 min).
h. Light alcoholic eosin stain (30 s).
i. Dehydration washes with 95% ethanol (5 min × 2).
j. Dehydration washes with 100% ethanol (5 min × 3).
k. Dehydration washes with Xylene (5 min × 3).

14. Cover slip slides with Permount or other neutral mounting medium.

4. Notes

1. When injecting mice with ^{3}H-thymidine/PBS solution, a bulge will occur in the skin of the mice. Remove the needle slowly so solution will not leak out of animal.
2. Proper temperatures, humidity, careful mixing, and cleanliness are essential for good autoradiographs. Ideally, one should use a dedicated darkroom for autoradiography.
3. When purchasing reagents for autoradiography, always use ACS or equivalent grade. Histology or technical grades often contain impurities that can ruin autoradiography.
4. Problems with autoradiographs and probable causes, *see* **Table 1**.

References

1 Bickenbach, J. (1981) Identification and behavior of label-retaining cells in oral mucosa and skin. *J. Dent. Res.* **60,** 1611–1620.
2. Morris, R. J. (2000) Keratinocyte stem cells: targets for cutaneous carcinogens. *J. Clin. Invest.* **106,** 3–8.

9

Method for the Harvest and Assay of In Vitro Clonogenic Keratinocytes Stem Cells From Mice

Wei-Yang Wu and Rebecca J. Morris

Summary

Primary adult mouse keratinocytes are harvested from the dorsal section of 6- to 8-wk-old mice. Euthanized mice are clipped and sterilized with serial washes in provodine iodine and ethanol solutions. The skin of the mice is removed and treated with trypsin to detach the dermis from the epidermis. Cells harvested from this proven method can be used for molecular biology, biochemistry, or a variety of cell culture techniques, including the clonogenic cell assay presented here.

Key Words:

Keratinocyte; harvest; primary; mouse; cell culture; skin.

1. Introduction

The skin is one of the largest organs of the body that serves a multitude of roles, including protection, thermal regulation, and water retention. Over the last 50 yr, research on the skin of mice has yielded new information on the structure and function of the skin as well as the mechanisms of carcinogenesis. Because of their usefulness in studies of hair follicle growth and carcinogenesis experiments, it is often desirable to isolate and culture primary epidermal keratinocytes from adult mice to use in conjunction with in vivo studies. The procedure presented here is a well-documented and successful method of harvesting primary keratinocytes from adult mice in addition to our assay for clonogenic keratinocytes is described ***(1–4)***.

2. Materials

2.1. Mice

Four to five adult female mice approx 6–8 wk of age should be obtained. Mice older than 8 wk will have entered into anagen, and the viability of cells from the preparation would be reduced. Additionally, epidermal trypsinization is more difficult if the mice are in the anagen stage of the hair-growth cycle. The harvesting procedure has been optimized for the thinner skin of female mice.

2.2. Fibroblast Cells

Swiss mouse 3T3 fibroblasts (ATCC, Rockville, MD; cat. no. CCL-92) should be obtained.

From: *Methods in Molecular Biology, vol. 289:*
Epidermal Cells: Methods and Protocols
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

2.3. Harvesting Tools

1. 500 mL Nalgene jar for washing mice (Fisher, Suwanee, GA; cat. no. 11-815-9E).
2. 10% Providone iodine surgical solution (Purdue Frederick Co., Stamford, CT; cat. no. NDC 0034-2100-87) (approx 150 mL).
3. Distilled or milliQ water. (We use freshly made double-distilled reverse osmosis water.)
4. 70% ethanol in water.
5. Autoclaved harvesting instruments in a beaker of 70% ethanol.
6. One pair of scissors (Biomedical Research Instruments, Rockville, MD; cat. no. 25-1050).
7. One pair of full-curve eye dressing forceps (Miltex, Bethpage, NY; cat. no. 18-784).
8. One pair of thumb dressing forceps (Miltex, Bethpage, NY; cat. no. 6-4).
9. No. 4 scalpel handles (Biomedical Research Instruments, Rockville, MD; cat. no. 26-1200).
10. No. 22 sterile stainless-steel blades (Biomedical Research Instruments, Rockville, MD; cat. no. 27-1330).
11. Sterile specimen cup (Fisher, Suwanee, GA; cat. no. 14-375-147).
12. Thin 100 × 10 sterile plastic Petri dishes (Fisher, Suwanee, GA; cat. no 08-747B).
13. Sterile conical tubes (50 mL, Falcon, Franklin Lakes, NJ; cat. no. 352068).
14. Disposable pipets (5 and 10 mL, Falcon, Franklin Lakes, NJ; cat. no. 5-mL pipet 357543; 10-mL pipet 357551).
15. Square sterile plastic Petri dishes (Fisher, Suwanee, GA; cat. no. 08-757-11A).
16. Microfuge tubes (Sarstedt, Newton, NC; cat. no. 72.608).
17. Glass funnel (Fisher, Suwanee, GA; cat. no. 10-384B).
18. 90-mm diameter Spectra-Mesh F, 70-µm pore size (Fisher, Suwanee, GA; cat. no. 08-670-198).
19. Nalgene jar (60 mL, Fisher, Suwanee, GA; cat. no. 11-815-10B) with a 1.5-inch stir bar with pivot ring (Fisher, Suwanee, GA; cat. no. 09-312-575).
20. 100 × 15 sterile plastic Petri dishes (Fisher, Suwanee, GA; cat. no. 08-757-12).
21. 70 µm teflon mesh (Fisher, Suwanee, GA; cat. no. 09-901C).
22. Oster Golden A5 small animal clipper with no. 40 blade (Miltex, Bethpage, NY; cat. no. 51-1000 (clipper) 51-1340 (blade).
23. Nalgene "Mr. Frosty" cell freezer (Fisher, Suwanee, GA; cat. no. 15-350-50).

2.4. Culturing Supplies

1. 150 cm^2 (T-150) culture flask (Corning, Corning, NY; cat. no. 430825).
2. 35-mm Sterile culturing dish (Corning, Corning, NY; cat. no. 430165).
3. 60-mm Sterile culturing dish (Corning, Corning, NY; cat. no. 430166).

2.5. Solutions

1. Dulbecco's phosphate-buffered saline, Ca^{2+} and Mg^{2+} free, sterile (Cambrex Biosciences, Walkersville, MD; cat. no. 17-515Q).
2. 0.25% Trypsin solution (Gibco, Rockville, MD; cat. no. 15050-057).
3. 0.4% Trypan blue in 0.9% saline. (Sigma, St. Louis, MO; cat. no. T-8154).
4. Antibiotics (penicillin–streptomycin, Sigma, St. Louis, MO; cat. no. P4333).
5. SMEM: Ca^{2+}- and Mg^{2+}-free minimal essential medium for suspension culture (Gibco, Grand Island, NY; cat. no. 11380-037).
6. Fetal bovine serum (FBS; Hyclone, Logan, UT; SH 30070.03).
7. Gentamycin (Cambrex, Walkersville, MD; cat. no. 17-518L).
8. Fibronectin (Sigma, St. Louis, MO; cat. no. F-1141).
9. Bovine serum albumin (BD Biosciences, Bedford, MA; cat. no. 354331).
10. Vitrogen collagen (Cohesion, Palo Alto, CA; cat. no. PC 0701).
11. 1 *M* HEPES (Cambrex, Walkersville, MD; cat. no. 17-737E).

12. Dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO; cat. no. D 2650).
13. Bovine calf serum (BCS) (Hyclone, Logan, UT; cat. no. SH 30073.03).
14. Williams E Medium (Gibco, Rockville, MD; cat. no. 125510-032).
15. Dulbecco's modified Eagle's medium (DMEM; Gibco, Rockville, MD; cat. no. 12100-061).

2.5.1. Harvesting Solutions

1. Phosphate-buffered saline (PBS) with 2X gentamycin: Dulbecco's PBS (500 mL) and gentamycin (2 mL).
2. Trypsin solution: PBS with 2X gentamycin (45 mL) and 2.5% trypsin (5 mL).

2.5.2. Cell Culture Solutions

1. Vitrogen–fibronectin dish-coating solution: cell culture medium (100 mL); fibronectin (1 mg); 1.0 mg/mL stock bovine serum albumin stock (10 mL); vitrogen collagen (1 mL); 1 *M* HEPES (1 mL); and 116 m*M* $CaCl_2$ (1 mL).
2. DMSO–DMEM cell-freezing solution: DMSO (2 mL) and DMEM with 10% BCS and Pen/Strepl (18 mL).

2.5.3. Medium

1. "Harvesting" medium: SMEM (500 mL); FBS (50 mL); and gentamycin (2 mL).
2. Medium used for mass culture is a high-calcium Williams E medium formulation with the following additives (*see* **Table 1**).
3. DMEM (formulated for 5% CO_2):
   a. place 8 L of distilled water into large, media flask.
   b. place flask on magnetic stirrer with stir bar and add DMEM powder while stirrer is on.
   c. rinse empty bottle of DMEM with distilled water and pour in flask.
   d. stir with a 25-mL pipet to aid dissolving of powder.
   e. measure out 22 g NaCl and 2 g $NaHCO_3$ and add to flask.
   f. add 2 L of distilled water to bring final volume to 10 L.
   g. adjust pH to 7.2–7.4.
   h. sterile filter with 0.22-µm filter and fill to 900-mL mark of 1-L media bottles.
4. 3T3 fibroblast medium: 900 mL DMEM; 100 mL BCS; and 10 mL penicillin–streptomycin.

3. Methods

3.1. 3T3 Feeder Layer

3.1.1. Initialization of Culture

1. Quickly thaw one vial containing frozen 3T3 cells from –80°C or liquid nitrogen canister by placing vial in 37°C water bath for 1–2 min. Remove vial when final sliver of ice has melted. Wipe the tube with 70% alcohol swab and very gently pipet cells three times with a 1-mL pipet in a biosafty cabinet or laminar flow hood. Pipet entire contents into a T-150 flask and rinse vial with 1 mL medium. Add 30 mL of warmed DMEM slowly to the cells. Gently rock flask to evenly spread the cells. Label flask with date and passage number of cells. Incubate at 37°C with 5% CO_2 and 100% humidified air.
2. Medium is changed 24 h later to remove dead cells and DMSO crypopreservation agent. Medium is changed twice a week. The cells are only allowed to proliferate to 80% confluency.

3.1.2. Subculture

1. Remove flask from incubator and wash twice with cold sterile PBS without gentamycin. Pipet 10 mL of prewarmed (37°C water bath) trypsin solution per T-150 flask. Place flask

Table 1
William's E Medium Supplements

Stock solution	Source[a]	Formulation
Epidermal growth factor	B #354001	100 µg/20 mL of H_2O
Glutamine	C #17-605B	14.5-mL aliquots
Hydrocortizone	B #354203	50 mg/50 mL ethanol
Insulin	S #I1882	200 mg/80 mL of H_2O
Linoleic acid–bovine serum albumin	B #354227	2.5 mg LA/500 mg BSA/25 mL of H_2O
Transferrin	S #T1147	1 g/200 mL of H_2O
Vitamin A (retinyl acetate)	S #R0635	1 mg/1 mL ethanol
Vitamin D_2	S #E8014	10 mg/1 mL ethanol
Formulating 500 mL medium, add:		
Epidermal growth factor	1 mL	
Glutamine	14.5 mL	
Hydrocortizone	0.5 mL	
Insulin	1 mL	
Linoleic acid–bovine serum albumin	0.5 mL	
Transferrin	1 mL	
Vitamin A (retinyl acetate)	57.5 µL	
Vitamin D_2	50 µL	
Penicillin/streptomycin	5 mL	
FBS	100 mL	

[a]The abbreviations used are: S, Sigma (St. Louis, MO); B, BD Biosciences (Bedford, MA); C, Cambrex (Walkersville, MD).

in incubator for 3–5 min. Remove flask and gently rap the sides of the flask to loosen any cells that are attached. Confirm cell detachment with inverted microscope. Trypsin solution should be cloudy with detached cells.

2. Pipet the solution five times washing the growth surface of the flask. Remove the entire contents and place in 30 mL DMEM in a 50-mL conical tube. Take 10 mL of this DMEM mixture and rewash the growth surface of the flask five times. Two T-150 flask contents can be placed in one 50-mL conical tube.
3. Centrifuge at 160*g* for 7 min at 4°C.
4. Aspirate supernatant and resuspend cell pellet with 5 mL of DMEM triturating gently 10 times. Add 25 mL more medium to bring total volume to 30 mL. Triturate again 10 times and place 5 mL of cell suspension into 6 T-150 flasks. The subculture ratio is 1:3.
5. Label flask with date and passage number.

3.1.3. Freezing 3T3

1. Follow methods of subculturing 3T3 cells up to **step 3**.
2. Remove tube from centrifuge and place on ice. Aspirate supernatant and resuspend cell pellet with 1 mL DMSO–DMEM cell mixture for each T-150 flask harvested.
3. Place 1 mL of the cell suspension per vial. Label vial with passage number, cell line (3T3), and date they were frozen.
4. Place vials into Nalgene cell freezer and place in a –70°C freezer for 5+ h. Remove vials and place in liquid nitrogen storage tank.

3.1.4. Irradiation and Seeding of 3T3 Feeder Layer

1. One week prior to irradiation of cells, allow cells in flasks to grow to 100% confluence. The cells used are normally within the passages of 120 to 130.
2. The cells within the flasks are irradiated with 5000 rads with a ^{137}Cesium Gamma-cell 40 unit. The entire flask with medium can be irradiated. Depending on the machine, this procedure can take up to an hour.
3. After irradiation, medium is aspirated and cells are washed twice with cold sterile PBS with gentamycin. Pipet 10 mL of pre-warmed (37°C water bath) trypsin solution per T-150 flask. Place flask in incubator for 3–5 min. Remove flask and gently rap the sides to loosen any cells that are attached. Confirm cell detachment with an inverted microscope. Trypsin solution should be cloudy with detached cells.
4. Pipet the solution five times washing the growth surface of the flask. Aspirate the entire contents and place in 30 mL DMEM in a 50-mL conical tube. Take 10 mL of this DMEM mixture and rewash the growth surface of the flask five times. Two T-150 flask contents can be placed in one 50-mL conical tube.
5. Centrifuge at 160*g* for 7 min at 4°C.
6. Aspirate supernatant and resuspend cell pellet with 5 mL of DMEM triturating gently 10 times. Add 25 mL more medium to bring total volume to 30 mL. Triturate again 10 times and remove approx 0.5 mL of the cell mixture and place into a small sterile tube. Remove 200 µL of this cell mixture and mix in 200 µL of 0.4% Trypan blue solution. Pipet this mixture three times gently. Place cells within a hemocytometer and count all nucleated cells. All dark or blue cells are scored as nonviable, whereas small gold cells are scored as viable.
7. Calculate number of cells and cell concentration and pipet 7×10^5 cells into 60-mm Petri dishes. Allow cells to attach for 24 h prior to seeding primary keratinocytes.

3.2. Keratinocyte Harvest and Seeding

1. Euthanize four to five mice with CO_2 inhalation for 1.5 min followed by cervical dislocation (*see* **Note 1**). Clip approx 15–18 cm^2 of the dorsal fur with electric animal clipper and place the mice in a jar with enough providone iodine solution to cover. Shake the jar well as to get even distribution of the solution over the mice. Pour off the solution and rinse with distilled or Milli-Q water until clear. Repeat again with another iodine wash followed by water rinse. Rinse two times with 70% ethanol accompanied with shaking. After final rinse, add enough 70% ethanol to cover the mice and let them soak for 5 to 10 min. Mice with light fur will retain a yellow color, while darker mice will not. The yellow color does not cause any obvious changes to cell viability or ability to grow in culture.
2. Within a laminar flow hood or a biosafty cabinet, remove the dorsal skin (clipped portion only) using thumb forceps and scissors and place skins in a cup with PBS with 2X gentamycin. Do not attempt to remove skin further below the sides as this could contaminate the harvest with unwanted mammary cells.
3. With forceps and scalpel, remove a skin and place with the hairy side facing down on a Petri dish (bottom or top can both be used). Scrape all subcutaneous tissue from the skin with the blade of the scalpel perpendicular to the skin until the skin is semitranslucent in a swift manner as to not allow the skins to dry out. Attempt to remove all traces of subcutaneous tissue, but do not scrape so hard as to tear the skin or to remove the hair follicles through the dermis. Place the skin back into PBS until all other skins are processed.
4. Take a skin on a Petri dish hairy side up and spread it out. Bisect the skin down the length and cut the skin into 0.5×1- to 1.5-cm strips.
5. Pour approx 15–25 mL of sterile trypsin solution into Petri dishes. Use forceps and float the skins hairy side up on the surface of the trypsin solution. Place Petri dishes in 32°C incubator for 2 h (*see* **Note 2**). During the incubation time, coat the dishes with the vitro-

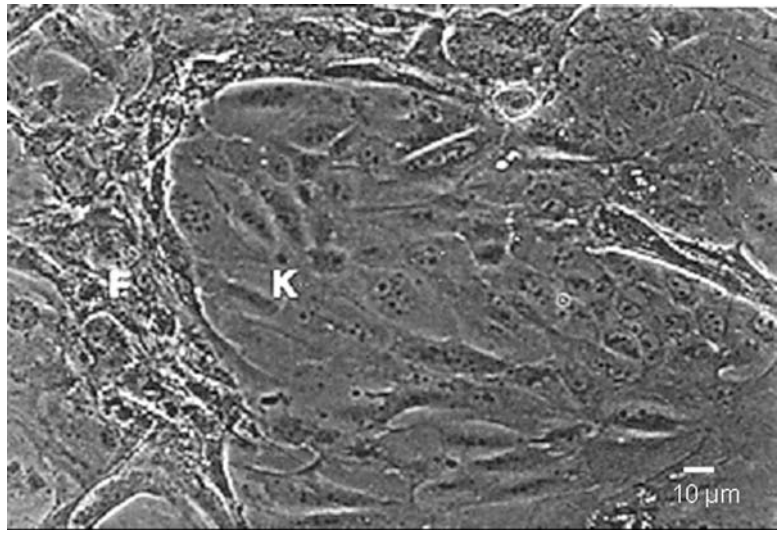


Fig. 1. Photomicrograph of keratinocyte colony on an irradiated 3T3 layer. Keratinocyte colony is denoted by (K). The fibrous cellular growth surrounding the dense cellular layer is the 3T3 fibroblast feeder layer denoted by (F).

gen-fibronectin coating solution and place them in a 37°C incubator for 1 h (*see* **Note 3**). After allotted time, aspirate off any remaining fluid from coating solution. Dishes do not need to dry.

6. Prepare a square Petri dish propped up on the lid at an angle (with a microfuge cap) with 10 mL SMEM. Remove a strip of skin with forceps and with a new scalpel blade scrap off the epidermis into the medium. Special attention needs to be paid to scraping the epidermis (*see* **Note 4**). Use sufficient force, but do not use excessive amount or the cell preparation will result in lower viability. Discard the dermis or retain it for confirmation of the full removal of the hair follicle with the epidermis.
7. Carefully pour the medium with the epidermis into a sterile 60 mL of Nalgene jar with a 1.5-in stir bar. Rinse the Petri dish with additional medium and bring the final amount in the jar to 30 mL and cover with screw cap. Stir at 100 rpm on a magnetic stirrer for 20 min at room temperature.
8. Place a sterile piece of 70-µm teflon mesh in a sterile funnel on top of a 50-mL conical tube. Pour contents from jar into mesh to strain out hair and sheets of stratium corneum. Use curved forceps and press the hair and materials within the mesh to allow trapped cells to flow into tube. Rinse jar with 5 mL SMEM and pour into mesh and repeat to remove additional cells within jar. Cap tube and centrifuge at 160*g* for 10 min in 4°C. If required, material within mesh could be used for further histological analysis. Mesh can be reused by washing with warm water and Liqui-Nox or other tissue culture detergent and rinsed well with distilled water.
9. Aspirate the supernatant, add 5 mL of refrigerated SMEM, and resuspend the cells by triturating 20–25 times with a 5-mL pipet. Be sure to keep the cells at 4°C to avoid aggregation of the cells. Add 25 mL of additional SMEM and triturate again for 20–25 times with a 5-mL pipet. Remove 1 mL of this cell suspension and add to 19 mL of SMEM for a 1 : 20 dilution of the cells and triturate again 20–25 times with a 5-mL pipet. This dilution is to ensure an accurate cell count.
10. Remove approx 0.5 mL of the 1 : 20 dilution cell mixture and place into a small sterile tube. Remove 200 µL of this cell mixture and mix in 200 µL of 0.4% Trypan blue solution. Pipet

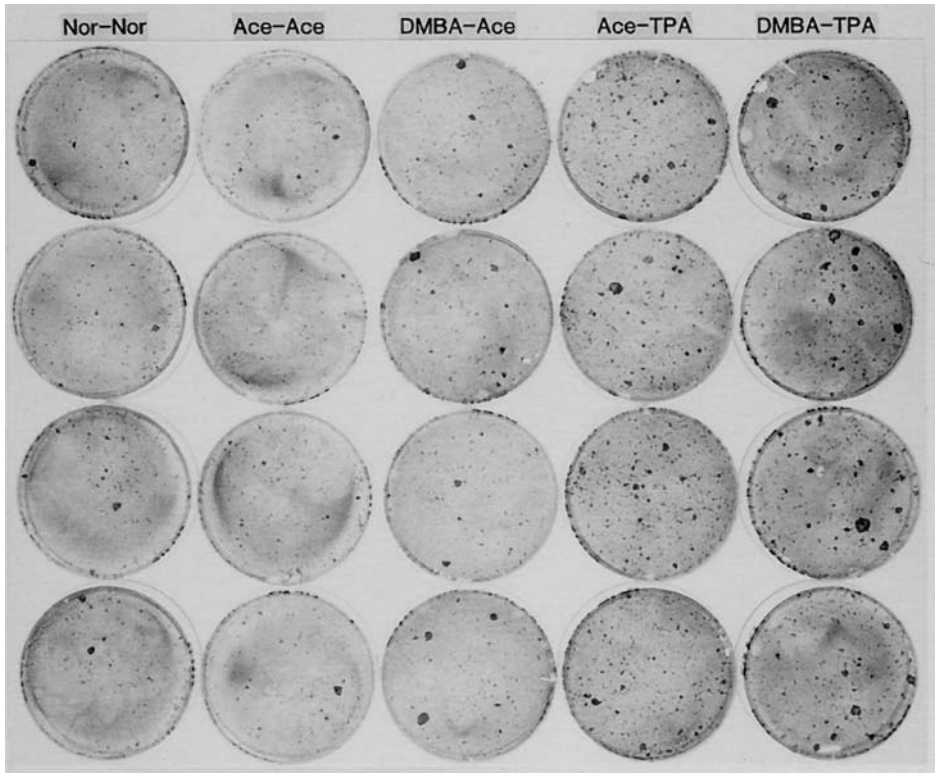


Fig. 2. Colony growth of keratinocytes from mice from different treatments. Keratinocyte colonies on 3T3 feeder layer in 60-mm dishes stained with 0.5% rhodamine B. Nor-Nor, Normal controls; Ace-Ace, treatment with acetone followed 24 h by second acetone treatment; DMBA–Ace, treatment with 7,12-dimethylbenz[a]anthracene (DMBA) followed 24 h by acetone treatment; Ace–TPA, treatment with acetone followed 24 h by 12-*O*-tetradecanoylphorbol-13-acetate (TPA); DMBA-TPA, treatment with DMBA followed 24 h by TPA.

this mixture three times, gently. Place cells within a hemocytometer and count all nucleated cells. All dark or blue cells and large gold ones are scored as non-viable, whereas small gold cells are scored as viable. Yields of cells should range from 20 to 25 $\times$ 10^6 cells per mouse.

11. Centrifuge original tube for 10 min at 1000 rpm. Resuspend cells in 5 mL of medium. Make appropriate dilutions of cells for seeding. Cells are usually seeded at 4 $\times$ 10^6 viable cells per 35-mm dish or 1.5 $\times$ 10^5–1.9 $\times$ 10^5 cells per cm^2 for mass culture or 1 $\times$ 10^3 cells per 60 mm dish for clonogenic cell assays (*see* **Note 5**). Place 2 and 4 mL of medium for 35-mm and 60-mm dishes, respectively.
12. The cultures are incubated in a 32°C, 100% humidified incubator with 5% CO_2. The medium is changed the day after initial seeding and three times weekly thereafter for mass cultures. For clonal cultures, the first medium change is 2 d after seeding and three times weekly thereafter.
13. Typically, clonal cultures **(Fig. 1)** are cultivated for 2- and 4-wk intervals. At these time points, the medium is aspirated and the cultures fixed in 10% buffered formalin overnight. After fixation, the cultures are stained with 0.5% rhodamine B in distilled water for 30 min. The stain is then removed and the dishes are rinsed in cold tap water for 10 s. After drying, the colonies are counted **(Fig. 2)**.

4. Notes

1. The outlined procedure can be used for single mice also. The only change to the procedure would be the final dilution of the cell suspension. Instead of 30 mL, use 6 mL for the main suspension.
2. Trypsinization time and temperature are critical for obtaining good yields of highly culturable cells. Although other methods may give good yields of viable cells, the culturablility of the keratinocytes has been less satisfactory in our hands.
3. Coating of culture dishes is very important for attachment, spreading, and ultimate growth of epidermal cells from adult mice.
4. During epidermis scraping step, it is imperative to keep the blade perpendicular to the skin. If the blade is angled toward the motion of the blade, a tendency to tear the tissue will occur. If the blade is angled away from the motion of the blade, insufficient amount of the epidermis will be removed.
5. When performing a quantitative clonal assay, we have found it helpful never to pipet less than 1 mL of cells. Thus, if cells in the first 20-mL dilution (*see* **Subheading 3.2., step 10**) are concentrated, then serial dilutions should be made to the cloning dilution.

References

1 Morris, R. J., Fischer, S. M., and Slanga, T. J. (1986) Evidence that a slowly cycling subpopulation of adult murine epidermal cells retains carcinogen. *Cancer Res.* **46,** 3061–3066.
2 Baer-Dubowska, W., Morris R. J., Gill, R. D., and DiGiovanni, J. (1990) Distribution of covalent DNA adducts in mouse epidermal subpopulations after topical application of benzo(a)pyrene and 7,12-dimethylbenz(a)anthracene. *Cancer Res.* **50,** 3048–3054.
3 Tani, H., Morris, R. J., and Kaur, P. (2000) Enrichment for murine keratinocyte stem cells based on cell surface phenotype. *Proc. Natl. Acad. Sci. USA* **97,** 10,960–10,965.
4. Trempus, C. S., Morris, R. J., Bortner, C. D., Cotsarelis, G., Faircloth, R. S., Reece, J. M., et al. (2003) Enrichment for living murine keratinocytes from the hair follicle bulge with the cell surface marker CD34. *J. Invest. Dermatol.* **120,** 501–511.

10

FACS Enrichment of Human Keratinocyte Stem Cells

Amy Li and Pritinder Kaur

Summary

Recent work from our laboratory has led to the development and validation of fluorescence-activated cell sorting (FACS)-based techniques to prospectively isolate viable keratinocyte stem cells from both human and murine skin. Here we describe a step-by-step method to apply our technique to isolate epidermal keratinocytes from skin tissue, process them for immunofluorescent staining for cell surface markers, and subject them to fluorescence-activated cell sorting to obtain the stem, transient amplifying, and early differentiating keratinocyte fractions. These viable cells can then be placed into culture for further analysis or directly into keratinocyte assays, such as organotypic cultures or in vivo transplantation. This method will be useful for the complete biological characterization of keratinocyte progenitors with respect to wound healing, carcinogenesis, and therapeutic manipulation.

Key Words:

Keratinocyte stem cells; FACS separation; immunofluorescent labeling; epidermis; cell surface markers; keratinocyte progenitors; integrin; CD71.

1. Introduction

Isolation of keratinocyte stem cells (KSCs) is central to the study of all aspects of skin biology, including tissue regeneration, wound healing, carcinogenesis, and the development of gene therapeutic approaches. KSCs represent between 1 and 10% of the basal cells ***(1–5)*** and are identified as slow cycling, [^{3}H]Tdr label-retaining cells ***(2,4,6,7)***. They reside in the basal epidermal layer along with their rapidly cycling but short-lived progeny, that is, transit-amplifying (TA) cells and early differentiating cells, and are ultimately responsible for the constant regeneration of terminally differentiated cells lost from the skin surface ***(1–3,8–11)***. These kinetic studies allowed the detection of KSCs in murine tissues *in situ*. The absence of specific cell surface markers for KSCs has hampered the development of techniques to identify and isolate human keratinocyte stem cells ex vivo. Pioneering studies used the proliferative capacity and differentiation fate of daughter cells to retrospectively identify human KSCs in culture ***(12)***. An important advance in identifying cell surface markers to distinguish basal keratinocytes with heterogeneous proliferative potential was made with the discovery that the level of β_1-integrin on human keratinocytes could be correlated with their colony forming efficiency ***(13,14)***. Although high levels of β_1 integrin expression were thought to identify KSCs, subsequent studies revealed that the β_1 integrin bright population comprised the majority of the proliferative basal cells (KSCs and

From: *Methods in Molecular Biology, vol. 289:*
Epidermal Cells: Methods and Protocols
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

TA cells) as well as some differentiating cells ***(15–17)***. Importantly, work from our laboratory has demonstrated that KSCs can be identified and isolated from human skin on the basis of two cell surface markers: α_6-integrin and CD71. Specifically, basal keratinocytes expressing high levels of α_6 and low levels of CD71 (designated $\alpha_6^{bri}CD71^{dim}$) fulfill many important criteria of stem cells (including quiescence; low incidence; greatest keratinocyte regenerative capacity in both short-term and long-term culture; small cell size; high nuclear-to-cytoplasmic ratio; absence of differentiation markers; **ref.** ***18***) and can regenerate a normal fully stratified epidermis in an in vitro organotypic culture system. Moreover, murine basal keratinocytes with the same cell surface phenotype are enriched for the label-retaining stem cells ***(19)***, providing the first direct link between a kinetically distinct in vivo stem cell population and cell surface phenotype. In this chapter, the isolation of this important subpopulation of basal keratinocytes and the TA ($\alpha_6^{bri}CD71^{bri}$) and differentiating (α_6^{dim}) cells on the basis of their cell surface phenotype using fluorescence-activated cell sorting (FACS) is described. This method permits the prospective isolation of these three classes of keratinocyte progenitors in a viable state for further characterization.

2. Materials

2.1. Antibodies for Immunolabeling (see also *Table 1)*

1. Mouse anti-CD49f (Serotec; cat. no. MCA956).
2. Biotinylated anti-human mouse CD71 (PharMingen BD; cat. no. 555535).
3. Mouse IgG2b isotype control (PharMingen BD; cat. no. 555740).
4. Biotinylated mouse IgG2a isotype control (PharMingen BD; cat. no. 555572).
5. Goat anti-mouse IgG2b-FITC (fluoroscein isothiocyanate; 1:80; Caltag; cat. no. M32501).
6. Strepavidin allophycocyanin (APC; 1:250; PharMingen BD; cat. no. 554067; *see* **Note 1** for alternative fluorochromes).

2.2. Instruments and Tubes

1. Dissecting scissors, curved, 115 mm (ProSciTech, Australia; cat. no. T1046).
2. Fine point-curved forceps (7A style; ProSciTech, Australia; cat. no. T67A).
3. Scalpel holder (ProSciTech, Australia; cat. no. T134) and blades (no. 22; ProSciTech, cat. no. LSB22).
4. 70-µm Cell strainers (Becton Dickson; cat. no. 352350).
5. Polypropylene round-bottom FACS tubes (5 mL, Becton Dickinson; cat. no. 352063).
6. Round-bottom tubes with cell strainer cap (5 mL, Becton Dickinson, cat. no. 352235).

2.3. Source of Human Skins

Human neonatal foreskin and split-thickness adult skin obtained from mammoplasty or abdominoplasty from consenting donors.

2.4. Reagents

All reagents were 0.22-µm filter-sterilized and stored at 4°C unless specified otherwise.

2.4.1. Processing of Skin Specimens

1. RPMI-AF (medium containing antifungal and antibiotic agents): RPMI-1640 (488.5 mL); penicillin/gentamycin (600 µg/mL and 8 mg/mL in Milli-Q H_2O; 10 mL); fluconazol (2 mg/mL solution; Pfizer Pty Ltd, France; 1.5 mL).

Table 1
Summary of the Controls Required for Flow Cytometry and the Primary Antibodies Used in the Immunofluorescent Labeling

	Number of cells	Antibodies	Final antibody concentration	Volume of staining
Unstained control	$1–2 \times 10^5$	N/A	N/A	100 µL
7-AAD control	$1–2 \times 10^5$	N/A	N/A	100 µL
α_6-FITC single-color control	$1–2 \times 10^5$	Mouse anti-CD49f	10 µg/mL	100 µL
CD71-APC single-color control	$1–2 \times 10^5$	Mouse anti-CD71-biotin	5 µg/mL	100 µL
Isotype matched controls	$1–2 \times 10^5$	Mouse IgG2b and mouse IgG2a-bio	10 µg/mL and 5 µg/mL, respectively	100 µL
Sample tube	$10^6–10^7$	Anti-CD49f and anti-CD71-biotin	10 µg/mL and 5 µg/mL, respectively	200 µL–800 µL[a]

[a]Adjust the volume according to cell number.

2. PBS-AF: Phosphate-buffered saline without Ca^{2+} or Mg^{2+} (488.5 mL); penicillin/gentamycin (600 µg/mL and 8 mg/mL in Milli-Q H_2O; 10 mL), fluconazol (2 mg/mL solution; Pfizer Pty Ltd, France; 1.5 mL).
3. Dispase solution: Neutral dispase II (Roche Diagnostics; cat. no. 165 859) at 4 mg/mL in PBS-AF. Dissolve in PBS and filter sterilize by passing through a 0.22-µm filter just prior to use.

2.4.2. Isolation of Keratinocytes From Skin Specimens

1. Trypsin–EDTA: 0.05% v/v trypsin and 0.025% v/v EDTA (Trace Biosciences; cat. no. 21-160-0100V).
2. Trypsin inhibitor: DMEM (500 mL), bovine serum albumin (BSA), tissue culture grade; Serologicals, GA; cat. no. 82-047-5; 0.1 g); soybean trypsin inhibitor (Sigma; cat. no. T9003; 0.01 g).
3. Trypan blue solution: 0.4% (Sigma; cat. no. T8154).

2.4.3. Immunofluorescent Labeling

1. Keratinocyte growth medium (KGM): KBM (Clonetic, CC-3101; 500 mL), penicillin/gentamycin (600 µg/mL and 8 mg/mL in Milli-Q H_2O; 5 mL), hydrocortizone (500 µg/mL in absolute EtOH; Sigma; cat. no. H-0135; 500 µL), insulin (5 mg/mL in 12 m*M* HCl; Sigma; cat. no. I-5500; 500 µL), epidermal growth factor (100 µg/mL in 0.22-µm filter-sterilized 10 m*M* acetic acid with 0.1% BSA; Sigma; cat. no. E-4127; 50 µL), bovine pituitary extract (35 mg/mL; Hammond Cell Tech; cat. no. 1077; 1 mL).

Hydrocortizone is stored at 4°C in a tightly sealed bottle, protected from light. Epidermal growth factor is reconstituted and stored at –80°C. All other supplements are prepared, 0.22-µm filter-sterilized, and aliquoted for storage at –20°C.

2. Blocking buffer: KGM (49 mL), BSA (1 g), fetal calf serum (CSL; 1 mL).
3. Labeling and wash buffer: KGM (500 mL), BSA (5 g).
4. 7-Aminoactinomycin D (7-AAD; Sigma; cat. no. A9400): prepare aliquots of 100 µg/mL in Milli-Q H_2O and store in the dark at –20°C.

3. Methods

3.1. Processing of Skin Samples

This section describes the procedure for preparing the skin specimens for keratinocyte isolation (*see also* **Note 2**).

3.1.1. Collection of Skin Samples

All skin samples from surgery are immediately immersed in normal saline or RPMI-AF and kept at 4°C. Specimens should be processed as soon as practicable. Cell isolation from tissue stored for more than 24 h is not recommended.

3.1.2. Processing of Adult Human Skin

To improve cell recovery from adult skin, it is preferable to reduce the skin to 1- to 2-mm thick dermatome slices before processing to remove all adipose tissue. Alternatively, the fatty tissue can be removed by trimming with a scalpel blade. Cut the skin into pieces of approx 2 × 3 cm (the equivalent of one foreskin) and rinse thoroughly in PBS-AF. Process the pieces following the steps outlined for foreskins in **Subheading 3.1.3.**

3.1.3. Processing of Human Neonatal Foreskins

Set up the following in a biohazard lamina flow hood: two 100-mm Petri dishes containing approx 15 mL of cold (4°C) PBS-AF for every foreskin; 10 mL of dispase solution per 60-mm Petri dish per two foreskins; autoclaved instruments; and beakers with 70% ethanol and sterile PBS-AF.

1. Rinse the skin thoroughly in a Petri dish of PBS-AF.
2. Place the skin dermal side up onto the sterile inverted lid of a Petri dish. Remove excess connective tissue by trimming with curved scissors while pressing the skin flat with a pair of forceps.
3. Using a scalpel blade, firmly scrape the underside of the skin to remove any remaining connective tissue.
4. Rinse the skin thoroughly in the second dish of PBS-AF.
5. Place the skin onto another sterile inverted lid and cut into small pieces of approx 2 × 2 mm using a new scalpel blade. Transfer the skin pieces into the dispase solution.
6. Seal the dish with parafilm and incubate overnight at 4°C, allowing the enzyme to cleave the basement membrane proteins thus dissolving epidermal–dermal attachments ***(20)***. The $\alpha_6\beta_4$ integrins remain intact during this process ***(21)***.

3.2. Isolation of Keratinocytes

This section describes the procedure for isolating keratinocytes from the dispase treated skin specimens. In a biohazard lamina flow hood, set up the following: dissecting instruments; beakers with 70% EtOH and sterile PBS-AF for the instruments; two 60-mm Petri dishes containing 10 mL of cold PBS-AF on ice per dish of dispase treated skin; sterile plastic transfer pipets; and trypsin inhibitor on ice.

1. Warm up 10 mL trypsin–EDTA for every dish of dispase treated skin to be processed in a 50-mL polypropylene tube in a 37°C water bath until use.
2. Place the skin pieces dermal side down on the inverted lid of a Petri dish, and while holding the dermal tissue, gently peel away the epidermis with another pair of forceps. Place the epidermal sheets directly into a petri dish containing PBS-AF.
3. Carefully aspirate the PBS-AF from the Petri dish using a plastic transfer pipet, then gather the epidermal pieces and transfer into the tube of prewarmed trypsin–EDTA with forceps. Actively trypsinise by pipetting up and down vigorously using a plastic transfer pipet for exactly 5 min, passing the epidermal sheets through the pipet as much as possible. The epidermal sheets remain intact during this process although basal cells are removed from them into the trypsin solution.
4. Quench the reaction by adding an equal volume (10 mL) of trypsin inhibitor. Mix gently and place on ice.
5. Repeat **steps 3** and **4** for the other dishes of skin.
6. Filter the digest through a 70-μm cell strainer into a 50-mL tube. Use one strainer per 20 mL of cell suspension.
7. Centrifuge at 400*g* for 5 min at 4°C.
8. Aspirate the supernatant and disperse the pellet by flicking the tube. Resuspend in 3 mL of wash buffer and transfer all the cells into one tube.
9. Dilute a small aliquot of the cell suspension with equal volume of 0.4% Trypan blue and count the viable and dead cells on a haemocytometer. Typically 2–3 $\times$ 10^6 cells can be obtained per foreskin with >90% viability.

3.3. Immunofluorescent Labeling of Keratinocytes

This section describes the process of labeling keratinocytes with anti-α_6 integrin and anti-CD71 antibodies. All labelling is performed on ice in 5-mL polypropylene round-bottom FACS tubes, and cells are handled aseptically in a biohazard lamina flow hood.

1. Centrifuge the cell suspension at 400*g* for 5 min at 4°C and resuspend the cells in 3 mL blocking buffer. Transfer the cell suspension into a 5-mL FACS tube, this is the sample tube. Incubate on ice for 15 min.
2. Transfer 1–2 $\times$ 10^5 cells into 5-mL FACS tubes for the controls required to set up the flow cytometer (*see* **Table 1**).
3. Pellet the cells and add the specified amount of antibody(s) to the dispersed cell pellet in each tube, mix well and incubate on ice for 45 min. Agitate every 15 min. The unstained and 7-AAD tubes are kept on ice until needed. When processing a large number of cells, that is, >10^7, the tube is placed on a rotator in the cold (4°C) room to prevent cell sedimentation and clumping.
4. Prepare a cocktail of the secondary antibodies, goat anti-mouse IgG2b-FITC (1 : 80), and Streptavidin-APC (1 : 250) in blocking buffer. The volume required per tube is the same as for the primary antibodies (*see* **Table 1**).
5. Wash the cells by adding 3 mL of wash buffer to the tubes, centrifuge at 400*g* for 5 min at 4°C, aspirate the supernatant, and disperse the cell pellet by flicking the tube. Repeat with another 3 mL of wash buffer.
6. Add the appropriate amount of the antibody cocktail to the dispersed cell pellet in each tube, mix well, and incubate on ice for a further 45 min. Agitate every 15 min.
7. Wash the cells twice (*see* **step 5**) and after the final wash, resuspend the cells in the sample tube to a final concentration of 3 $\times$ 10^6 cells/mL in wash buffer. The controls are resuspended in 500 μL of wash buffer.

8. If cell clumps are observed, filter the cell suspension through to a 5-mL round bottom tube with cell strainer cap to remove them since these can block the flow cytometer.
9. Just prior to sorting, add 7-AAD (2 µg/mL final) to all the tubes except the unstained tube.

3.4. Flow Cytometry

This section describes the basic approach to setting up the cell sorter for the analysis and sorting of immunofluorescent labeled keratinocytes. All samples are analysed on a Becton Dickinson FACS Vantage™, in which forward light scatter (FSC) is collected through a 488-nm band pass 10 filter, and a one decade neutral density filter in the forward light scatter path, and side scatter (SSC) is collected through a 488/10 band pass filter at 90°. Fluorescin isothiocyanate (FITC) and 7-AAD are excited by the 488-nm laser light and their fluorescence emissions are collected though the 530DF30 filter in fluorescence channel 1 (FL1), and the 670 long-pass filter in FL3 respectively. APC is excited by the 647-nm laser light and its fluorescence emissions are collected through the 670DF14 filter in FL6. For data analysis, a minimum of 10,000 viable events are acquired for each list mode file using the CellQuest software (Becton Dickinson). For samples labeled with two or more fluorochromes, the single-color controls (i.e., cells stained with one fluorochrome), in addition to the unstained and 7-AAD controls, are required to set up of the flow cytometer correctly (*see* **Note 3**).

1. Create an analysis template by setting up the following dot-plots: forward scatter (FSC) vs side scatter (SSC); FSC vs 7-AAD; FITC vs APC, place a quadrant at 10^1; and FITC vs 7-AAD, place a quadrant at 10^1.
2. Run the unstained cells. Adjust the FSC and SSC settings until the cells appear in the middle of the FSC vs SSC dot plot similar to that shown in **Fig. 1A**. Then adjust the PMT voltage of the FITC, APC, and 7-AAD detectors until the cells appear within the lower left quadrant of the different dot-plots (e.g., *see* **Fig. 1C**), thus setting the negative fluorescence levels in these parameters.
3. Change to the 7-AAD control tube. 7-AAD is a deoxyribonucleic acid staining dye that readily passes through compromised cell membranes of dead and dying cells. Subtract the 7-AAD fluorescence emissions detected by the FITC detector by increasing the amount of FL1-%FL3 until the 7-AAD cells appear negative in the FITC fluorescence parameter in the FITC vs 7-AAD dot-plot. Correct compensation is achieved when the median value of cells in the lower left quadrant is equal to the median value of cells in the upper left quadrant in the FITC (FL1) parameter. 7-AAD compensation is not required for the APC detector (*see* **Note 3**). Place the sort region R1 around the negative, viable cells as shown in **Fig. 1B**.
4. Change to the CD49f-FITC single-color tube. Two discrete populations of α_6-expressing cells should be observed **(Fig. 1D)**. Subtract the FITC fluorescence emissions detected by the 7-AAD detector by increasing the amount of FL3-%FL1 until the cells appear negative in the 7-AAD fluorescence parameter. Verify that the correct amount of compensation was applied by ensuring that the median value of cells in the lower right quadrant and the lower left quadrant in the 7-AAD (FL3) parameter are equal. FITC compensation is not required for the APC detector (*see* **Note 3**).
5. Change to the CD71-APC single colour tube. The typical CD71-APC expression in a FITC vs APC dot plot is shown in **Fig. 1E**. APC compensation is not required for the FITC or 7-AAD detectors (*see* **Note 3**).
6. Run the isotype control tube. This is used to determine the nonspecific binding of the isotype-matched primary antibodies. In general, a few events of nonspecific binding are observed (*see* **Fig. 1C**).

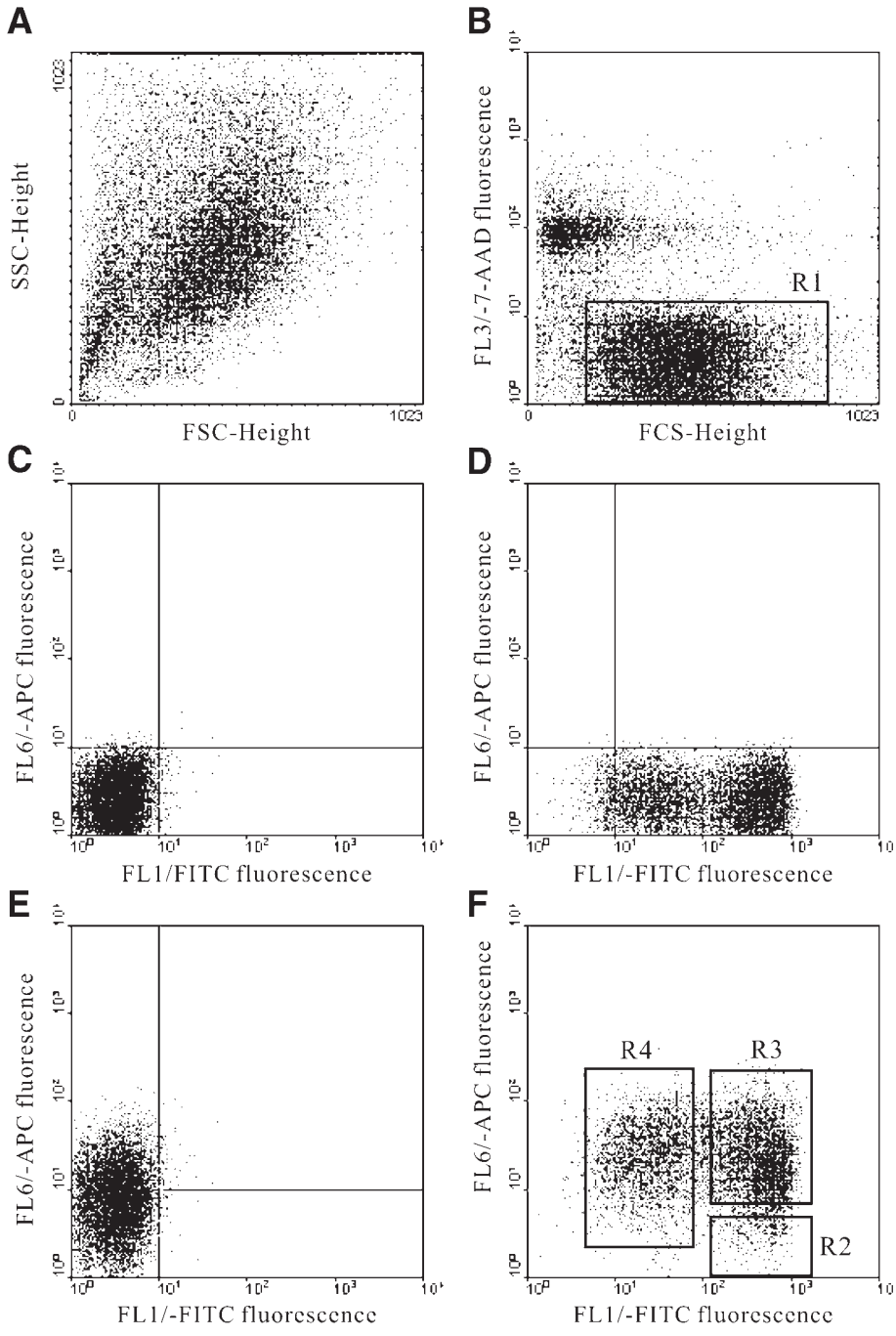


Fig. 1. Fluorescence profiles of human foreskin basal keratinocytes stained with α_6-integrin and CD71 and controls. **A,** Light scatter plot of foreskin basal keratinocytes. **B,** Dot-plot of FSC vs 7-AAD fluorescence showing the negative (viable) cells, which is gated in region R1, excluding the population of 7-AAD-positive (dead) cells. **C,** Two color dot-plot of keratinocytes stained with the isotype matched control antibodies. **D,E,** Fluorescence profile of the α_6-FITC and the CD71-APC single-color control, respectively. **F,** Two-color fluorescence dot-plot of keratinocytes labeled simultaneously for α_6-FITC and CD71-APC. R2 = $\alpha_6^{bri}CD71^{dim}$ = KSCs; R3 = $\alpha_6^{bri}CD71^{bri}$ = TA cells; and R4 = α_6^{dim} = differentiating cells.

7. Run the α_6/CD71 dual-stained sample. Apply the viability gate defined by R1 to the FITC vs APC dot-plot to exclude cell debris and nonviable cells from analysis. The highly reproducible (n > 300) two-color fluorescence profile of basal keratinocytes stained for α_6 and CD71 is shown in **Fig. 1F**. Draw sorting regions to demarcate the keratinocyte stem cells (R2 = α_6^{bri}CD71^{dim}); the TA cells (R3 = α_6^{bri}CD71^{bri}); and the differentiating cells (R4 = α_6^{dim}). Given that the KSCs are located in the CD71 low-negative region of the α_6^{bri} population of cells, the KSCs sort region is positioned to include the majority of cells defined by this phenotype while allowing sufficient separation from the TA cells.
8. Collect these keratinocyte fractions into 5-mL FACS tubes containing 1 mL KGM-2% BSA. Prior to cell collection, invert the collection tubes a few times to coat the inside of the tubes with KGM–2% BSA.
9. Transfer a small aliquot (10–20 µL) of the collected samples into fresh tubes containing 200 µL of wash buffer and reanalyze the cells on the flow cytometer to determine purity of the cells collected (*see* **Note 4**). Use the same parameter settings determined for the cell sorting, and flush the cytometer thoroughly with sheath fluid (Isoton II) between samples to minimize cross-contamination.

4. Notes

1. Keratinocytes express CD71 at low to medium levels; therefore, a fluorochrome with a bright emission, such as APC, PE-cy5, or PE (phycoerythrin R) is recommended for detecting CD71 antibody binding. Note, some flow cytometers are not equipped to detect APC emissions. We have chosen the combination of FITC, APC, and 7-AAD because they present minimal compensation issues.
2. Human specimens are potentially infectious and must be processed aseptically in a biohazard hood. During processing, the tissues are kept on ice where possible to maintain cell viability. To ensure sterility, the dissecting instruments are rinsed first in 70% EtOH and then PBS-AF before being applied to the tissues. All instruments must be soaked in bleach after experimentation, washed thoroughly and autoclaved. Disposal of waste human tissue is as per institutional guidelines.
3. For samples labeled simultaneously with two or more fluorochromes, spectral overlaps may occur. This results in the detection of fluorescence emissions from one colour by other fluorescence channels, giving rise to unwanted signals. These signals must be subtracted from the detector in these channels using the electronic compensatory network. Only after all the necessary compensations have been performed will the fluorescences detected on the sort sample reflect the 'true' fluorescence intensities on these cells. By using the combination of FITC, APC, and 7-AAD for our immunofluorescent labeling, minimal compensation is required between FITC and 7-AAD, and there are no compensation issues between FITC/7-AAD and APC because these fluorochromes are excited by different laser lights and their fluorescence emissions are collected sequentially as cells pass through the first (488 nm) and then the second (647 nm) laser. However, if PE is used to detect CD71 binding, there are significant spectral overlaps between FITC, PE, and 7-AAD. Therefore, appropriate compensations must be performed. The approach is similar to that described in **Subheading 3.4.** Briefly, run the 7AAD control, followed by the α_6-FITC, and then the CD71-PE single-color controls, performing the appropriate compensation adjustments in all fluorescence parameters with each sample.
4. When sorted cells are reanalyzed to determine their sort purity, some events will fall outside of the original sort region used to collect the cells. In particular, when α_6^{bri}CD71^{dim} cells are reanalyzed, a slight upward shift of the population in the CD71 parameter is often

observed. This phenomenon is more apparent when PE is used to detect CD71, but is minimized to within acceptable limits with APC.

Acknowledgments

The authors would like to thank Andrew Fryga for providing expert technical flow cytometric details and critical reading of the flow cytometry section in this chapter.

References

1 Morris, R. J., Fischer, S. M., and Slaga, T. J. (1985) Evidence that the centrally and peripherally located cells in the murine epidermal proliferative unit are two distinct cell populations. *J Invest. Dermatol.* **84(4),** 277–281.
2 Mackenzie, I. C. and Bickenbach, J. R. (1985) Label-retaining keratinocytes and Langerhans cells in mouse epithelia. *Cell Tissue Res.* **242,** 551–556.
3 Bickenbach, J. R., McCutecheon, J., and Mackenzie, I. C. (1986) Rate of loss of tritiated thymidine label in basal cells in mouse epithelial tissues. *Cell Tissue Kinet.* **19,** 325–333.
4 Morris, R. J. and Potten, C. S. (1994) Slowly cycling (label-retaining) epidermal cells behave like clonogenic stem cells in vitro. *Cell Prolif.* **27,** 279–289.
5. Potten, C. S. and Hendry, J. H. (1973) Letter: clonogenic cells and stem cells in epidermis. *Int. J. Radiat. Biol.* **24,** 537–540.
6 Bickenbach, J. R. and Holbrook, K. A. (1987) Label-retaining cells in human embryonic and fetal epidermis. *J. Invest. Dermatol.* **88,** 42–46.
7 Bickenbach, J. R. and Mackenzie, I. C. (1984) Identification and localization of label-retaining cells in hamster epithelia. *J. Invest. Dermatol.* **82,** 618–622.
8 Potten, C. S. (1975) Epidermal cell production rates. *J. Invest. Dermatol.* **65,** 488–500.
9. Potten, C. S. (1986) Cell cycles in cell hierarchies. *Int. J. Radiat. Biol.* **49,** 257–278.
10 Allen, T. D. and Potten, C. S. (1974) Fine-structural identification and organization of the epidermal proliferative unit. *J. Cell Sci.* **15,** 291–319.
11 Christophers, E. (1971) Cellular architecture of the stratum corneum. *J. Invest. Dermatol.* **56,** 165–169.
12 Barrandon, Y. and Green, H. (1987) Three clonal types of keratinocyte with different capacities for multiplication. *Proc. Natl. Acad. Sci. USA* **84,** 2302–2306.
13 Jones, P. H., Harper, S., and Watt, F. M. (1995) Stem cell patterning and fate in human epidermis. *Cell* **80,** 83–93.
14 Jones, P. H. and Watt, F. M. (1993) Separation of human epidermal stem cells from transit amplifying cells on the basis of differences in integrin function and expression. *Cell* **73,** 713–724.
15 Kaur, P. and Li, A. (2000) Adhesive properties of human basal epidermal cells: an analysis of keratinocyte stem cells, transit amplifying cells, and postmitotic differentiating cells. *J. Invest. Dermatol.* **114,** 413–420.
16 Bickenbach, J. R. and Chism, E. (1998) Selection and extended growth of murine epidermal stem cells in culture. *Exp. Cell Res.* **244,** 184–195.
17 Albert, M. R., Foster, R. A., and Vogel, J. C. (2001) Murine epidermal label-retaining cells isolated by flow cytometry do not express the stem cell markers CD34, Sca-1, or Flk-1. *J. Invest. Dermatol.* **117,** 943–948.
18 Li, A., Simmons, P. J., and Kaur, P. (1998) Identification and isolation of candidate human keratinocyte stem cells based on cell surface phenotype. *Proc. Natl. Acad. Sci. USA* **95,** 3902–3907.

19 Tani, H., Morris, R. J., and Kaur, P. (2000) Enrichment for murine keratinocyte stem cells based on cell surface phenotype. *Proc. Natl. Acad. Sci. USA* **97),** 10,960–10,965.

20 Stenn, K. S., Link, R., Moellmann, G., Madri, J., and Kuklinska, E. (1989) Dispase, a neutral protease from Bacillus polymyxa, is a powerful fibronectinase and type IV collagenase. *J. Invest. Dermatol.* **93,** 287–290.

21. Poumay, Y., Roland, I. H., Laclercq-Smekens, M., and Leloup, R. (1994) Basal detachment of the epidermis using dispase: tissue spatial organization and fate of integrin $\alpha_6\beta_4$ and hemidesmosomes. *J. Invest. Dermatol.* **102,** 111–117.

11

Isolation, Characterization, and Culture of Epithelial Stem Cells

Jackie R. Bickenbach

Summary

It is well accepted that homeostasis of continuously renewing adult tissues, such as the epidermis, is maintained by somatic stem cells. These are undifferentiated, self-renewing cells, which also produce daughter transit amplifying (TA) cells that make up the majority of the proliferative cell population in the tissues. Although still proliferative in nature, it is thought that TA cells can undergo only a finite number of cell divisions before they commit to leave the proliferative compartment and move toward terminal differentiation. Stem cells, on the other hand, have been assumed to persist throughout the lifetime of the organism. We directly demonstrated the presence of putative stem cells in the proliferative compartment of murine epithelia in 1981 when we identified a small population of label-retaining cells (LRCs) in mouse stratified squamous epithelia. Since then, we have developed the method described here to isolate this population of epidermal stem cells (EpiSC). We showed that EpiSC are all keratin 14+ and thus of keratinocyte origin and not of mesenchymal or hematopoietic origin. We have also demonstrated that EpiSC can regenerate the epidermis, that they can permanently express a recombinant gene in the regenerated tissue, and that while the majority of EpiSC reside in the G1 phase of the cell cycle, they are not held out of the cell cycle, that they express proliferating genes and the mitotic cyclin B1 protein. Recently, we have shown that EpiSC have the capacity to alter their cell fate in vivo if placed into stress environments, i.e. after irradiation or wounding or when injected into a developing blastocyst environment. Thus being able to isolate EpiSC is critical for testing their use in cell and gene therapy.

Key Words:

Stem cells; epidermis; plasticity; skin.

1. Introduction

Homeostasis of continuously renewing epithelial tissues is maintained by somatic stem cells ***(1–3)***. These are undifferentiated, self-renewing cells, that also produce daughter transient amplifying (TA) cells, which make up the majority of the proliferative population. TA cells undergo a finite number of cell divisions before leaving the proliferative compartment and moving toward terminal differentiation, whereas it has been assumed that the stem cells persist throughout the lifetime of the organism. Through a series of labeling experiments with tritiated thymidine, we previously showed that stem cells from adult mouse skin did not divide as often as the other basal cells, but they did divide at a steady rate in vivo ***(4,5)***. We also showed that they continued to proliferate in vivo throughout life and that they have a high proliferative potential in vitro. However, isolation of stem cells has been problematic. We combined two

From: *Methods in Molecular Biology, vol. 289:*
Epidermal Cells: Methods and Protocols
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

dissimilar methods to achieve this result: a Hoechst and propidium iodide dye method published to isolate hematopoietic stem cells ***(6)*** and a method showing that cell size contributed to the highest proliferative potential for epithelial cells ***(7)***. In combination with specifically defined gating, we have sorted mouse epidermal basal cells into three fractions, identified as stem, TA, and nonproliferative basal cells ***(8)***. More than 90% of freshly isolated stem cells showed a G0/G1 cell cycle profile, whereas greater than 20% of the TA cells were actively dividing. Recently, we showed that these stem cells proceed through the cell cycle during normal tissue regeneration ***(9)***. Although both stem and TA cells retained proliferative capacity, only the stem cells formed larger, more expandable colonies in culture. Both populations could be transduced with a retroviral vector and used to bioengineer an epidermis. However, only the epidermis from the stem cell population continued to grow and express the reporter gene for 6 mo in organotypic culture. The epidermis from the transient amplifying cell fraction completely differentiated by 2 mo. Last year, we determined that these epidermal stem cells also have the remarkable ability to participate in the formation of the other tissues, a plasticity similar to that of embryonic stem cells and a few other somatic stem cells ***(10)***. The somatic epidermal stem cells isolated from neonatal mouse skin incorporated into a variety of tissues and altered their phenotype after injection into blastocysts. Thus the fate determination of these stem cells appears to be in direct response to their environment. Very recently, we have determined that this sorting technique also yields viable stem cells from adult mouse skin and palate epithelia, adult human skin, gingiva, alveolar mucosa, and palate. Thus, this novel sorting method yields pure viable epithelial stem cells that can be used to bioengineer tissues and to test permanent recombinant gene expression for potential gene and cell therapy.

2. Materials

2.1. Tissue and Reagents

1. ICR mice from Harlan Labs.
2. C57BL/6J and C57BL/6-TgN(ACTbEGFP)1Osb mice (original breeders from Jackson Labs).
3. Discarded adult human pathology samples of skin from breast reduction and general surgery.
4. Discarded adult human gingival, alveolar, and palatal samples from periodontal procedures.
5. All media, additives, and enzymes from Life Technologies (K-FSM, DK-FSM, DMEM, SMEM, penicillin streptomycin antinomycin D (PSA), trypsin, trypsin inhibitor, phosphate-buffered saline [PBS]).
6. Dispase II and collagen type IV from Collaborative Biomedical.
7. Hoechst 33342 and propidium iodide (PI) from Sigma.
8. Becton Dickinson FACS DiVa with three excitation sources (argon ion laser at 488 nm, dye laser at 595 nm, krypton laser at 351-364 nm or 406 nm ultraviolet light), nine fluorescent channels, and two light scatter channels. It can sort at rates up to 50,000 cells per second.

2.2. Stock Solutions

1. Hoechst 33342 in SMEM (1 mg/mL); store aliquots at –80°C in the dark. Do not refreeze. In 9.9 mL SMEM, add 100 µL HEPES, 50 µL Hoechst stock (final conc. = 5 µg/mL), filter through 0.22-µ filter to sterilize, and store at 4°C. Make fresh each day.
2. PI in SMEM (1 mg/mL); store at 4°C in the dark for several months. In 9.9 mL SMEM, add 100 µL HEPES, 5 µL stock PI (final conc. = 0.5 µg/mL), filter through 0.22-µ filter to sterilize, and store at 4°C. Make fresh each day.
3. 25 mg/mL HEPES buffer; store at 4°C until used up.

2.3. To Coat Culture Dishes With Collagen Type IV

1. Thaw collagen IV stock (1 mg bottle) very slowly at 4°C overnight (may take longer). Vortex when thawed.
2. Thoroughly wipe down hood with alcohol, including walls, remove dirty pipets, and so on. Wipe down metal trays with alcohol (to get rid of dust).
3. Mix 0.5 mL 37% HCl into 100 mL sterile ddiH_2O (this gives you 0.05 *N* HCl).
4. Add 1 mg collagen IV to 100 mL 0.05 *N* HCl.
5. Coat culture dishes at a concentration of 1 µg/cm^2.
6. Let sit 1 h in the tissue culture hood.
7. Remove all the liquid.
8. Rinse with PBS, then with ddiH_2O, and allow dishes to air-dry thoroughly.
9. Store coated dishes at 4°C. May store for several months.

3. Methods

3.1. Primary Isolation of Keratinocytes From Mouse Skin and Oral Mucosa

1. Sterilize forceps and scissors by flaming in ethanol.
2. Remove skin from mice with sterile scissors and forceps, or cut away palate from teeth with sterile scalpel with a no. 11 blade.
3. Treat mouse skin and palate samples the same.
4. Place tissues in 10% PSA in SMEM for 1 h at room temperature.
5. If needed, cut pieces into strips of no more than 4-mm wide and 40-mm long.
6. In a sterile Petri dish, float the pieces connective tissue down in dispase II overnight at 4°C. The next morning, remove the dispase from the dish.
7. Using sterile forceps, separate the epithelia from the connective tissue. This is easiest using a dissecting microscope.
8. Place the epithelia into 50-mL centrifuge tubes, cover each sample with 0.25% trypsin and incubate in 37°C water bath for 30 min. After the 30 min, shake the tube vigorously.
9. Add an equal volume of trypsin inhibitor (0.25 mg/mL) to stop the trypsin reaction.
10. Pass the trypsinized cell suspension through a 70-µm sterile cell strainer to remove the large particles.
11. Count the cells. Centrifuge the cell solutions at 400*g* for 10 min. Remove the supernatant.

3.2. Primary Isolation of Keratinocytes From Human Skin and Oral Mucosa

1. Discarded skin samples from the surgical pathology lab or discarded gingival, alveolar, and palatal samples from periodontal procedures are used within 2 d of surgical procedure. Store tissue until needed in medium at 4°C.
2. Soak samples in 90% Dulbecco's modified Eagle's medium + 10% PSA for 1 h at room temperature to ensure sterility.
3. Sterilize forceps, scissors, and scalpels by flaming in ethanol.
4. Sterilely cut skin samples into long, narrow (less than 4-mm wide) strips. (This ensures that the dispase II can completely penetrate between the connective tissue and the epidermis.) Rinse in PBS.
5. Add dispase II to Petri dishes until all of the tissue is covered. Incubate overnight at 4°C. The next morning, remove the dispase II from the dish.
6. Using sterile forceps, separate the epithelia from the connective tissue. This is easiest using a dissecting microscope.
7. Place the epithelia into 50-mL centrifuge tubes, cover each sample with 0.25% trypsin, and incubate in 37°C water bath for 30–40 min. Then, shake the tube vigorously.
8. After the trypsin incubation, add an equal amount of trypsin inhibitor (0.25 mg/mL).

9. Pass the trypsinized cell suspension through a 70-µm sterile cell strainer to remove the large particles.
10. Count the cells. Centrifuge the cell solutions at 400*g* for 10 min. Remove the supernatant.

3.3. Preparing Cells for Sorting

1. Resuspend 1×10^6 cells per 1 mL Hoechst dye medium.
2. Incubate cells in Hoechst dye medium for 90 min at 37°C in the dark (wrap tube in foil). Every 30 min, shake tube gently to resuspend cells because they will fall to the bottom of the tube.
3. Centrifuge cells and resuspend 5×10^6–1×10^7 cells in PI medium. Filter the cell suspension through a 70-µm sterile cell strainer.
4. Place tubes in ice bucket and take to flow cytometry for cell sorting. *Note:* Take 15-mL conical tubes with 5 mL culture medium for collecting the cells.

3.4. Cell-Sorting Method

The test tube containing the single cell suspension is placed on the Becton Dickinson DiVa instrument and the sample is pneumatically driven down silastic tubing where it is injected into a high velocity jet nozzle of 100–130 µ in diameter. The nozzle, containing sheath fluid under pressure, hydrodynamically focuses the cells so that they exit the nozzle in single file and are constrained to the center of the stream resulting in laminar coaxial flow. This provides a predictable trajectory from the jet for each cell that passes through the instrument. Laser light is then focused onto the stream where each cell is interrogated as it passes through the laser.

Photo multiplier tubes and solid-state detectors (photodiodes) collect the photon emissions from each cell and convert them to analog voltages. The analog signals are then digitized by analog to digital converters and stored on magnetic medium for analysis. Optical filters are placed before the detectors so that only wavelengths of light corresponding to specific fluorochrome emissions are collected by each detector (e.g., FITC emits in the green region; therefore, a 525-nm bandpass filter is used to collect light from this fluorochrome).

Light scattered at the same wavelength and in the same direction as the laser light, primarily from the surface of the cell, correlates with relative cell size (forward angle light scatter). Light scattered 90 degrees to the laser (orthogonal scatter or side scatter) from internal structures correlates with granularity. By correlating these two parameters, subpopulations of cells corresponding to relative specific size can be isolated or excluded. Unwanted signal, such as cell debris or cell aggregates, can also be detected and excluded. Cells stained with Hoechst and/or PI or fluorochromes directed at deoxyribonucleic acid can be detected and analyzed as to amount of stain. Data are displayed and analyzed using histogram representation on a networked computer system (*see* **Fig. 1** for example).

Subpopulations of cells can be sterilely isolated for further experimentation by using the instrument's cell sorting capability. A pizo-electric quartz crystal connected to the jet nozzle drives the cell stream into oscillation causing droplets to form. The droplets are formed at a high enough frequency so that any given droplet will contain only one cell. When a cell meets the criteria set by the investigator and defined by sort windows of gates, the sorting electronics place a charge on the cell stream at exactly the time when the droplet containing the cell of interest is breaking off from the stream. The droplet passes between two metal plates that have a very high electric potential across them. The resulting magnetic field forces the charged droplet to be deflected toward

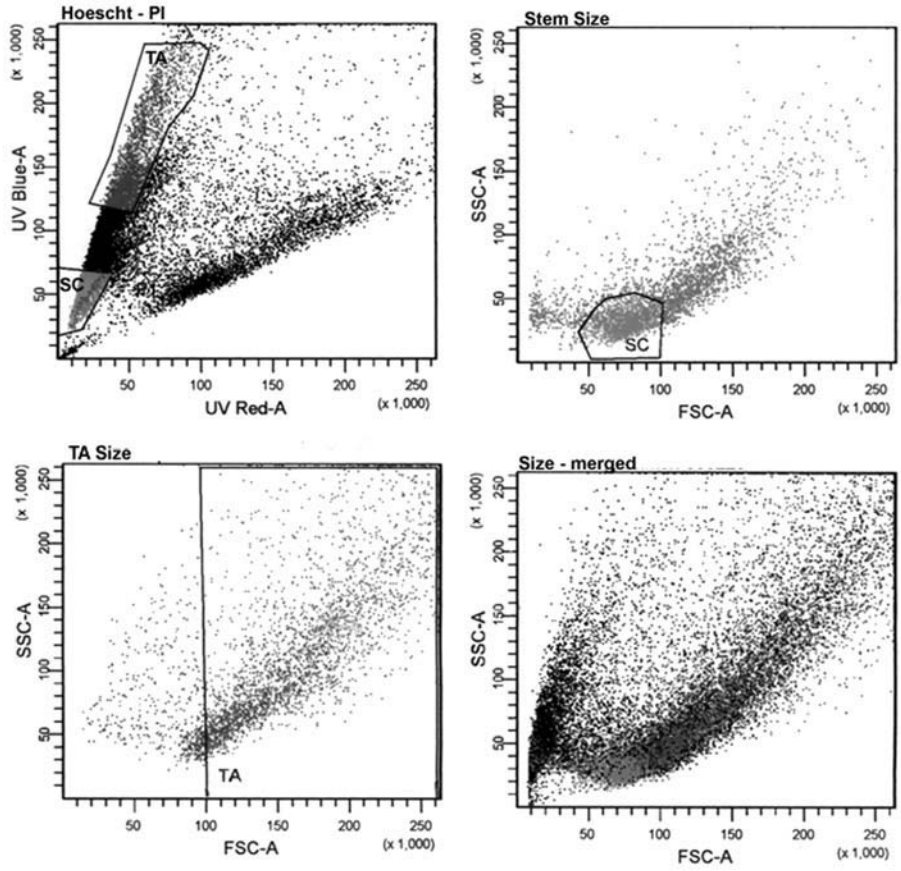


Fig. 1. Scatter diagrams of epidermal stem and TA cell sort parameters. Stem cells are first sorted according to low Hoechst red/blue fluorescence (green [SC] in panel 1) and then sorted by small size (green [SC] in panel 2). TA cells are sorted by high Hoechst red/blue fluorescence (purple [TA] in panel 1), then by size (purple [TA] in panel 3). Panel 4 shows relative sizes of all populations.

one of the plates, the direction depending on whether the charge on the droplet is positive or negative. By placing a collection vessel in the deflected droplet's path, cell populations up to 99% purity can be collected at rates of several thousand per second.

For our purposes, we collect two populations of cells based upon their size and their ability to exclude Hoechst and PI. Stem cells (SC in **Fig. 1**) are cells that are small and can exclude both dyes. TA cells (TA2 in **Fig. 1**) are larger than stem cells and are moderately stained by Hoechst dye, but exclude the PI dye.

3.5. Culture of Sorted Cells

1. After sorting, centrifuge the stem and TA (transit amplifying) cells, and resuspend in culture medium.
2. Plate approx 2×10^3 cells per cm^2 on collagen type IV-coated culture dishes.
3. Grow at 36.5°C in a humid CO_2 incubator.
   a. Sorted neonate and adult mouse skin and oral mucosal keratinocyte stem cells will grow for several passages in DK-FSM, made according to manufacturer's directions.
   b. Sorted adult human skin keratinocyte stem cells will grow for at least eight passages in K-SFM, made according to manufacturer's directions.
   c. Sorted adult human alveolar, gingival, and palatal keratinocyte stem cells will grow for at least three passages in DK-FSM, made according to manufacturer's directions.

4. Notes

1. Overall, the most important thing to keep consistent is the cell pattern obtained from the Flow Cytometer (*see* **Fig. 1**). If you note wide variation, make sure that your Hoechst dye is fresh or that it has not been frozen, thawed, and refrozen.
2. Do not worry about having too many dead cells; they can be gated out in the first gate. Usually, dead cells are a result of incubating too long in trypsin.
3. To distinctly differentiate between stem and transit amplifying cells, keep both the fluorescent gates and the size gates away from each other (*see* **Fig. 1**).
4. Cells may be cultured with other substrates or with a 3T3 feeder layer.
5. Cells cultured as described previously can be induced to differentiate if the calcium level is raised.
6. Finally, each flow cytometer is slightly different. Thus, to establish this type of system for sorting cells, you will have to establish routine Hoechst/PI and size gates, then adjust the gain to ensure that your cells are gated properly and consistently each time.

Acknowledgments

The author would like to thank the present and past members of the Bickenbach laboratory who have contributed to the development of the isolation and culture procedures, especially Dr. Luchuan Liang, Ann Tomanek-Chalkley, Tony Manuel, Martine Dunnwald, and Dana Alexandrunas; and the members of the UI Flow Cytometry Core, Justin Fishbaugh and Gene Hess.

References

1 Cairns, J. (1975) Mutation selection and the natural history of cancer. *Nature* **255,** 197–200.
2 Potten, C. S., Owen, G., and Booth, D. (2002) Intestinal stem cells protect their genome by selective segregation of template DNA strands. *J. Cell Sci.* **115,** 2381–2388.
3. Potten, C. S. (1997) Stem Cells. London: Academic Press.
4 Bickenbach, J. R. (1981) Identification of label-retaining cells in oral mucosa and skin. *J. Dent. Res.* **122C,** 1611–1620.
5. Bickenbach, J. R., McCutcheon, J., and Mackenzie, I. C. (1986) Rate of loss of tritiated thymidine label in basal cells in mouse epithelial tissue. *Cell Tissue Kinet.* **19,** 325–333.
6 Goodell, M. A., Brose, K., Paradis, G., Conner, A. S., and Mulligan, R. C. (1996) Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J. Exp. Med.* **183,** 1797–1806.
7 Barrandon, Y. and Green, H. (1985) Cell size as a determinant of the clone-forming ability of human keratinocytes. *Proc. Natl. Acad. Sci. USA* **82,** 5390–5394.
8 Dunnwald, M., Tomanek-Chalkley, A., Alexandrunas, D., Fishbaugh, J., and Bickenbach, J. R. (2001) Isolating a pure population of epidermal stem cells for gene use in tissue engineering. *Exp. Dermatol.* **10,** 45–54.
9 Dunnwald, M., Chinnathambi, S., Alexandrunas, D., and Bickenbach, J. (2003) Mouse epidermal stem cells proceed through the cell cycle. *J. Cell Physiol.* **195,** 194–201.
10. Liang, L. and Bickenbach, J. R. (2002) Somatic epidermal stem cells can produce multiple cell lineages during development. *Stem Cells* **20,** 3–10.
11. Liang, L., Chinnathambi, S., Stern, M., Tomanek-Chalkley, A., Manuel, T. D., and Bickenbach, J. R. (2004) As epidermal stem cells age they do not substantially change their characteristics. *J. Invest. Dermatol. Montagna. Supplement.* (in press).
12. Bickenback, J. R. and Chinnathambi, S. (2004) Plasticity and potential of epidermal stem cells, in *Progress in Stem Cell Research* (Emura, M. ed.), Nova Science Publishers, Inc., Hauppauge, NY.

12

Keratin 19 as a Stem Cell Marker In Vivo and In Vitro

Danielle Larouche, Cindy Hayward, Kristine Cuffley, and Lucie Germain

Summary

The skin is a dynamic tissue in which terminally differentiated keratinocytes are replaced by the proliferation of new epithelial cells that will undergo differentiation. The rapid and continual turnover of skin throughout life depends on a cell population with unique characteristics: the stem cells. These cells are relatively undifferentiated, retain a high capacity for self-renewal throughout their lifetime, have a large proliferative potential, and are normally slow cycling. The long-term regeneration of grafted cultured epidermis indicates that epidermal stem cells are maintained in cultures. In animals they can be identified with ^{3}H-thymidine or bromodeoxyuridine based on their property of slow cycling. The development of markers such as keratin 19 also permits their study in human tissues. In this chapter, protocols to study skin stem cells using their property of slow cycling and their expression of keratin 19 will be described in detail. The methods include the double labeling of tissues for keratin 19 and label-retaining cells (autoradiography of ^{3}H-thymidine) *in situ*. The labeling of keratin 19 by immunofluorescence of by flow cytometry is described for cells in vitro.

Key Words:

Stem cells; keratin; keratin 19; epidermis; hair follicles; human skin; flow cytometry.

1. Introduction

The skin is a dynamic tissue in which terminally differentiated keratinocytes are replaced by the proliferation of new epithelial cells that will undergo differentiation. The rapid and continual turnover of skin throughout life depends on a cell population with unique characteristics: the stem cells. These cells are relatively undifferentiated, retain a high capacity for self-renewal throughout their lifetime, have a large proliferative potential, and are normally slow cycling. The long-term regeneration of grafted cultured epidermis indicates that they are maintained in cultures ***(1–3)***. In animals, they can be identified with ^{3}H-thymidine or bromodeoxyuridine based on their property of slow cycling. The development of markers such as keratin 19 also permits their study in human tissues ***(4)***. In this chapter, protocols to study skin stem cells using their property of slow cycling and their expression of keratin 19 will be described in detail ***(3–6)***. The methods include the labelling of tissues in situ and of cells in vitro.

From: *Methods in Molecular Biology, vol. 289: Epidermal Cells: Methods and Protocols*
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

2. Materials

2.1. Antibodies for Immunofluorescence Staining

2.1.1. Primary Antibodies

1. Guinea pig anti-mouse keratin 19: a site-directed polyclonal antibody was raised in guinea pigs against the specific amino acid sequence 391-404 (NH_2-Glu-Ala-His-Tyr-Asn-Asn-Leu-Pro-Thr-Pro-Lys-Ala-Ile-OH) of mouse keratin 19 *(7)*. Sera were tested and used at dilutions of 1 : 500 to 1 : 2000.
2. Mouse monoclonal anti-human K19 (cat. no. 03-61010, Ks 19.1, American Research Product, Belmont, MA).
3. Mouse monoclonal anti-human K18 (cat. no. 03-61009, Ks 18.174, American Research Product, Belmont, MA).
4. Mouse monoclonal anti-human K20 (cat. no. 03-61054, Ks 20.10, American Research Product, Belmont, MA).

2.1.2. Secondary Antibodies

1. FITC-tagged goat anti-guinea pig IgG(H+L) (cat. no. 106-095-003, Jackson ImmunoRes Laboratories, West Grove, PA). Alternatively, Texas red-conjugated affinipure goat anti-Guinea pig IgG (H+L) (cat. no. 106-075-003, Jackson ImmunoRes Laboratories) or Fluorescein (DTAF)-conjugated AffiniPure goat anti-guinea pig IgG (H+L) (cat. no. JPG015003, Accurate Chemical & Scientific Corporation, Westbury, NY) can be used.
2. FITC-tagged goat anti-mouse IgG(H+L) (cat. no. Ap-130F, Chemicon, Temecula, CA).
3. Rhodamine-tagged goat anti-mouse IgG-IgM (cat. no. Ap-130R, Chemicon, Temecula, CA).

2.2. Animals and Radioactive Probe

1. SENCAR adult and newborn mice (Harlan Sprague-Dawley, Indianapolis, IN; from Charles River).
2. [Methyl-^{3}H]thymidine, specific activity 40–85 Ci/mmol (NEN-Dupont). Dilute [methyl-^{3}H]thymidine in sterile 0.85% NaCl to prepare a solution to inject 15 µL per mouse containing 5 µCi/g of body weight.
3. Alzet osmotic minipumps (model 2002, Alza Corp., Palo Alto, CA).

2.3. Tissue Preservation

1. Optical cutting temperature (OCT) Compound (Miles Inc., Elkhart, IN).
2. Small container with N_2L (liquid nitrogen).
3. Superfrost glass slides (cat. no. 12-550-15, Fisher Scientific, Nepean, Ont).

2.4. Immunofluorescence Staining and Flow Cytometry Analysis

1. dH_2O: distilled water.
2. Phosphate-buffered saline (PBS)–Ca: 137 m*M* NaCl, 2.7 m*M* KCl, 6.5 m*M* Na_2HPO_4, 1.5 m*M* KH_2 PO_4, 0.5 m*M* $MgCl_2$, 0.9 m*M* $CaCl_2$. To make 1 L, dissolve 8 g NaCl, 0.2 g KCl, 0.92 g Na_2HPO_4, 0.2 g KH_2 PO_4, and 0.17 mL $MgCl_2$•$6H_2O$ of a 2.8 *M* solution kept at –20°C, 0.131 g $CaCl_2$•$2H_2O$. Complete to 1 L with dH_2O. Verify that pH is 7.4 (*see* **Note 1***).*
3. PBS–calcium (Ca)–bovine serum albumin (BSA): PBS–Ca containing 1% BSA. Dissolve 1 g BSA in 100 mL PBS–Ca.
4. Liquid mounting media: 137 m*M* NaCl, 0.1 *M* glycine, 22 m*M* NaN_3, 8.75 m*M* NaOH. To make 100 mL, dissolve 0.85 g NaCl, 0.725 g glycine, 0.05 g NaN_3, and 0.035 g NaOH, in

40 mL dH_2O. Adjust pH to 8.6. Complete to 50 mL with dH_2O. Add 50 mL glycerol. This medium is used for temporary mounting because the slides can be unmounted easily.

5. Solid mounting media: 137 m*M* NaCl, 2.7 m*M* KCl, 6.5 m*M* Na_2HPO_4, 1.5 m*M* KH_2PO_4, 22 m*M* NaN_3, 1.5% gelatin. To make 62 mL, dissolve 0.8 g of gelatin in hot water. Add 0.224 g NaCl, 0.0056 g KCl, 0.0258 g Na_2HPO_4, 0.0056 g KH_2PO_4, 0.04 g NaN_3, and add 25 mL dH_2O. Adjust pH to 7.6 using pH paper. Complete to 40 mL with dH_2O. Add 12 mL glycerol.

2.5. Double-Labeling Procedure for Colocalization of K19 and Thymidine Label-Retaining Cells

1. Security lamp (Kodak Safelight) with a red filter Kodak Safelight no. 2 (cat. no. 152-1525, Kodak, Rochester, NY).
2. Lab-Tek Slide container (microscope slide mailer; cat. no. 25608-874, VWR, Montreal, Qc; Sakura Finetek 4310).
3. Black plastic microslide box (cat. no. 48444-004, VWR, BD Biosciences 423843).
4. Emulsion solution NTB-2 (cat. no. KP 165-4433, Kodak, Rochester, NY).
5. Staining dishes (cat. no. 4456, Somagen, Edmonton, Alberta).
6. Aluminum foil, scotch tape, Kleenex, gloves, and drierite.
7. Kodak Dektol developer solution (cat. no. KP 146-4726, Kodak).
8. Fixing solution (Kodak rapid fixer, cat. no. KP 146-4106, Kodak). Stock fixing solution: 49.8 mL solution A, 5.47 mL solution B, complete to 200 mL with dH_2O. Working fixing solution: Dilute 1 stock solution: 3 dH_2O.
9. Developing solution: warm dH_2O to 38°C. Dissolve Dektol in warm water and complete to 3.8 L. Working developing solution: Dilute 1 stock solution: 1 dH_2O.

2.6. Flow Cytometry Analysis

1. Saline: 137 m*M* NaCl. To make 1 L, dissolve 8.5 g NaCl in 1 L dH_2O.
2. 1X PBS: 137 m*M* NaCl, 2.7 m*M* KCl, 6.5 m*M* Na_2HPO_4, 1.5 m*M* KH_2PO_4. To make 2 L, dissolve 16 g NaCl, 0.4 g KCl, 1.85 g Na_2HPO_4, and 0.4 g KH_2PO_4. Complete to 2 L with dH_2O. Verify that pH is 7.4 (*see* **Note 2**).
3. PBS–BSA: PBS containing 1% BSA. Dissolve 1 g BSA in 100 mL PBS.
4. The labeling is performed in 15-mL conic tubes (cat. no. Falcon BD-35 2096, VWR, Montreal).

3. Methods

Subheadings 3.1. to **3.4.** describe the double labeling for keratin 19 by indirect immunofluorescence and for [^{3}H]thymidine by autoradiography to detect label-retaining cells and identify stem cells.

3.1. In Vivo [^{3}H]Thymidine Labeling

1. Inject 15 µL [methyl-^{3}H]thymidine solution with a 25-µL Hamilton syringe subcutaneously or intraperitoneally (*see* **Note 3**).
2. Be careful not to disturb the mother so that she does not reject her offsprings.
3. Repeat these two steps twice daily, every 12 h, for the first 7 d of postnatal life.
4. Sacrifice the mice 28 d after birth and excise pieces of skin (back or other anatomic sites) and process for tissue preservation immediately.

3.2. Tissue Preservation and Sectioning

1. Cut the mouse or human skin samples from different anatomical sites into small pieces (1- to 5-mm wide rectangles).

2. Deposit OCT on a tissue holder and use forceps to immerse it for 2–3 s in N_2L, removing it before the OCT is completely white.
3. Take a piece of tissue using forceps and place it on the liquid OCT on the tissue holder.
4. Add OCT on top of the sample to embed the tissue and prevent freeze-drying.
5. Take the holder with forceps and immerge it in liquid nitrogen for approx 15 s. The OCT should be white but should not crack.
6. Store the blocks at –70°C until use.
7. Cut the tissue in a cryostat (4-µm thick sections) and place the tissue sections on glass slides (*see* **Note 4**).
8. Identify each slide with a lead pencil.
9. Dry the sections on a warm plate at 37°C for 30 min.
10. Process for immunofluorescence staining or store the slides at –20°C (they can be kept for a few weeks).

3.3. Immunofluorescence Staining

1. Take the sections from the freezer and leave them at room temperature for 5 min to remove excess humidity. If the sections are small they can be encircled by etching with a diamond pencil.
2. Fix the sections by immersion in acetone for 10 min at –20°C.
3. Wash three times in PBS–Ca for 5 min. Put the slides in a rack and immerse in three baths of PBS–Ca.
4. Incubate with the primary antibody. Process rapidly to avoid drying the sections: Aspirate the liquid surrounding the sections. Cover each section with 25 to 50 µL of antibody diluted in PBS–Ca–BSA. Put PBS–Ca–BSA or an unrelated antibody on the control sections. Incubate 45 min at room temperature (RT).
5. Wash three times in PBS–Ca for 5 min. Put the slides in the rack and immerse in three baths of PBS–Ca. Be careful to avoid detachment of the sections from the slides.
6. Incubate with the secondary antibody. Process rapidly to avoid drying the sections: Aspirate the liquid surrounding the sections. Cover with 25 to 50 µL of antibody diluted in PBS–Ca–BSA. Put PBS–Ca–BSA or an unrelated antibody on the control sections. Incubate 30 min at RT in the dark.
7. Wash three times in PBS-Ca for 5 min. Put the slides in the rack and immerse in three baths of PBS. Be careful to avoid detachment of the sections from the slides.
8. For autoradiography, keep the slides in PBS–Ca in the dark until the emulsion is added; do not dry the sections. To verify the fluorescent labeling, one or more slides can be mounted with a liquid mounting medium. After microscopic observation, the slides are unmounted by immersing them in PBS for a few minutes, until the cover slip slides off easily.
9. For single indirect immunofluorescence staining, *see* **Subheading 3.5.**

3.4. Autoradiography

3.4.1. Preparation of the Darkroom

1. Make sure that the darkness is complete. The crucial steps can be performed in complete darkness. Alternatively, a security lamp with a red filter could be used but at a distance of at least 1.2 m from the working surface.
2. Clean all plastic and glassware used for the emulsion to ensure that they are free of old emulsion.
3. Warm the water bath to 42°C. Make sure that the level of water reaches the upper level of the emulsion. Hide the red light from the water bath with aluminum foil. Do not unplug the water bath during the process to avoid electrical sparks.

4. Prepare the aluminum foil, scotch tape, Kleenex, gloves, and drierite.
5. Prepare a wood support to let the slides dry. Place it within a Styrofoam box for drying the slides in the dark (by adding the cover and putting it in a black plastic garbage bag).
6. Prepare a small black microslide box (10 or 25 slides) for dark storage of the coated slides. Place drierite absorbent (calcium sulfate) in a Kleenex and put it between two blank slides to hold it in the box. Close the box until ready to place the emulsion-coated slides within.
7. Prepare the slides in order, with the sections all on the same side, to ensure putting the emulsion on the right side while in the dark.

3.4.2. Preparation of the Emulsion Stock

1. Warm a beaker with dH_2O in the bath to 42°C. Make sure that the water level reaches the upper level of the emulsion.
2. Put a label on each Lab-Tek slide container at the 20-mL level.
3. Add 10 mL dH_2O to each Lab-Tek.
4. Turn off all lights except the security lamp.
5. Add the cold emulsion with a clean spoon to the 20-mL level (10-mL emulsion).
6. Put two layers of aluminum foil over each container to keep the emulsion in the dark. Keep the emulsion at 4°C, far from all radioactive sources. It is best stored in a clean lead-lined box.

3.4.3. Preparation of the Emulsion

1. Prepare the darkroom as described previously.
2. Warm the emulsion to 42°C. Make sure that the water level reaches the upper level of the emulsion. Keep the aluminum foil in place to avoid any exposure to light.

3.4.4. Coating With the Emulsion

1. In the dark, remove the aluminum foil.
2. Mix the emulsion gently to avoid bubbles. Always keep the emulsion in the water bath.
3. Test the emulsion with a slide to ensure that the emulsion is well mixed.
4. Dip the slide into the emulsion twice, slowly and regularly, at a constant speed (you can count to ensure the constant speed) to ensure a uniform thickness of emulsion. Dry the back of the slide with a Kleenex and drain it on a paper.
5. Check a blank slide for uniform emulsion coating outside the room. Make sure that no light enters the room.
6. Put the emulsion on each slide, one by one.
7. Place the slide on the wood rack vertically, the frosted end within the split.
8. Close the Styrofoam box and place it in the plastic bag.
9. Let dry for 1–3 h at RT.
10. Transfer the slides into the black microslide box with the drierite. Put aluminum foil around it and place it in black thick plastic bags. Keep at 4°C until development (7 or 14 d), far from any radioactive source.
11. Clean up the dark room and the emulsion container.

3.4.5. Development of the Emulsion

1. Warm the microslide box and the solutions to room temperature (at least 30 min).
2. Make the following solutions and place 200 mL of each in separate staining dishes: dilute the Kodak Dektol revealing stock solution 1 : 1 in dH_2O and dilute the fixing stock solution 1 : 3 in dH_2O.
3. Turn off the light in the dark room.
4. Dip slides in the following baths: 8 min in revealing solution; 30 s in water; 4 min in fix; 4 min in fix. Turn on the room light. Rinse the slides in running tap water (or change the

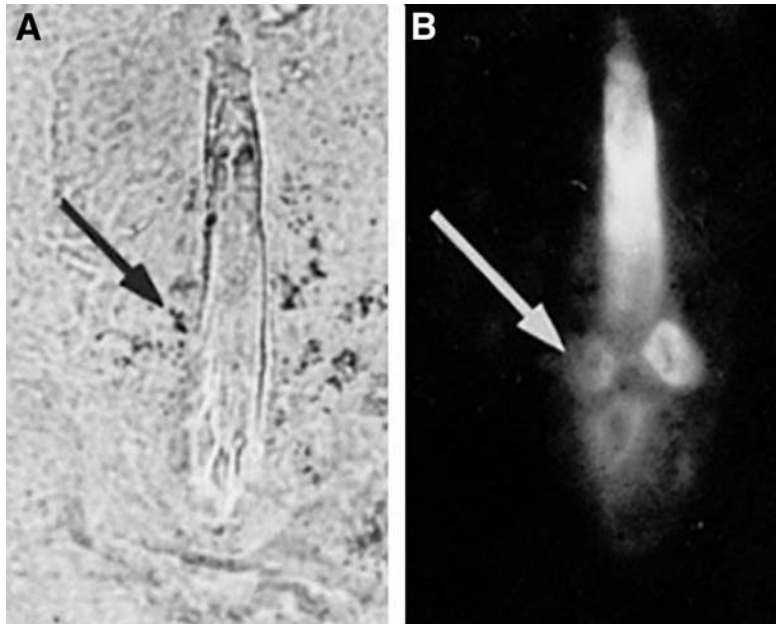


Fig. 1. Double labeling of label-retaining cells **(A)** and keratin 19 **(B)** in the skin tissue.

bath every 5 min) for 30 min. Mount the slides with the solid mounting medium (as described in **Subheading 3.5.**). Observe under an epifluorescence microscope. An example of the results of double labeling of label-retaining cells **(Fig. 1A)** and keratin 19 **(Fig. 1B)** is presented in **Fig. 1** (*see* **Note 5**).

3.5. Single Immunofluorescence Staining of Keratin 19

This section describes the indirect immunofluorescent staining method used to detect the expression of keratin 19, in both murine and human tissues as well as isolated and cultured cells ***(3)***. The same method could be used to detect keratin 18 and keratin 20, which are useful markers for evaluating the Merkel cell subpopulation that can also co-express K19 ***(6)***.

3.5.1. Tissue Sections

For tissue sections, perform **steps 1** to **7** from **Subheading 3.3.** Continue with steps from **Subheadings 3.5.3.** and **3.5.4.**

3.5.2. Cells

For immunofluorescence staining of keratins in cells, keratinocytes cultured on plastic culture dishes or on glass cover slips are fixed with ethanol for 10 min at –20°C. Perform **steps 3** to **7** from **Subheading 3.3.** Continue with steps from **Subheadings 3.5.3.** and **3.5.4.**

3.5.3. Counterstaining With Hoechst

1. The sections or cells can be counterstained with Hoechst (nuclei staining) as follows: wash in dH_2O for 2 min: put the slides in the rack and immerse in two baths of dH_2O. Be careful to avoid detachment of the sections from the slides.

2. Cover with 25 to 50 µL of the Hoechst solution: 0.05 g Hoechst 33258 diluted in 1 L dH_2O.
3. Incubate with the Hoechst for 10 min in the dark.
4. Wash in dH_2O for 2 min: put the slides in the rack and immerse in three baths of dH_2O. Be careful to avoid detachment of the sections from the slides.

3.5.4. Mounting Slides

1. Warm the solid mounting media in warm water or in the microwave to liquefy it. Be careful to avoid too elevated a temperature for the sections.
2. For tissues on slides, place one drop of mounting media on each tissue section.
3. Place a cover slip on top gently, avoiding bubbles.
4. For cells on cover slips: identify the slides. Place 1 drop of mounting media on the slide.
5. Gently place the cover slip with cells in contact with the drop, avoiding bubbles.
6. Remove the excess media by draining on paper or Kleenex.
7. Store at 4°C in the dark to avoid any bleaching of the fluorescence.

The sections are examined under a microscope (e.g., Nikon Optiphot) equipped with epifluorescence.

3.6. Flow Cytometry Analysis

This method is an immunofluorescence staining of cells in suspension and allows the analysis of a great number of cells in suspension by flow cytometry. For keratin 19, cells must be fixed to allow the penetration of the antibodies because keratin is a cytoplasmic protein.

1. Freshly isolated cells are counted and centrifuged (300*g*, 10 min). Subconfluent cell cultures are trypsinized, counted, and centrifuged (300*g*, 10 min).
2. Cells are resuspended in saline at 4°C at a concentration of 1×10^6 cells in 100 µL.
3. Prepare 15-mL tubes with caps by adding 4.5 mL of 70% ethanol at –20°C. Fix the cells by adding them (500 µL with a micropipet) to the cold ethanol. Do not put more than 1×10^6 cells per mL of ethanol to avoid cell aggregation.
4. Fix 30 min at –20°C. Keep at –20°C until use.
5. The labeling is performed in 15-mL conic tubes, 4×10^5 cells in 2 mL PBS–BSA (first wash).
6. Wash: add 2 mL PBS–BSA. Centrifuge (300*g*, 7 min). Remove supernatant.
7. Wash again with 2 mL PBS–BSA.
8. Incubate with the primary antibody. Dissociate the pellet in 40 µL of primary antibody appropriately diluted in PBS–BSA. Put 40 µL PBS–BSA or an unrelated antibody in the control tubes. Agitate manually every 10 min to resuspend the cells. Incubate 45 min at RT.
9. Three washes with 2 mL PBS–BSA. Add 2 mL PBS–BSA. Centrifuge (300*g*, 7 min). Remove supernatant.
10. Incubate with the secondary antibody. Dissociate the pellet in 40 to 70 µL of secondary antibody appropriately diluted in PBS–BSA. Agitate manually every 10 min to resuspend the cells. Incubate 30 min at RT.
11. Three washes with 2 mL PBS–BSA.
12. Resuspend the pellet in 200 µL. Transfer to smaller tubes (e.g., Falcon 2058) and analyze immediately with the flow cytometer (e.g., Becton-Dickinson FACScan®).
13. At least 10,000 events are acquired in list mode for each sample according to three parameters: forward scatter (FSC), side scatter, and green fluorescence. The FSC is gated at 200 (arbitrary units) to exclude false-positive differentiated cells (a variable background staining of differentiated keratinocytes was obtained with unrelated antibodies such as anti-digoxigenin).

14. The percentage of K19-positive cells present in the small- and medium-sized keratinocyte populations (less differentiated, lower FSC) is evaluated by subtracting the control from the labelled cell profile in an FSC gated (channels 0–200 kept and 200–256 excluded) population, using the Consort 30 program, overlaid histogram (Becton-Dickinson) and evaluating the proportion of the cells under the peak or shoulder.

4. Notes

1. Five times concentrated PBS solution can be prepared and kept at 4°C, but the $CaCl_2$ should be omitted and added only after dilution of PBS to 1X.
2. Ten times concentrated solution can also be prepared and kept at 4°C.
3. The labeling of slow-cycling cells in adult mice (4-wk-old SENCAR mice) can be performed by implanting two Alzet osmotic minipumps, each loaded with 200 µCi of [methyl-^{3}H]thymidine. Each pump should deliver 10 µCi/d for a total dose of 20 µCi of [methyl-^{3}H]thymidine per animal per day. The labeling is performed for 14 continuous days. The pumps are then removed. The animals are kept for 4 to 8 more weeks ***(8)***.
4. To improve the adhesion of the tissue, glass slides can be treated with gelatin (immersed in 0.5% gelatin in dH_2O and dried), or Superfrost slides can be used.
5. Counterstain the slides if desired. Be careful not to mask the fluorescence.

Acknowledgments

The authors would like to thank other present and past members of the LOEX laboratory who have contributed to developing the foregoing protocols.

References

1 Green, H., Kehinde, O., and Thomas, J. (1979) Growth of cultured human epidermal cells into a multiple epithelia suitable for grafting. *Proc. Natl. Acad. Sci. USA* **76,** 5665–5668.
2. Germain, L., Rouabhia, M., Guignard, R., Carrier, L., Bouvard, V., and Auger, F. A. (1993) Improvement of human keratinocyte isolation and culture using thermolysin. *Burns* **2,** 99–104.
3. Germain, L., Michel, M., Fradette, J., Xu, W., Godbout, M-J., and Li, H. (1997) Skin stem cell identification and culture: a potential tool for rapid epidermal sheet production and grafting, in *Skin Substitute Production by Tissue Engineering: Clinical and Fundamental Applications.* (Rouabhia, M., ed.), Landes Bioscience, Austin, TX, pp. 177–210.
4 Michel, M., Török, N., Godbout, M.-J., Lussier, M., Gaudreau, P., Royal, A. et al. (1996) Keratin 19 as a biochemical marker of skin stem cells in vivo and in vitro: keratin 19 expressing cells are differentially localized in function of anatomic sites, and their number varies with donor age and culture stage. *J. Cell. Sci.* **109,** 1017–1028.
5 Michel, M., L'Heureux, N., Auger, F. A., and Germain, L. (1997) From newborn to adult: phenotypic and functional properties of skin equivalent and human skin as a function of donor age. *J. Cell Phys.* **171,** 179–189.
6 Fradette, J., Larouche, D., Fugère, C., Guignard, R., Beauparlant, A., Couture, V., et al. (2003) Normal human Merkel cells are present in epidermal cell populations isolated and cultured from glabrous and hairy skin sites. *J. Invest. Dermatol.* **120,** 313–317.
7 Lussier, M., Ouellet, T., Lampron, C., Lapointe, L., and Royal, A. (1989) Mouse keratin 19: complete amino acid sequence and gene expression during development. *Gene* **85,** 435–444.
8. Cotsarelis, G., Sun, T. T., and Lavker, R. M. (1990) Label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogenesis. *Cell* **61,** 1329–1337.

III

Analysis of Epidermal Differentiation

13

Immunolocalization in the Epidermis

Tammy-Claire Troy, Ramtin Rahbar, Bilge Diker, and Kursad Turksen

Summary

Immunohistochemistry is an efficient means of localizing specific proteins to their relative expression compartment in the epidermis thereby providing evidence as to their functionality. This chapter therefore describes a dependable method for immunolocalization within the epidermis.

Key Words:

Immunohistochemistry; epidermis; differentiation; keratins; antibody.

1. Introduction

In the epidermis, immunohistochemistry is an efficient means of localizing specific proteins to their relative expression compartment; namely the basal, suprabasal, and stratum corneum layers. The precise localization within the epidermis of a particular protein lends clues toward its functional role within the epidermis. In this chapter, we describe a reliable method for immunolocalization within the epidermis modified for both frozen and paraffin sections that we use very routinely in our laboratory ***(1,2)***. Paraffin sections generally provide much better morphology, hence, superior results and photographs; however, not all antibodies will work with the harsh fixation and treatment involved in their processing. Therefore, the protocol for frozen sectioning is also included. Within paraffin sectioning, two fixation protocols are described (Bouin's and paraformaldehyde); the choice of fixative will be directly related to the antibody specifications and may require another fixing method.

2. Materials

2.1. Tissue Specimen Slide Preparation

2.1.1. Mouse Dissection

1. Electric razor (e.g., commercially available Braun, Phillips or Remington).
2. Dissecting tools: fine-tipped Dumont forceps no. 5 (Fine Science Tools, North Vancouver, Canada; cat. no. 11251-30); Straight Fine Scissors 10.5 cm (Fine Science Tools; cat. no. 14094-11).
3. Paper towels (brown; Grand & Toy, Toronto, Canada; cat. no. WW405306).

2.1.2. Fixation and Paraffin Block Preparation

1. Saturated picric acid (Sigma–Alderich, Oakville, Canada; cat. no. 925-40).
2. Formaldehyde solution (Fisher Scientific, Nepean, Canada; cat. no. F79-500).

From: *Methods in Molecular Biology, vol. 289:*
Epidermal Cells: Methods and Protocols
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

3. Glacial acetic acid (Fisher; cat. no. A38-500).
4. Bouin's fixative solution (*see* **Note 1**). For 50 mL: 37.5 mL saturated picric acid, 10 mL formaldehyde and 2.5 mL glacial acetic acid. Use at room temperature.
5. Paraformaldehyde (Fisher; cat. no. 04042-500).
6. 0.1 *N* NaOH. For 100 mL: 0.4 g sodium hydroxide (NaOH) in 100 mL H_2O.
7. 10X Phosphate-buffered saline (PBS), 1 L: dissolve 11.5 g sodium phosphate dibasic (Na_2HPO_4) and 2.0 g potassium phosphate monobasic (KH_2PO_4) in 700 mL H_2O. Add 80 g sodium chloride (NaCl) and 2.0 g potassium chloride (KCl). Top up the final volume to 1 L with H_2O.
8. 50-mL Falcon Tubes (VWR International, Mississauga, Canada; cat. no. 21008-940).
9. 4% paraformaldehyde (*see* **Note 1**). For 100 mL: using a stirrer/hotplate and stir bar, heat 60 mL H_2O to 65°C in the fumefood. Add 4 g paraformaldehyde and stir. Add 60 µL 0.1 *N* NaOH and stir until the solution is clear, remove from heat. Add 10 mL 10X PBS and bring final volume to 100 mL with H_2O. Mix well and pour into 2X 50-mL Falcon tubes; cool on ice for at least 20 min.
10. 17 × 100-mm polypropylene sterile culture tubes (Fisher Scientific, Nepean, Canada; cat. no. 14-956-1J).
11. Parafilm (VWR; cat. no. 52858-000).
12. Rocker (e.g., Hoefer RedRocker model no. pR50-115V; Hoefer Scientific Instruments, San Francisco, CA).
13. 1X PBS (diluted from 10X; *see* **Subheading 2.1.2., item 7**).
14. 30%, 50%, 70%, 90%, and 100% ethanol.
15. Toluene (Fisher; cat. no. T323-4).
16. Embedding cassettes (Fisher; cat. no. 15-182-500).
17. Fisherbrand Superfrost Plus Microscope Slides (Fisher; cat. no. 12-550-15).

2.1.3. Frozen Sectioning

1. Isopentane (2-methylbutane, Fisher; cat. no. 03551-4).
2. Wide-mouth container (e.g., a thermos).
3. Dry ice.
4. Cryomolds 24 mm × 24 mm × 5 mm (Fisher; cat. no. 22-038-218).
5. Cryomatrix frozen specimen embedding medium (Fisher; cat. no. 28-600-51).
6. Fine tipped Dumont forceps no. 5 (Fine Science Tools; cat. no. 11251-30).
7. Serrated Jaw Forceps 8-in (VWR; cat. no. 25607-120).
8. Aluminium foil.
9. Airtight plastic container (e.g., a Rubbermaid container).
10. Fisherbrand Superfrost Plus Microscope Slides (Fisher; cat. no. 12-550-15).
11. Slide box (Fisher; cat. no. 03-448-10).
12. Parafilm (VWR, cat. no. 52858-000).

2.2. Immunolocalization

2.2.1. Dewaxing Paraffin Sections

1. Slide rack (Fisher; cat. no. 08-812-1B) with metal handle (Fisher; cat. no. 08-812-1C).
2. Hybridization oven heated to 50°C.
3. Aluminium foil.
4. Staining dish with cover (Fisher; cat. no. 08-812-1A).
5. Toluene (Fisher; cat. no. T323-4).
6. 100%, 95%, and 70% ethanol.
7. Sodium citrate dihydrate (Fisher; cat. no. S279-500).
8. Citric acid monohydrate (Fisher; cat. no. A104-500).

9. 1X Citrate buffer (diluted from 10X solution). For 10X solution: dissolve 24.1 g sodium citrate dihydrate and 3.78 g citric acid monohydrate into 900 mL H_2O. Autoclave and store at room temperature (*see* **Note 2**).
10. Polypropylene beaker (1 L) (Fisher; cat. no. 02-591-17E).
11. Microwave oven.
12. 1X PBS (diluted from 10X; *see* **Subheading 2.1.2., item 7).**

2.2.2. Fixing Frozen Sections

1. Slide rack (Fisher; cat. no. 08-812-1B) with metal handle (Fisher; cat. no. 08-812-1C).
2. Staining dish with cover (Fisher; cat. no. 08-812-1A).
3. Methanol (–20°C) (Fisher; cat. no. A452SK-4).
4. 1X PBS (diluted from 10X; *see* **Subheading 2.1.2., item 7**).

2.2.3. Hematoxylin and Eosin (H&E) Staining

1. Slide rack (Fisher; cat. no. 08-812-1B) with metal handle (Fisher; cat. no. 08-812-1C).
2. Staining dish with cover (Fisher; cat. no. 08-812-1A).
3. Shandon Instant Hematoxylin (Fisher; cat. no. 28-601-02). To make stock solution: to 800 mL H_2O, add bottle A, mix, then add bottle B. Store at room temperature for up to 2 wk protected from light.
4. Hematoxylin (working solution): dilute stock solution 1:3. For 450 mL: dilute 150 mL stock solution in 300 mL H_2O (*see* **Note 3**).
5. 80%, 95%, and 100% ethanol.
6. Glacial acetic acid (Fisher; cat. no. A38-500).
7. Eosin (Fisher; cat. no. E-511).
8. Stock eosin solution: dissolve 10 g eosin in 100 mL H_2O and add 800 mL 95% ethanol.
9. Working eosin solution: combine 200 mL stock Eosin solution with 600 mL 80% ethanol and 4 mL glacial acetic acid.
10. Toluene (Fisher; cat. no. T323-4).
11. Permount Mounting Media (Fisher; cat. no. SP15-100).
12. Fisherfinest™ Premium cover glass (22 × 50) (Fisher; cat. no. 12-548-5E).

2.2.4 Immunostaining

1. PAP Pen (The Binding Site, Birmingham, UK; cat. no. AD100.1).
2. 15-cm Pyrex dish (Fisher; cat. no. 08-747F).
3. Filter paper (Fisher; cat. no. 09-801D).
4. Humidified chamber (*see* **Note 4**).
5. 1X PBS (diluted from 10X; *see* **Subheading 2.1.2., item 7**).
6. Bovine Serum Albumin (BSA, fraction V, Sigma; cat. no. A-9647).
7. Gelatin (IGSS quality; Amersham Biosciences, Baie d'Urfé, Canada; cat. no. RPN416V).
8. For 1 L washing buffer: dissolve 8 g BSA into 50 mL 1X PBS, top the volume to 1 L with 1X PBS, and add 1 mL gelatin.
9. Goat serum (Invitrogen, Burlington, Canada; cat. no. 16210-064). To heat inactivate: thaw the bottle of serum overnight at 4°C, warm in a 57°C water bath for 7 min with constant mixing, then continue to incubate for 30 min with occasional mixing. Aliquot and store at –20°C.
10. Blocking buffer. For 1 mL: add 50 µL heat-inactivated goat serum to 950 µL washing buffer.
11. Slide rack (Fisher; cat. no. 08-812-1B) with metal handle (Fisher; cat. no. 08-812-1C).
12. Staining dish with cover (Fisher; cat. no. 08-812-1A).
13. For 1 mL incubation buffer: add 10 µL heat-inactivated goat serum to 990 µL washing buffer.

14. 1° and 2° antibodies (commercially available or custom-generated).
15. Mowiol 4-88 (Polysciences Inc.; cat. no. 17951).
16. Glycerol (Fisher; cat. no. BP229-1).
17. 0.2 *M* Tris-HCl, pH 8.5. For 100 mL, dissolve 2.42 g Tris in 70 mL H_2O, pH to 8.5 with HCl and top the volume to 100 mL with H_2O. Autoclave and store at room temperature.
18. 50-mL Falcon Tubes (VWR International, Mississauga, Canada; cat. no. 21008-940).
19. 50°C water bath.
20. Disposable 10-mL pipets (Fisher; cat. no. 13-678-11E).
21. Nalgene centrifuge tubes (Fisher; cat. no. 05-562-14D).
22. DABCO (1, 4-diazabicyclo[2. 2. 2]octane; Sigma; cat. no. D-2522).
23. Mowiol+. For 20 mL: add 2.4 g Mowiol 4-88 to 6 g glycerol and stir for 2 h at room temperature. Add 6 mL dH_2O and stir for several hours at room temperature. Add 12 mL 0.2 *M* Tris, pH 8.5, and heat to 50°C for 10 min with occasional mixing. Using a disposable pipet, transfer solution to a Nalgene tube and spin at 5000*g* for 15 min. Transfer solution to a fresh tube and add 2.5% DABCO as an anti-fade agent. Stir to dissolve. Prepare 1-mL aliquots and store at –20°C. As needed, a tube is thawed and may be stored for several weeks at 4°C.
24. Fisherfinest™ Premium cover glass (22 × 50) (Fisher; cat. no. 12-548-5E).

3. Methods

3.1. Tissue Specimen Slide Preparation

3.1.1. Mouse Dissection

1. Using an electric razor, gently shave fur from the desired area of backskin (*see* **Note 5**).
2. As per animal care guidelines, sacrifice the mouse. Working quickly to minimize tissue damage, harvest the skin, and gently lie flat on a piece of paper towel (*see* **Note 6**). Trim the paper towel to the edges of the skin and if necessary, cut the tissue section into manageable pieces (*see* **Note 7**). Proceed directly to fixation (*see* **Subheading 3.1.2.**) or freezing (*see* **Subheading 3.1.3.**).

3.1.2. Fixation and Paraffin Block Preparation

1. Prepare Bouin's or 4% paraformaldehyde fixative directly prior to fixation. Dispense 15 mL into labeled polypropylene culture tubes.
2. Immerse tissue in the appropriate fixative solution; cap the tube and seal with parafilm. Place tubes on a rocker and incubate overnight with agitation (*see* **Note 8**). When fixing with Bouin's solution incubate at room temperature and when using 4% paraformaldehyde incubate at 4°C.
3. After fixation, rinse tissue samples with a few changes of 1X PBS at room temperature.
4. Dehydrate samples through serial ethanol washes at room temperature: (1) 30% ethanol, 2 × 30 min, 15 mL per wash; (2) 50% ethanol, 2 × 30 min, 15 mL per wash; (3) 70% ethanol, 2 × 30 min, 15 mL per wash.
5. After the last wash, replace with a fresh 15 mL 70% ethanol. Samples can be stored at 4°C in 70% ethanol until processing for paraffin sectioning.
6. Prior to paraffin embedding, samples are further dehydrated: (1) 90% ethanol, 2 × 30 min, 15 mL per wash; (2) 100% ethanol, 2 × 30 min, 15 mL per wash.
7. Dehydrated tissues are washed 2 × 30 min in toluene, then are encased in embedding cassettes and submerged for 30 min in paraffin heated to 56°C (*see* **Note 9**) to infiltrate the tissue.
8. Paraffin blocks are prepared such that tissues are in an optimal orientation (*see* **Note 10** and **Note 11**). Sections are cut to an optimal thickness of 7–10 μm and mounted onto

Fisherbrand Superfrost Plus slides (*see* **Note 12**). Cut section slides and uncut paraffin blocks can be stored for future need at room temperature.

3.1.3. Frozen Section Block Preparation

1. Pour isopentane into a wide mouth container and add dry ice to make it cold (*see* **Note 13**).
2. On a flat surface, fill cryomold with cryomatrix embedding medium and using a pair of fine forceps, orientate the skin within the embedding media.
3. Using a pair of serrated jaw forceps, immediately submerge the cryomold into the cold isopentane for 30 s until it is frozen solid (*see* **Note 14**).
4. Remove frozen tissue block from cryomold and wrap in aluminium foil and place in an airtight plastic container to store at –20°C until sectioning (*see* **Note 15**).
5. Frozen sections of skin are cut at –25°C, 7–10 μm in thickness and are mounted onto Fisherbrand Superfrost Plus slides (*see* **Note 12**). Preprepared slides may be stored in a parafilm wrapped slide box at –20°C (*see* **Note 15**).

*3.2. Immunolocalization (***see** ***Note 16)***

3.2.1. Dewaxing Paraffin Sections

1. Place slides in a slide rack, wrap with aluminium foil and incubate at 50°C for 15 min (*see* **Note 17**).
2. Quickly unwrap the slide rack and incubate in three changes of toluene, 5 min each (*see* **Note 18**). After the third incubation in toluene, wash slides briefly in 100% ethanol before proceeding (*see* **Note 19**).
3. Incubate in three changes of 100% ethanol, 5 min each.
4. Rehydrate samples: (1) 95% ethanol, 5 min; (2) 70% ethanol, 5 min; (3) H_2O, 2 × 5 min. Proceed directly to H&E (*see* **Subheading 3.2.3.**) from this step or continue with the following steps for immunohistochemistry (*see* **Subheading 3.2.4.**).
5. Pour 500 mL 1X citrate buffer into a 1-L plastic beaker and immerse slide rack (without the metal handle). Microwave at high power for 2 × 5 min (*see* **Note 20**).
6. Transfer slide rack to a staining dish containing warm tap water for 5 min to gradually reduce the working temperature.
7. Incubate slides in three changes of 1X PBS, 5 min each wash.
8. Proceed directly to immunostaining (*see* **Subheading 3.2.4.** and **Note 21**).

3.2.2. Fixing Frozen Sections

1. Remove slides from –20°C and place in a slide rack. Incubate 5 min at room temperature (*see* **Note 22**).
2. Fix sections in cold 100% methanol at –20°C for 10 min (*see* **Note 23**).
3. Wash sections 3 × 5 min in three changes of 1X PBS at room temperature.
4. Proceed directly to H&E (*see* **Subheading 3.2.3.**) or immunohistochemistry (*see* **Subheading 3.2.4.**; *see* **Subheading 3.2.4.** and **Note 24**).

*3.2.3. H&E Staining (***see** ***Note 25)***

1. Stain slides in working hematoxylin solution for 2 min (*see* **Note 3**). Wash in H_2O, 5 min.
2. Stain slides in working eosin solution for 3 min (*see* **Note 3**).
3. Wash in two changes of 95% ethanol, 2 min each followed by two changes in 100% ethanol, 2 min each.
4. Incubate 2 × 2 min in toluene. Remove excess toluene and mount with a few drops of permount and apply a cover slip (*see* **Note 26**).

3.2.4. Immunohistochemistry

1. Remove slide from PBS and, using a tissue paper, dry the glass surrounding the specimen (*see* **Note 27**). With a PAP pen, demarcate the area of the specimen to prevent cross-contamination of antibodies between specimens.
2. Place slides in a humidified chamber (*see* **Note 4**) and incubate sections with 100 µL blocking solution for 30 min (*see* **Note 28** and **Note 29**).
3. Transfer slides to a slide rack and wash for 5 min in washing buffer.
4. Remove excess washing buffer surrounding the specimen (*see* **Note 27**) and return slides to the humidified chamber. Incubate sections with 50 µL of appropriately diluted 1° antibodies and incubate for 1 h at room temperature (*see* **Notes 28–30**).
5. Return slides to the slide rack and wash in three changes of washing buffer, 10 min each.
6. Remove excess washing buffer surrounding the specimen (*see* **Note 27**), cover the specimen with 50 µL of appropriately diluted 2° antibodies, and incubate for 1 h at room temperature in a humidified chamber (*see* **Notes 28–30**).
7. Wash in three changes of washing buffer, 15 min each.
8. Wash in three changes of 1X PBS, 5 min each.
9. Remove all excess liquid surrounding the specimen (*see* **Note 27**) and wipe the PBS away from the back of the slide. Apply 60 µL Mowiol$^+$ to the slides and carefully cover with a cover slip (*see* **Note 31**).

4. Notes

1. Fixative solutions should be prepared with disposable or designated plastic and glassware.
2. 10X citrate buffer tends to become contaminated; therefore it is important to check it prior to starting the protocol.
3. Staining solution is prepared freshly and is used for 1 wk to a maximum of 30 slides to ensure quality staining.
4. A humidified chamber is prepared by placing a wet piece of filter paper into a 15-cm glass dish with a lid.
5. The electric razor minimizes skin tissue damage as compared with a disposable razor or blade. Waxing is not recommended because it will bruise the area.
6. Laying the skin onto a piece of paper towel will prevent the skin from rolling up.
7. In order for complete and consistent fixation and easier sectioning, the tissue piece should be no larger than 1 cm^2.
8. Fixation should be approx 16 to 18 h; shorter/longer fixation periods are not recommended.
9. If the paraffin is too hot or if the incubation period is too long, the antigenicity of the tissue may be compromised.
10. Skin sections should be orientated such that the resulting sections display either complete hair follicles (from tip to bulb region) or partial structures (showing all concentric layers).
11. If tissue is poorly oriented in the final block, the block can be melted down and the tissue re-embedded.
12. Superfrost Plus slides are used because they electrostatically charged to assure that tissues remain adhered to the slides and that they do not fold or crease.
13. When the bubbling stops it is at the right temperature.
14. The embedding media changes from a transparent liquid to an opaque white solid upon freezing.
15. We have found that long-term storage at –20°C does not preserve tissue integrity; therefore storage beyond 2 wk is not recommended.
16. The following steps are performed with slides in a slide rack and solutions in rectangular staining dishes with lids. An immunolocalization workstation may be set up in your laboratory with separate staining dishes designated to all of the individual solutions required. This

minimizes set up and clean up time. The solutions are simply refreshed each day prior to staining, with the exception of citrate buffer, PBS and washing buffer, which must be prepared fresh daily.

17. For incubation at 50°C, we use a hybridization oven switched on at least 1 h before use.
18. This step should be carried out in a fume hood, as toluene fumes are hazardous to your health.
19. A white precipitate will form in the ethanol rinse.
20. Between incubations open the door briefly to cool the oven.
21. Paraffin section slides are much less delicate than frozen section slides; therefore washes can be done more vigorously and shaking the slide to remove excess liquid is acceptable.
22. Bringing slides to room temperature increases the adhesiveness of the sections to the glass.
23. A 500-mL bottle of methanol and a staining dish is stored at –20°C for this purpose.
24. Frozen section slides must be treated more delicately than paraffin section slides as they have a greater tendency to fold and/or detach from the slide; therefore all washes should be done very gently and shaking the slide to remove excess liquid should be avoided.
25. It is recommended to stain one slide of each specimen to verify tissue integrity and orientation before continuing with immunohistochemistry in order to reduce the costs associated with proceeding using substandard sections.
26. Be careful not to use too much permount as it will leak out of the cover slip and damage the microscope stage; allow the permount to harden at least 1 h at room temperature before observation.
27. Be careful not to touch the specimen.
28. Working quickly, wipe away excess liquid and apply blocking buffer/antibodies one slide at a time to prevent drying.
29. Make sure that sections are fully covered so that they do not dry out during the incubation period; drying will increase the background.
30. Incubation temperatures and times may vary for specific antibodies; consult your antibody specification sheet for specific requirements.
31. Lower the cover slip slowly onto the slide to minimise the generation of bubbles between the specimen and the cover slip.

Acknowledgments

This work was sponsored by grants from the Canadian Institutes of Health Research (CIHR). We would like to thank Mr. Peter Ripstein MLT., ART., Mr. William Parks MLT. and Ms. Josephine Urbisci MLT. from the Ottawa Hospital for their unfailing technical support over the years.

References

1 Turksen, K. and Troy, T. C. (2003) Overexpression of the calcium sensing receptor accelerates epidermal differentiation and permeability barrier formation in vivo. *Mech. Dev.* **120,** 733–744.

2. Turksen, K. and Troy, T. C. (2002) Permeability barrier dysfunction in transgenic mice overexpressing claudin 6. *Development* **129,** 1775–1784.

14

Epidermal Cell Analysis by RT-PCR

Tammy-Claire Troy, Robert Man-Kit Cheung, and Kursad Turksen

Summary

Reverse transcription polymerase chain reaction is used for the semiquantitative analysis of epidermal gene expression, particularly when immunolocalization is not feasible because of the lack of antibodies available for novel genes. This chapter is therefore devoted to the delineation of a reliable reverse transcription polymerase chain reaction method to analyze gene expression both in vivo and in vitro.

Key Words:

RNA; gene expression; RT-PCR; epidermal cells; differentiation.

1. Introduction

The analysis of epidermal gene expression by reverse-transcription polymerase chain reaction (RT-PCR) is favored as it provides a valuable overall sense of gene expression levels especially when there are no antibodies yet available for novel genes. In this protocol, we describe the RT-PCR method that we routinely and very successfully employ to analyze gene expression both in vivo and in vitro ***(1,2)***.

2. Materials

2.1. RNA Extraction (see Note 1)

2.1.1. From Cultured Cells

1. 1X phosphate-buffered saline (PBS): diluted from 10X PBS. To prepare 10X PBS (1 L): dissolve 11.5 g sodium phosphate dibasic (Na_2HPO_4) and 2.0 g potassium phosphate monobasic (KH_2PO_4) in 700 mL H_2O. Add 80 g sodium chloride (NaCl) and 2.0 g potassium chloride (KCl). Top up the final volume to 1 L with H_2O.
2. TRIzol® Reagent (100 mL, Invitrogen, Burlington, Canada; cat. no. 15596-026).
3. Cell Scrapers (Falcon, VWR International, Mississauga, Canada; cat. no. 15621-010).
4. 17- × 100-mm Polypropylene Sterile Culture Tubes (Fisher Scientific, Nepean, Canada; cat. no. 14-956-1J).
5. Chloroform (500 mL, Sigma-Aldrich, Oakville, Canada; cat. no. C-2432).
6. Parafilm (VWR; cat. no. 52858-000).
7. High-performance centrifuge with appropriate centrifuge rotors and adaptors (i.e., Beckman Coulter Avanti™ J-25I, Beckman Coulter, Mississauga, Canada).
8. Isopropanol (500 mL, Fisher, cat. no. A416-500).
9. 75% ethanol (–20°C), diluted in diethyl pyrocarbonate water (DEPC–H_2O).

From: *Methods in Molecular Biology, vol. 289:*
Epidermal Cells: Methods and Protocols
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

10. DEPC-H_2O (diethyl pyrocarbonate water); DEPC (5 mL, Sigma; cat. no. D-5758).
11. RNase-free microtubes.
12. –80°C freezer.

2.1.2 From Mouse Tissue

Previously listed items (except **item 3**) are required in addition to the following:

1. Dissecting tools: Fine-tipped Dumont forceps no. 5 (Fine Science Tools, North Vancouver, Canada; cat. no. 11251-30); Straight Fine Scissors 10.5 cm (Fine Science Tools; cat. no. 14094-11).
2. Aluminum foil.
3. Liquid nitrogen.
4. Serrated jaw forceps 8 (VWR; cat. no. 25607-120).
5. 50-mL Falcon tubes (VWR; cat. no. 21008-940).
6. 75% ethanol (room temperature) diluted in DEPC–H_2O.
7. Homogeniser (i.e., Polytron® System PT 1300D; Kinematica AG, Luzern, Switzerland).
8. Microcentrifuge located at 4°C.

2.2. Purifying RNA Samples (see Note 1)

1. RNase-free microtubes.
2. DEPC–H_2O.
3. 10X DNase buffer: 200 m*M* Tris-HCl, pH 8.4, 20 m*M* $MgCl_2$, 500 m*M* KCl; for 100 mL, combine 50 mL 1 *M* KCl, 1.5 mL 1 *M* $MgCl_2$, 10 µL of gelatin, and 10 mL Tris-HCl, pH 8.4. Aliquot and store at –20°C.
4. RNase Inhibitor (20 U/µL, Perkin–Elmer Applied Biosystems, Foster City, CA; cat. no. N808-0119).
5. DNase I (256 U/µL, Invitrogen, cat. no. 18047-019).
6. Phenol, saturated solution (100 mL, Amresco, Solon, OH; cat. no. 0945-100ML).
7. Chloroform (500 mL, Sigma; cat. no. C-2432).
8. Isoamyl alcohol (500 mL, Fisher; cat. no. A393-500).
9. 3 *M* Sodium acetate. For 100 mL: dissolve 24.6 g of sodium acetate in 80 mL of DEPC–H_2O and top volume to 100 mL.
10. Anhydrous ethyl alcohol (–20°C).
11. 70% EtOH (–20°C), diluted in DEPC–H_2O.

2.3. RT-PCR (see Note 1)

1. MicroAmp Reaction Tubes (Perkin–Elmer; cat. no. N801-0840).
2. Thermocycler (i.e., Model 2400, Perkin Elmer; cat. no. 0993-6057).
3. DEPC–H_2O.
4. 25 m*M* $MgCl_2$ (Perkin–Elmer, cat. no. N808-0010).
5. 10X PCR buffer II (Perkin–Elmer, cat. no. N808-0010).
6. 100 m*M* dNTP set (Invitrogen; cat. no. 10297-018).
7. 10 m*M* dNTPs are required for RT. Dilute stock dNTP's 1:10 with DEPC–H_2O.
8. RNase Inhibitor (20 U/µL, Perkin–Elmer; cat. no. N808-0119).
9. MuLV Reverse Transcriptase (50 U/µL, Perkin–Elmer, cat. no. N808-0018).
10. Random hexamers (50 µ*M*, Perkin–Elmer, cat. no. N808-0127).

2.4. PCR

1. MicroAmp reaction tubes (Perkin–Elmer; cat. no. N801-0840).
2. Thermocycler (model 2400, Perkin–Elmer; cat. no. 0993-6057).

3. 10X PCR buffer containing 15 m*M* $MgCl_2$, supplied with *Taq* polymerase.
4. 100 m*M* dNTP set (Invitrogen; cat. no. 10297-018).
5. 2.5 m*M* dNTPs are required for PCR. Combine 250 µL of each stock dNTP (100 m*M*) into a sterile microtube and mix (=25 m*M*). Aliquot 100 µL into each of 10 tubes and add 900 µL sterile H_2O and mix (=2.5 m*M*).
6. PCR primers diluted to 25 pmol/µL with sterile H_2O.
7. *Taq* polymerase (5U/µL, Perkin–Elmer, cat. no. N808-0160).
8. Agarose gel apparatus and reagents for running the gel (agarose, TBE, ethidium bromide).

3. Methods

*3.1. RNA Extraction (***see** ***Note 1)***

*3.1.1. From Cultured Cells (*see ***Notes 2** *and* **3)***

1. Remove culture media and rinse cells three times with 1X PBS. Lyse cells in the tissue culture dish by adding TRIzol® (for a 35-mm dish, use 1 mL; 60-mm dish, use 3 mL; and for a 100-mm dish, use 6 mL) (*see* **Note 4**).
2. Using a cell scraper, collect cells into the TRIzol® and transfer to a sterile culture tube. Incubate the samples for 5 min at room temperature.
3. Add 0.2 mL chloroform/mL of TRIzol® used (i.e., for a 35-mm dish, use 200 µL; 60-mm dish, use 600 µL; and for a 100-mm dish, use 1.2 mL). Cap the tubes shake vigorously for 15 s, then incubate at room temperature for 3 min. Replace the cap with parafilm during the incubation and place tubes in centrifuge adaptors (*see* **Note 5**).
4. Centrifuge at 12,000*g* for 15 min at 4°C (*see* **Note 6**). The mixture will separate into a lower phenol : chloroform phase, an interface, and a colorless upper aqueous phase (*see* **Note 7**).
5. Transfer the aqueous phase into a fresh tube. Precipitate the RNA by adding 0.5 mL of isopropanol/mL TRIzol® originally used (i.e., for a 35-mm dish, use 0.5 mL; 60-mm dish, use 1.5 mL; and for a 100-mm dish, use 3 mL) and vortex briefly. Incubate the samples for 10 min at room temperature, replace the cap with parafilm, and place the tubes in a centrifuge adaptor (*see* **Note 5**).
6. Centrifuge at 12,000*g* for 10 min. The RNA forms a pellet on the bottom and the side of the tube.
7. Remove the supernatant and wash the pellet with RNase-free 75% ethanol (–20°C). Use 1 mL ethanol/mL of TRIzol® used originally (i.e., for a 35-mm dish, use 1 mL; 60-mm dish, use 3 mL; and for a 100-mm dish, use 6 mL). Vortex the sample and centrifuge at 7500*g* for 5 min.
8. Air-dry the pellet and resuspend it in 20–30 µL DEPC–H_2O (*see* **Note 8**).
9. Read the absorbance of the RNA at 260 nm and 280 nm (*see* **Note 9**). Use the following equation to calculate the concentration:

$$[\text{RNA}] \text{ in } \mu g/\mu L = (A_{260} \times 40 \times \text{dilution factor}) / 1000$$

10. Store at –80°C.

3.1.2. From Tissues

3.1.2.1. Mouse Dissection

1. Shave fur from desired area of mouse using an electric razor (*see* **Note 10**).
2. Euthanize the mouse per your animal care regulations.
3. Quickly and precisely harvest tissue (i.e., skin) from mouse and wrap in aluminum foil. The tissue piece should be approx 100 to 200 mg cut up into small pieces (*see* **Note 11**). Label the foil with a permanent marker and using a pair of long serrated jaw forceps immerse in a container of liquid nitrogen until frozen (~60 s).

4. Transfer frozen tissue wrapped in foil to the –80°C freezer until you are ready to extract RNA.

3.1.2.2. RNA Extraction (*see* **Notes 1–3**)

1. Clean homogenizer thoroughly by washing 10 s each in three changes of H_2O, 10 s each in three changes 70% ethanol, and finally 10 s each in three changes of DEPC–H_2O. Wipe away excess H_2O with a fresh tissue.
2. Add 4 mL TRIzol® to a sterile culture tube and place the tube on ice protected from light until required (*see* **Note 4**).
3. Unwrap foil packet and carefully transfer its contents to the TRIzol® containing tube. Label tube immediately as to ensure no confusion of its contents (*see* **Note 12**).
4. Homogenize at medium speed for 60 s or until the tissue is adequately homogenized (*see* **Note 13**). After each sample, ensure that there is no remaining tissue chunks trapped in the homogenizer, wipe homogenizer with a tissue and clean as in **step 1** (*see* **Note 14**).
5. Add 800 µL chloroform to each tube (0.2X the volume of TRIzol® used). Tightly cap and shake vigorously for 15 s. Incubate 3 min at room temperature. While waiting, cap with parafilm and place in centrifuge adapter (*see* **Note 5**).
6. Centrifuge at 12,000*g*, 15 min at 2–8°C (*see* **Note 6**).
7. Transfer the aqueous phase (clear upper layer) to a fresh and labeled culture tube (*see* **Note 7**). Add 2 mL of isopropanol to each tube (0.5X the volume TRIzol® used). Tightly cap and briefly vortex then incubate at room temperature for 10 min. While waiting, cap with parafilm and place in centrifuge adapter (*see* **Note 5**).
8. Centrifuge at 12,000*g* for 10 min to precipitate RNA.
9. Carefully aspirate the supernatant and wash pellet with 1 mL cold (–20°C) 75% ethanol and transfer to a labeled microfuge tube. Rinse culture tube with another 0.5 mL to ensure all RNA is transferred. Vortex briefly.
10. Centrifuge at 7500*g* for 20 min at 4°C.
11. Aspirate the ethanol and invert the microtube to air dry the pellet for 15–30 min.
12. Dissolve the pellet in 20–50 µL of DEPC–H_2O (as required; *see* **Note 8**).
13. Quantify RNA as in **Subheading 3.1.1., step 9** and store samples at –80°C.

3.2. Removal of Genomic DNA From RNA Samples (see Notes 1 and 3)

1. Digest 30–100 µg RNA so that RNA + DEPC–H_2O = 87 µL. Add 10 µL 10X DNase buffer; 2 µL RNase inhibitor; and 1 µL DNase I.
2. Mix and incubate for 15 min at room temperature.
3. Prepare chloroform : isoamyl alcohol (24 : 1): In a 50-mL Falcon tube put 12 mL chloroform + 0.5 mL isoamyl alcohol (*see* **Note 15**).
4. Add an equal volume of phenol : chloroform to RNA sample (*see* **Note 15**). For example: for a 100-µL sample, add 50 µL phenol and 50 µL chloroform : isoamyl alcohol (24 : 1).
5. Mix vigorously by inversion until an emulsion forms and spin at maximum speed at room temperature for 5 min.
6. Transfer the aqueous phase (the top phase) to a fresh microtube.
7. Ethanol precipitate by adding a 1 : 10 dilution of 3 *M* NaAc. For example: for a 100-µL sample, add 10 µL 3 *M* NaAc.
8. Add 2X volume 100% EtOH (–20°C). For example: for a 100-µL sample, add 200 µL 100% EtOH.
9. Mix vigorously and incubate tube at –80°C for 1 h.
10. Spin immediately at maximum speed for 20 min at 4°C.
11. Wash the pellet with 200 µL 70% EtOH (RNase free, –20°C).
12. Spin again for 10 min at 4°C.

13. Air dry the pellet (~15 min) and resuspend the pellet in DEPC–H_2O (*see* **Note 8**).
14. Quantify RNA as in **Subheading 3.1.1., step 9**.
15. Dilute samples to 1 µg/µL with DEPC–H_2O in order to be used for RT-PCR and store at –80°C.

3.3. RT-PCR

3.3.1. Denature RNA

1. Mix 1 µL RNA with 2 µL DEPC–H_2O in a 100-µL PCR tube. Vortex and pulse.
2. Place tubes in a Perkin–Elmer thermocycler and run the RNA at 65°C for 5 min then hold the temperature at 4°C. The reaction must hold at 4°C for at least 5 min before proceeding to the RT reaction.

3.3.2. RT

1. Prepare RT mix: 25 m*M* $MgCl_2$ (4 µL); 10X PCR buffer II (2 µL); 10 m*M* **each** dNTP (2 µL); RNase inhibitor 1 µL; MuLV reverse transcriptase 1 µL; and random hexamers 1 µL.
2. Add 17 µL of the RT mix to the RNA and vortex briefly.
3. Place the tubes in the thermocycler and run at 42°C for 15 min, 99°C for 5 min, then hold at 4°C. The reaction must hold at 4°C for at least 5 min before proceeding to PCR.

3.3.3. PCR

1. Prepare the PCR reaction mix (50 µL final volume): 0.5 µL template DNA (~10 ng); 5.0 µL 2.5 m*M* dNTPs; 1.0 µL 10X PCR buffer; 1.0 µL 3′ primer (25 pmol/µL); 1.0 µL 5′ primer (25 pmol/µL); 0.5 µL *Taq* polymerase; and 37 µL dH_2O.
2. Run samples as per the following:

   94°C, 2 min

   94°C, 30 s
   49°C, 30 s* } 35 cycles
   72°C, 30 s

   72°C, 7 min
   4°C, 5 min
   Hold at 4°C

   *The temperature of the extension step will depend upon the primers being used.
3. Analyze results by running PCR samples on ethidium bromide containing agarose gels and view under ultraviolet light. Relative band intensities can be estimated using a variety of computer software programs available through many scientific supply companies.

4. Notes

1. It is crucial that during this extraction procedure that RNase-free instruments and solutions are used and that gloves are often changed in order to minimize the risk of RNase contamination. Use sterile plasticware and/or baked glassware for all applications and make all solutions using RNase-free designated chemicals and DEPC–H_2O.
2. The following procedure has been derived from the manufacture's protocol.
3. Work quickly to minimize RNA degradation using RNAse-free conditions and chemicals.
4. If possible use TRIzol® only in the fumehood because breathing the vapors is a health hazard.
5. Be careful not to use an excess of parafilm; otherwise, the tube may get jammed into the centrifuge adaptor.

6. The running temperature of the centrifuge should be set at between 2 and 8°C for all spins described in this protocol.
7. The RNA is in the aqueous phase and is about 60% of the original amount of TRIzol® added.
8. If the RNA pellet does not easily dissolve, incubate for 10 min at 55–60°C.
9. The $A_{260}:A_{280}$ should be between 1.6 and 2 for good-quality RNA.
10. The electric razor minimizes skin tissue damage as compared to a disposable razor or blade.
11. Large tissue pieces will be too difficult to homogenize when frozen.
12. Note one sample should be processed at a time until **Subheading 3.1.2.2., step 5** to minimize degradation.
13. Using the Polytron®, generally 60% of maximum speed is sufficient.
14. Samples can be stored on ice, protected from light until all samples are homogenized before continuing on to the next step.
15. If possible, use chloroform and phenol only in the fumehood since breathing the vapors is not good for your health.

Acknowledgments

This work was sponsored by grants from the Natural Sciences and Engineering Council of Canada (NSERC).

References

1 Turksen, K., and Troy, T. C. (2002) Permeability barrier dysfunction in transgenic mice overexpressing claudin 6. *Development* **129,** 1775–1784.
2. Turksen, K. and Troy, T. C. (2003) Overexpression of the calcium sensing receptor accelerates epidermal differentiation and permeability barrier formation in vivo. *Mech. Dev.* **120,** 733–744.

15

Whole-Mount Assays for Gene Induction and Barrier Formation in the Developing Epidermis

Carolyn Byrne and Matthew J. Hardman

Summary

The skin as a surface organ is uniquely accessible for whole embryo/fetal analyzes of developmental changes, such as gene induction, protein expression, formation of epidermal-derived appendages such as hair follicles, and formation of the protective barrier. Such analyses have emphasized the heterogenous nature of skin development, perhaps not surprising because epidermal development is programmed by heterogenous underlying mesenchyme. It is necessary to account for this heterogeneity by precisely matching body sites when correlating sequential events during development, for example, the activation of gene expression. In this chapter, protocols designed to assay whole-mount *in situ* hybridization and whole-mount barrier formation are presented. Formation of the protective barrier is the endpoint of epidermal terminal differentiation and defects in this process are reflected in failure, acceleration, or delay in barrier formation. Hence, these latter assays are of particular value as a rapid initial assay for epidermal developmental defects in genetically modified organisms.

Key Words:

Whole-mount *in situ* hybridization; gene induction; dye-penetration; dye-exclusion barrier assays.

1. Introduction

Whole-mount assays for epidermal gene induction, protein expression, and barrier function can be applied to epidermis, uniquely, until very late in gestation without penetration problems associated with other tissues. Whole-mount approaches are crucial when studying skin development caused by the heterogeneity of developing skin and hair follicles. This heterogeneity must be taken into account when comparing sequential developmental events or interpreting mutant/transgenic phenotypes. In this chapter, whole-mount *in situ* hybridization, modified from standard protocols for use on embryonic/fetal epidermis, is described. The robustness and sensitivity of the whole-mount *in situ* hybridization technique, compared with section *in situ* hybridization, is probably caused by a lack of tissue manipulation and consequent cellular damage and RNA degradation before hybridization. Hence, whole-mount RNA analysis linked to highly sensitive cellular level RNA detection by posthybridization sectioning is described. Methods for comparison of RNA whole-mount patterns with protein localization via recently published whole-mount immunohistochemical techniques are also included.

From: *Methods in Molecular Biology, vol. 289: Epidermal Cells: Methods and Protocols*
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

The outcome of epidermal development is the formation of the protective barrier; therefore, whole-mount assays for barrier function ***(1)***, described in this chapter, give results on defects in epidermal development and are being applied, increasingly, as a rapid screen for epidermal developmental change in genetically modified animals.

2. Materials

1. Transcription buffer (5 or 10X) is available with most commercial preparations of RNA-dependent DNA polymerases. A typical 1X transcription buffer may contain 40 m*M* Tris-HCl, pH 7.5; 10 m*M* NaCl, 6 m*M* $MgCl_2$, 1 m*M* spermidine, and 50 µg/mL BSA ***(2)***.
2. 10X nucleotide labeling mix for riboprobe production: 10 m*M* ATP, 10 m*M* CTP, 10 m*M* GTP, 3.5 m*M* UTP, 6.5 m*M* hapten-labeled UTP (e.g., digoxygenin-11-UTP, Roche Applied Sciences, Indianapolis, IN; cat. no. 1209256; fluoroscein-12-UTP, Roche Applied Sciences, Indianapolis, IN; cat. no. 1427857; biotin-16-UTP, Roche Applied Sciences, Indianapolis, IN; cat. no. 1388908) in 10 m*M* Tris HCl, pH 7.6, or purchase premixed nucleotides (e.g., Dig RNA labeling mix, Roche Applied Sciences, Indianapolis, IN; cat. no. 1175025; Fluorescein RNA labeling mix Roche Applied Sciences, Indianapolis, IN; cat. no. 1685619; or Biotin RNA labeling mix, Roche Applied Sciences, Indianapolis, IN; cat. no. 1685597).
3. Enzymes for riboprobe production: T7, T3, or SP6 RNA polymerases (from a variety of commercial sources, e.g., Promega, Madison, WI; cat. no. P2075, P2083, P1085 respectively); RNasin ribonuclease inhibitor (e.g., Promega, Madison, WI; cat. no. N2611).
4. 4 *M* LiCl (Sigma, St. Louis, MO; cat. no. L 9650) for RNA precipitation: 16.96 g of LiCl is dissolved in 100 mL diethylpyrocarbonate (DEPC)-treated water (*see* **Note 1**) then aliquoted for –20°C storage.
5. 10X PBS buffer (phosphate-buffered saline, *see* **Note 1**): per liter mix 80 g of NaCl, 2 g of KCl, 11.5 g of Na_2HPO_4, 2 g of KH_2PO_4, pH to 7.4 with NaOH.
6. 1X PBST (phosphate-buffered saline with Tween-20: Sigma, St. Louis, MO; cat. no. P 1379): 1X PBS with 0.1% Tween-20.
7. Proteinase K solution for permeabilisation of embryos/fetuses: for convenience premade solutions can be purchased, for example, Roche Applied Sciences, Indianapolis, IN; cat. no. 1373196.
8. 2 mg/mL Glycine (Sigma, St. Louis, MO; cat. no. G 2879) for proteinase K inactivation: prepare freshly for each use in DEPC-treated 1X PBST (*see* **Note 1**).
9. 4% paraformaldehyde and 0.2% glutaraldehyde in PBS for refixation of embryos/fetuses after proteinase K treatment: Prepare by heating DEPC-treated PBS (*see* **Note 1**) to approx 80–90°C, then add 4 g paraformaldehyde (Sigma, St. Louis, MO; cat. no. P 6148) per 100 mL. Paraformaldhyde is dissolved on a heater-stirrer. Dissolution can be assisted by adding a few drops of 1 *M* NaOH. After cooling, 50% glutaraldehyde (Sigma, St. Louis, MO; cat. no. G 7651, store frozen) is added to 0.2% (e.g., 0.4 mL per 100 mL).
10. 0.1% sodium borohydride (Sigma, St. Louis, MO; cat. no. S 9125) in 1X PBST for blocking free aldehyde groups after fixation is freshly prepared. This solution will bubble and evolve hydrogen so should be prepared in tubes with loose-fitting caps.
11. 10X TBS buffer (Tris-buffered saline; *see* **Note 2**): per 1 L, mix 80 g NaCl, 2 g KCl, 30 g Tris base, pH to 7.6 with HCl.
12. 1X TBST (Tris-buffered saline with Tween-20; *see* **Note 2**): 1X TBS with 0.1% Tween-20.
13. 1X TBST containing 2 m*M* levamisole (Sigma, St. Louis, MO; cat. no. L 9756) is used when inhibition of endogenous alkaline phosphatases is required.
14. 10X PE buffer: 100 m*M* Pipes, pH 6.8, 10 m*M* EDTA: per liter mix 30 g Pipes (Sigma, St. Louis, MO; cat. no. P 6757), 3.7 g EDTA (disodium salt), pH to 6.8.

15. Hybridization buffer: 50% formamide, 1X PE buffer, 0.75 *M* NaCl, 1% sodium dodecyl sulfate (SDS), 0.05% heparin (Sigma, St. Louis, MO; cat. no. H 3393), 100 µg/mL tRNA (Sigma, St. Louis, MO; cat. no. R 8759), 0.1% bovine serum albumin (New England Biolabs, Beverly, MA; free with restriction enzyme purchases).
16. Hybridization washes (***3***, *see* **Note 3**):
    Wash 1: 1X PE, 1% SDS, 300 m*M* NaCl
    Wash 2: 1X PE, 0.1% SDS, 50 m*M* NaCl
    Wash 3: 50% formamide, 1X PE, 1% SDS, 300 m*M* NaCl
    Wash 4: 50% formamide; 1X PE, 0.1% Tween-20, 150 m*M* NaCl
    Wash 5: 1X PE; 0.1% Tween-20, 500 m*M* NaCl
17. Embryo powder ***(2)***: Embryo powder should be prepared from the same species as being hybridized and from a similar or later developmental stage. However, for very late gestation fetuses, use midgestation embryos (e.g., E14.5 in mouse; *see* **Note 4**) to include tissue differentiation products but avoid inclusion of excessive fat.
    Prepare embryo powder exactly as in **step 2**:
    a. Homogenize in minimum cold PBS using a dounce homogenizer or similar.
    b. Add 4 vol of ice-cold acetone and incubate on ice for 30 min.
    c. Spin on a bench top centrifuge to pellet and remove acetone.
    d. Wash pellet by resuspending in ice-cold acetone and recentrifuging.
    e. Air-dry pellet with continuous grinding (e.g., with a smooth glass rod) to produce a fine powder.
    f. Store in an air-tight container at –20°C.
    Before use, embryo powder should be heat-inactivated. For every 5 mL of diluted antibody solution used (*see* **Subheading 3.1.5., step 1**) 1–3 mg of embryo powder is weighed into 1 *M* of 1X TBST, then heated at 70°C for 30 min.
18. Antibodies to hapten-labeled probes: Hybridization is detected with enzyme-conjugated antibodies to the hapten incorporated into the RNA probe (riboprobe). Enzymes commonly used are alkaline phosphatase or horseradish peroxidase. Highest sensitivity is attained with alkaline phosphatase-conjugated antibodies, for example alkaline phosphates-conjugated antidigoxigenin antibody (Roche Applied Sciences, Indianapolis, IN; cat. no.1093274) or antifluorescein antibody (Roche Applied Sciences, Indianapolis, IN; cat. no.1426338). Alkaline phosphatase catalyses production of a purple/brown insoluble precipitate from the colorless substrates nitroblue tetrazolium and 5-bromo,4-chloro,3-indolyl phosphate (*see* **step 22**). Use of alternative substrates for alkaline phosphatase, including HistoMark® Red (KPL, Gaithersburg, MD); cat. no. 55-69-00), Vector Red™ (Vector Laboratories, Burlingame; cat. no. SK-5100) have been described in applications for two-color *in situ* hybridization ***(4)*** involving sequential detection of digoxigenin and fluorescein-labeled riboprobes. Horseradish peroxidase-conjugated antibodies (e.g., horseradish peroxidase-conjugated antidigoxigenin antibody (Roche Applied Sciences, Indianapolis, cat. no. 1207733) or antifluorescein antibody (Roche Applied Sciences, Indianapolis, IN; cat. no. 1426346) can be used with diaminobenzidine substrates to give a black/brown stain or, with greater sensitivity, with TrueBlue™ substrate (KPL, Gaithersburg, MD); cat. no. 71-00-64) to give blue stain ***(4)***. This reaction can be used in multiple labelling applications involving simultaneous detection of digoxygenin and fluorescein-labelled riboprobes ***(4)***.
19. Nitroblue tetrazolium (NBT, Sigma, St. Louis, MO; cat. no. N 6639, N5514) is made to 75 mg/mL in 75% *N′N′*dimethylformamide (Sigma, St. Louis, MO; cat. no. D 4551) and stored at –20°C.
20. 5-Bromo, 4-chloro,3-indolyl phosphate (BCIP, Sigma, St. Louis, MO; cat. no. B 6777, B 0274) is made to 50 mg/mL in 100% *N′N′*dimethylformamide (Sigma, St. Louis, MO; cat. no. D 4551) and stored at –20°C.

21. NTMT solution: 100 m*M* NaCl, 100 m*M* Tris HCl, pH 9.5; 50 m*M* $MgCl_2$; and 0.1% Tween-20. This solution is mixed from stock solutions just prior to use to prevent precipitation.
22. For detecting alkaline phosphatase activity on whole embryos, mix 45 µL NBT solution and 35 µL BCIP to 10 mL NTMT solution just before use. Alternatively, premixed alkaline phosphatase substrates can be bought from a variety of suppliers. Stop reaction by rinsing with TBST.
23. PVA solution for alkaline phosphatase detection on sections ***(5)***: add 10% (w/v) polyvinyl alcohol 70–100 kDa (Sigma, St. Louis, MO; cat. no. P1763) to 90°C 100 m*M* Tris-HCl, pH 9.0; 100 m*M* NaCl to dissolve. Allow to cool.
24. For detecting alkaline phosphatase activity on sections using PVA enhancement mix 45 µL of NBT solution, 35 µL of BCIP solution, and 100 µL 1 *M* $MgCl_2$ to 20 mL of cooled PVA solution just before use ***(5)***.
25. DAB staining solution: 0.5 mg/mL 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma, St. Louis, MO; cat. no. D5637) in PBT. 0.01% H_2O_2 is added just before use. Alternatively, because DAB is a potent carcinogen, it may be preferable to buy preweighed tablets or premixed solutions (e.g., DAB Substrate Kit, Vector Laboratories, Burlingame; cat. no. SK4100; Liquid DAB Substrate Kit, Novocastra, UK; cat. no. NCL-L-DAB).
26. For detecting horseradish peroxidase activity on whole embryos incubate in DAB staining solution and monitor color development. Stop reaction by rinsing with PBST or TBST. Alternatively, use premixed staining solutions or TrueBlue™ (KPL, Gaithersburg, MO; cat. no. 71-00-64) staining solution according to the manufacturer's instructions.
27. Serum from the same species as the antibody for hapten detection is used as a blocking agent, e.g., for antidigoxigen antibodies raised in sheep use sheep/lamb serum (e.g., Sigma, St. Louis, MO; cat. no. S 2263). Heat-inactivate serum by diluting to 10% in 1X TBST, then heating at 70°C for 30 min. Heat-inactivated serum can be stored in aliquots at –20°C.
28. Staining mix for endogenous glycosidases (barrier assay): 100 m*M* sodium phosphate, pH 5.5; 1.3 m*M* magnesium chloride; 3 m*M* $K_3Fe(CN)_6$, 3 m*M* $K_4Fe(CN)_6$; 1 mg/mL X-gal (5-bromo, 4-chloro, 3-indolyl β-D-galactopyranoside, Sigma, St. Louis, MO; cat. no. B4252: dilute from a 20 mg/mL stock in *N*,*N*-dimethylformamide), 0.1% sodium deoxycholate (Sigma, St. Louis, MO; cat. no. D 6750), 0.2% Igepal Ca-630 (Sigma, St. Louis, MO; cat. no. I 8896).
29. 1% Toluidine blue solution for barrier assay: dissolve 1 g Toluidine blue O (Sigma, St. Louis, MO; cat. no. T 3260) in 100 mL water. It is important to dissolve in water. Commercial buffered Toluidine blue solutions or stocks made in salt solutions should not be used.

3. Methods

3.1. Whole-Mount In Situ *Hybridization (*see *Note 5)*

This section describes whole-mount *in situ* hybridization protocols ***(2,3)*** modified from the version of Conlan ***(3)*** for use on embryonic/fetal epidermis, effective until the protective barrier forms at embryonic d 16–17 in mouse and E17 in chick. At this point riboprobes and antibodies are excluded from the epidermis by newly formed stratum corneum. For skin analyses, proteinase K levels are much lower than in standard protocols as it is unnecessary to penetrate underlying tissues while fragile surface ectoderm has to be protected from damage. In addition, greater gentleness is required in manipulation steps to protect ectoderm.

3.1.1. Riboprobe Generation

Riboprobes are generated by transcription using bacteriophage T7, T6, or SP6 DNA-dependent RNA polymerases from cDNAs cloned downstream from bacteriophage

promoters. A wide range of commercially available cloning vectors contain these promoter sequences flanking the multiple cloning site. cDNAs available from Expressed Sequence Tag (EST) collections (e.g., I.M.A.G.E. clones, http://image.llnl.gov) are usually in this format. Transcription is more efficient if plasmids are linearized downstream of the inserted cDNA by restriction enzyme digestion prior to transcription.

Alternatively, DNA templates for transcription can be generated by polymerase chain reaction from an appropriate source using primers modified to incorporate a T7, T3, or SP6 promoter sequence 5′ to the homology region.

DNA should be purified prior to transcription (e.g., Qiagen MinElute Cleanup Kit, Qiagen, Valencia) and collected in water at approx 1 µg/µL.

Riboprobe RNA can be synthesized using commercially available kits with detailed instruction manuals (e.g., Promega, Madison, MI; http://www.promega.com; Roche Applied Sciences, Indianapolis, IN; http://www.roche-applied-science.com) using hapten-labeled nucleotides, most commonly digoxygenin, fluorescein or biotin-labeled UTP (Roche Applied Sciences, Indianapolis, IN). Alternatively, the following protocol can be used.

Production of riboprobes by in vitro transcription.

Mix as follows:

1. H_2O (not diethylpyrocarbonate treated) with volume adjusted to 20 µL.
2. 4 µL 5X transcription buffer.
3. 2 µL 10X nucleotide labeling mix.
4. 2 µL 0.1 *M* DTT (available with most commercial polymerase preparations).
5. 1 µL RNasin ribonuclease inhibitor (10–50 U/µL).
6. 1 µL DNA-dependent RNA polymerase (T7, T3, or SP6 RNA polymerase).
7. 1 µg linerarized template or 0.1–0.3 µg amplified DNA.

Incubate at 37°C for 2–4 h (40°C when using SP6). Stop reaction by adding 2 µL of 0.2 *M* EDTA, pH 8.0. Unincorporated nucleotide can be removed for estimation of yield or riboprobe can be added directly to the hybridization mixture without further "clean up." For removal of unincorporated nucleotide precipitate RNA by adding 2.5 µL of 4 *M* LiCl and 75 µL of ethanol. Mix and hold at –20°C for 5 min to overnight and collect RNA by centrifugation at top speed in a microfuge for 20 min. Alternatively, use a commercial column, for example, Qiagen RNeasy Cleanup Kit (Qiagen, Valencia) according to the manufacturer's instructions.

Yield can be estimated by running an aliquot (e.g., 1 µL) of reaction mix before and after reaction on a 1% agarose gel and visualizing the synthesized RNA under ultraviolet light. An RNA band that appears after reaction with an intensity 10 times that of the plasmid template indicates approx 10 µg RNA was synthesized ***(2)***. It is unnecessary to degrade template with DNases.

*3.1.2. Collection of Embryos (*see ***Note 5****)*

1. Freshly excised embryos are fixed in ice-cold 4% paraformaldehyde in PBS for 4 h to overnight with very gentle rocking on ice.
2. Embryos/fetuses are dehydrated through a methanol series: 25% methanol/75% PBST for 5 min; 50% methanol/50% PBST for 5 min; and 75% methanol for 5 min (*see* **Note 6**).
3. Embryos are rinsed 3X in 100% methanol for 10 min and can be stored at –20°C for several months.

3.1.3. Pretreatment of Embryos/Fetuses

1. Embryos/fetuses are incubated (all incubations at room temperature unless stated otherwise) in five parts methanol : one part H_2O_2 for 1–2 h with gentle rocking to inactivate endogenous peroxidases plus bleach the pigments from skin.
2. Embryos are rehydrated through a methanol series: 75% methanol for 5 min; 50% methanol/50% PBST for 5 min; 25% methanol/75% PBST for 5 min; then washed in PBST three times for 5 min.
3. Embryos are incubated with varying concentrations of proteinase K in PBST for exactly 10 min (*see* **Note 7**). Proteinase K Concentrations have to be determined empirically for each batch of proteinase K. A sample protocol for CD1 mouse embryos is shown: E9.5 (2 µg/µL); E 11 (3 µg/µL); E 13 (6 µg/µL); E 14 (12 µg/µL); E 15 (24 µg/µL); and E 16 (32 µg/µL).
4. Wash with freshly prepared glycine (2 mg/mL) in 2X PBST for 5 min to inactivate proteinase K.
5. Refix embryos in 4% paraformaldehyde, 0.2% glutaraldehyde in PBS for 20 min, then wash with 3X PBT, 5 min per wash.
6. Treat with freshly prepared 0.1% sodium borohydride in PBST for 20 min (this step blocks free aldhehyde groups), then wash with 3X PBST, 5 min per wash (note that tube lids have to be loose during this step to release evolving gas).
7. Wash twice with hybridization buffer containing formamide. After equilibration in formamide embryos/fetuses can be stored at –20°C until ready to hybridize.

3.1.4. Hybridization (see ***Note 3*** Regarding Optional Posthybridization Ribonuclease Treatment, **ref. 4**)

1. Prehybridize at 63°C for at least 1 h (*see* **Note 8**).
2. Replace with fresh hybridization buffer and add probe to approx 1–2 µg/mL. Hybridize at 63°C overnight with gentle rotation.
3. Rinse several times with wash 1, then rotate gently in wash 1 twice at 63°C for 30 min.
4. Rinse several times with wash 2, then rotate gently in wash 2 twice at 50°C for 30 min.
5. Rinse with wash 3, then rotate gently in wash 3 at 50°C for 30 min.
6. Rinse with wash 4, then rotate gently in wash 4 at 50°C for 30 min.
7. Rinse several times with wash 5 (to remove formamide from wash 4 before raising temperature), then incubate in wash 5 at 70°C for 20 min. This step is to inactivate endogenous phosphatases if using a phosphatase-linked enzyme for riboprobe detection.

3.1.5. Antibody Detection of Hybridized Probe

1. The antibody to hapten-labeled probe is preabsorbed with embryo powder to remove antiembryo activity. Antibody is diluted 1/2000–1/5000 in cold TBST (*see* **Note 9**), 2 m*M* levamisole, 1% serum plus inactivated embryo powder and incubated at 4°C for 30 min with gentle rotation, then centrifuged at 4°C for 10 min in a bench centrifuge to pellet and remove embryo powder.
2. Embryos/fetuses are blocked with heat-inactivated 10% serum in TBST containing 2 m*M* levamisole for 1 h with gentle rocking.
3. Embryos/fetuses are incubated with preabsorbed secondary antibody overnight at 4°C with gentle rocking.
4. Rinse three times with TBST 2 m*M* levamisole at room temperature.
5. Wash with TBST 2 m*M* levamisole six times for 1 h. It is often convenient to let the final wash go overnight.

3.1.6. Color Development

For detection of alkaline phosphatase-conjugated secondary antibodies rinse three times with freshly prepared NTMT, 2 m*M* levamisole for 10 min with rocking. Incubate in NBT/BCIP in NTMT (*see* **Subheading 2., step 22**). Perform color development in the dark in glass dishes. Monitor reaction and terminate by washing in TBST then transferring to 4% paraformaldehyde. Store at 4°C indefinitely.

For detection of peroxidase-conjugated secondary antibodies incubate with DAB or TrueBlue™ staining mix until color develops (*see* **Subheading 2., item 26**). Wash with PBST or TBST then transfer to 4% paraformaldehyde. Store at 4°C indefinitely.

3.1.7. Photography of Embryos/Fetuses

Secure embryos/fetuses to the base of a Petri dish with 1% agarose in water. Either position the embryos on a thin layer of molten agarose or pour agarose plates, then use a hot wire/glass rod to melt the surface locally before attaching the embryos. Adhesion to agarose prevents vibration during photography and permits photography at different angles. Cover with TBS or PBS as appropriate and photograph through the liquid. It is necessary to completely submerge the embryo/fetuses for photography to prevent reflection from shiny surfaces and dehydration of skin. Photograph under a dissecting microscope with fiber optic illumination.

3.1.8. Posthybridization Sectioning of Skin

Whole-mount *in situ* hybridization provides a very robust and sensitive method of detecting RNA compared to section *in situ* hybridization, possibly from reduced RNA degradation as dissection and handling prior to hybridization is minimized. Posthybridization sectioning is used to locate RNA to cells.

Wax sections of hybridized embryos/fetuses can be prepared by standard techniques with the caveat that some color precipitates are partially alcohol or xylene soluble, resulting in reduced signal.

Frozen sections provide the quickest and simplest method for locating RNA at the cellular level after whole-mount *in situ* hybridization, with some sacrifice of tissue integrity. For greater sensitivity, prepare frozen sections after posthybridization washes but prior to antibody detection (i.e., complete **Subheading 3.1.4.**, freeze and section embryos, then resume blocking and antibody detection as in **Subheading 3.1.5.**). Antibody detection is conducted as for whole embryos on the frozen sections, giving a stronger signal, probably because of facilitated reagent access.

Alkaline phosphatase detection on sections, rather than whole fetuses, permits inclusion of high molecular weight polyvinyl alcohol to the BCIP/NBT color reaction ***(5)***, substantially enhancing and concentrating signal and permitting detection of rare mRNAs (**ref. *5***; *see* **Note 9**).

3.1.9. Comparison of Whole-Mount RNA and Protein Signal

The whole-mount antibody staining method of Nishioka et al. ***(6)*** using microwave permeabilization of embryonic/fetal epidermis provides a simple and efficient method for whole-mount immunohistochemical localization of protein. Comparison of RNA and protein patterns is carried out on age-matched fetuses. Alternatively, with older gestational ages single fetuses can be bisected saggitally after brief (30 min) fixation in

paraformaldehyde/PBS to provide rigidity. Separate halves of the fetus are used for *in situ* hybridization and immunohistochemistry. Protocols for simultaneous or sequential *in situ* hybridization and immunohistochemistry have been described ***(4)***.

3.2. Barrier Assays (see Note 10)

Whole-mount dye penetration or dye exclusion barrier assays report defects/acceleration in developmental barrier formation or barrier defects in adult skin. The first assay modifies the skin by brief treatment with methanol. This procedure probably removes skin surface lipid. Entry of histological dyes is then dependent on barrier integrity. The second assay detects endogenous glycosidases, most probably lysosomal in origin, active at pH 3.0 to 6.0. Entry of the artificial substrate, 5-bromo,4-chloro, 3-indolyl β-D galactopyranoside or X-gal, is dependent on a dysfunctional barrier. It is necessary to use controls, for example, postnatal skin as barrier positive control, pre-16 d murine skin or wounded adult skin as a negative control.

Formation of the protective barrier in mice is strain-dependent and correlates more closely with fetal weight than estimated gestational age. In CD1 strain mice barrier forms at embryonic d 16, corresponding to a fetal weight of 0.6–0.9 g. However, in hybrid offspring of C57Bl6 × CBA mice, often used to generate transgenic mice, barrier forms at E17 over a similar weight range. In Sprague-Dawley rats barrier forms at E18 ***(1)*** and in chicken barrier forms at d 17 (Byrne and Hardman, unpublished).

3.2.1. Dye Penetration Barrier Assay, Method 1

This barrier assay modifies skin to permit barrier-dependent penetration by histological dyes. The nature of the modification is unknown but likely involves the extraction of surface polar lipids. This assay can be carried out on tissue previously fixed with a variety of standard fixatives (e.g., formaldehyde, glutaraldehyde, Bouins fixative).

1. Tissue is transferred up, then down a methanol gradient, about 30 s to 1 min per step; (1) 25% methanol in water; (2) 50% methanol in water; (3) 75% methanol in water; (4) 100% methanol; (5) 75% methanol in water; (6) 50% methanol in water; (7) 25% methanol in water; and (8) equilibrate in PBS or water.
2. If using embryos/fetues add to dye mix (1% Toluidine blue in water, *see* **Subheading 2., item 29**), destain in PBS pH 7.4 until the pattern appears. The high pH of PBS facilitates the rapid destain necessary to see the pattern.
3. If using skin pieces seal the dermal side by placing skin piece (embedding slightly so that only the epidermal side is exposed) in a Petri dish containing vaseline gel. Either smear the gel onto the dish, or add vaseline gel to the plate then melt in a microwave to get a smooth surface upon setting, like a bacterial agar plate. Add dye mix topically (e.g., use freshly prepared 1% Toluidine blue in water), then destain in PBS pH 7.4 until the pattern appears.
4. Photograph as in **Subheading 3.1.7.** as soon as possible to prevent diffusion of dye and blurring of pattern.

3.2.2. Dye-Penetration Barrier Assay, Method 2

This barrier assay exploits endogenous glycosidase activity present in murine skin at low pH (pH 3.0–6.0, with an optimum at approx pH 5.5). This glycosidase activity will cleave the substrate X-gal to produce a blue, insoluble precipitate provided X-gal can penetrate the skin barrier. Hence, the glycosidase activity is only accessible topically if barrier is unformed or dysfunctional. Do not go up and down a methanol series, simply

add the embryos/fetuses to the solution or seal the dermal side as above, add the solution topically and incubate (reaction proceeds faster at 37°C; however, tissue morphology will be poorer than a room temperature incubation). The endogenous glycosidases are sensitive to fixative so this assay should be carried out on unfixed or partly fixed fresh tissue (*see* **Note 11**). Tissue can be fixed post-staining and staining is stable. Photograph as in **Subheading 3.1.7.**

4. Notes

1. Aqueous solutions are pretreated in diethylpyrocarbonate (DEPC, Sigma, St. Louis, MO; cat. no. D 5758) 0.1% for 2 h to overnight to inactivate ribonucleases, followed by autoclaving under standard conditions for 40 min to breakdown diethylpyrocarbonate.
2. Tris-buffered saline replaces phosphate-buffered saline in later stages of the wholemount in situ procedure when alkaline phosphatase enzymic reaction is to be used. This replacement is to remove inhibitory phosphate. Tris solutions are not treated with DEPC but made up in DEPC-treated reagents. Levamisole is added to washes to suppress endogenous phosphatase activity.
3. Many authors include an RNase treatment during the posthybridization washes between wash two and three to remove unincorporated RNA ***(2,3)***. This step improves background, but it can be omitted for many skin applications. To perform RNase treatment embryos/fetuses are rinsed a few times in RNase buffer (10 m*M* Pipes, pH 7.2, 0.5 *M* NaCl, 0.1% Tween-20) then incubated with heat-treated RNase A (e.g., ribonuclease A from bovine pancrease, Sigma, St. Louis, MO; cat. no. R 4875 resuspended in TE at 10 mg/mL and heat-treated by boiling for 10 min, then cooled aliquots stored at –20°C) at 50 µg/mL at 37°C for 30 min ***(3)***.
4. Mouse and rat embryos are staged so that the day the vaginal plug is detected is d 0.5 or E0.5. Chick embryos/fetuses are stages so that start of incubation is d 0 (E0).
5. Embryonic epidermis is particularly fragile. Manipulation steps must be minimized and rocking carried out gently. Smaller embryos can be transferred by suction using the wide end of an inverted Pasteur pipet or plastic pipet tip to avoid damage to the surface ectoderm.
6. Longer equilibration times are for the larger late gestation fetuses and are probably unnecessary for smaller embryos.
7. Other protocols use fixed concentrations of proteinase K for varying periods of time. The concentration of proteinase K is crucial for success of this method and had to be determined empirically for each batch.
8. Hybridization and posthybridization washes can be performed in a standard rotating hybridization oven with a rotor or attachment for 50-mL sterile, disposable plastic tubes. For hybridization with small volumes, embryos/fetuses can be placed in smaller tubes within the 50-mL tube. Posthybridization washes can be performed directly in 50-mL tubes.
9. When conducting antibody detection on sections of hybridized embryos the washing times can be reduced to at least half. Use of high-molecular-weight PVA solutions when performing alkaline phosphatase color reaction with whole embryos/fetuses results in unacceptable levels of background and this type of signal enhancement can only be used with sections.
10. Whole-mount dye penetration or colorimetric barrier assays report major defects or changes in barrier. In reality, the barrier develops and is refined until quite late in development and, possibly, during early postnatal life. Late or subtle defects in barrier formation may not be detected with these assays. In order to detect these changes transepidermal water loss, a measure of barrier function, can be assayed quantitatively on newborn or young (hairless) animals using instruments (e.g., Tewometer, CK Electronics GmbH, Berlin; Evaporimeter, Servo-Med, Cairo) that measure the water vapor pressure gradient at the skin surface.

11. When detecting endogenous glycosidase activities (and transgene-encoded β-galactosidase activity) crosslinking fixatives, such as glutaraldehyde and formaldehyde, will reduce enzyme activity as the enzymes also become crosslinked. Omission of fixatives, or fixation delay until color development is complete, will reduce tissue integrity. However, epidermis is a particularly robust tissue and epidermal keratinocytes and epidermal strata can still be clearly distinguished after sectioning even when tissue is unfixed or poorly fixed.

References

1 Hardman, M. J., Sisi, P., Banbury, D. N., and Byrne, C. (1998) Patterned acquisition of skin barrier function during development. *Development* **125,** 1541–1552.

2. Xu, Q., and Wilkinson, D. G. (1997) In situ hybridization of mRNA with hapten labelled probes, in *In Situ Hybridization, A Practical Approach*, 2nd ed. (Wilkinson, D. G., ed.), IRL Press, Oxford, pp. 87–106.

3 Conlon, R. A. and Rossant, J. (1992) Exogenous retinoic acid rapidly induces anterior ectopic expression of murine Hox-2 genes in vivo. *Development* **116,** 357–368.

4. Jowett, T. (1997) Two colour in situ hybridisation, in *In Situ Hybridization, A Practical Approach*, 2nd ed. (Wilkinson, D. G., ed.), IRL Press, Oxford, pp. 107–126.

5 De Block, M. and Debrouwer, D. (1993) RNA-RNA in situ hybridization using digoxigenin-labeled probes: the use of high-molecular-weight polyvinyl alcohol in the alkaline phosphatase indoxyl-nitroblue tetrazolium reaction. *Anal. Biochem.* **215,** 86–89.

6. Nishioka, E., Tanaka, T., Yoshida, H., Matsumura, K., Nishikawa, S., Naito, A., et al. (2002) Mucosal addressin cell adhesion molecule 1 plays an unexpected role in the development of mouse guard hair. *J. Invest. Dermatol.* **119,** 632–638.

16

Analysis of Early Epidermal Development in Zebrafish

Ashley E. Webb and David Kimelman

Summary

The zebrafish *(Danio rerio)* is a member of the teleost (bony fish) lineage that diverged from the mammalian lineage 420 million years ago. Despite this ancient divergence, there are important similarities between the epidermis of fish and mammals. In addition, recent work suggests that the genes required for epidermal development in the zebrafish also are essential for this process in higher vertebrates, indicating that analysis of the zebrafish system will contribute to understanding mouse and human development. The zebrafish is a very useful genetic system, and many mutants with epidermal defects have been obtained in large-scale genetic screens. In addition, zebrafish embryos are transparent, develop externally, and can be collected in abundance. The aim of this chapter is to provide the researcher with basic protocols to examine gene expression, cell proliferation, and cell death in the developing zebrafish. Although only a few specific epidermal markers have been identified in the zebrafish thus far, we also provide a list of useful genes for epidermal analysis.

Key Words:

Epidermis; zebrafish; *in situ* hybridization; bromodeoxyuridine; TUNEL; immunocytochemistry.

1. Introduction

1.1. Adult Zebrafish Epidermis

The structure and function of zebrafish skin can be directly compared with that of higher vertebrates. Although dehydration is not a problem for fish, the skin must serve as a protective layer between the internal organs of the fish and the environment. As in mammals, the fish skin is composed of a dermal layer and a superficial epidermis. In the fins of teleost fish, including the zebrafish, these layers are closely apposed, separated only by a basement membrane. In contrast, throughout the body of the zebrafish, dermal sclerifications (scales) are embedded in the dermal layer, and the epidermis is draped over the scales, contacting the dermis only occasionally (**Fig. 1**). The epidermis varies in thickness between 2 and 12 cells in different regions of the body and appears to consist mostly of a single, filament-containing cell type ***(1,2)***. This organization is a simplification of the mammalian epidermis because the fish epidermis is not a true stratified epithelium and the most superficial epidermal layer is not a stratum corneum, as in mammals, but instead consists of nucleated cells ***(3)***.

Renewal of the mammalian epidermis is achieved by cell proliferation in the most basal layer of the epidermis and sloughing of the outermost epidermal layer. Experiments in the goldfish *(Carassius auratus)* have revealed that cell proliferation in

From: *Methods in Molecular Biology, vol. 289:*
Epidermal Cells: Methods and Protocols
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

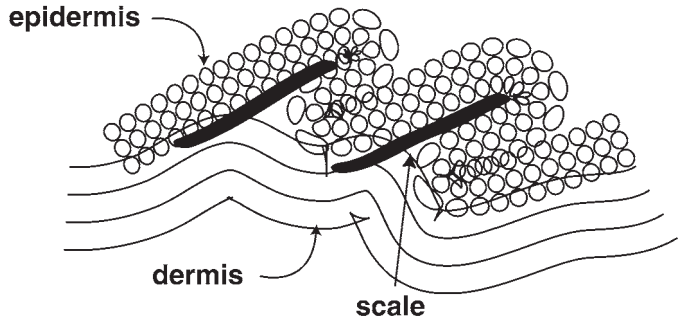


Fig. 1. The structure of fish skin. The epidermis is composed of several layers of cells. The scales are underneath the epidermis and connected to the dermis.

the adult teleost epidermis is not restricted to the most basal cells but occurs throughout the epidermal layer ***(3)***. Despite this organizational difference, the genes required for epidermal maintenance are likely to be conserved between fish and mammals. For example, the p63 protein, which has been implicated in epidermal proliferation and maintenance in mammals, is also present in the adult zebrafish epidermis ***(4,5)***.

The zebrafish epidermis, like the mammalian epidermis, contains abundant amounts of keratin ***(6,7)***. A recent study in zebrafish using a panel of antibodies suggested that the epidermis contains two type I keratins and four type II keratins ***(6)***. It is not yet known whether the different keratins are expressed in different regions of the epidermis, as is the case in mammalian skin, or whether all epidermal cells contain the same complement of keratins.

1.2. Epidermal Development in the Zebrafish

According to the zebrafish fate map, epidermal cells originate from the ventral side of the embryo, near the animal pole ***(8)***. These cells express the epidermal markers *gata2* and Δ*Np63*, both encoding transcription factors ***(4,9)***. The role of Δ*Np63* in mammalian epidermal development and maintenance has been studied extensively (reviewed in **refs. *10*** and ***11***). Interestingly, disruption of the p63 locus in the mouse results in an absence of epidermis ***(12,13)*** and mutations in the human p63 gene result in a variety of skin defects ***(14–16)***. Δ*Np63* has been shown to be required for epidermal development in the zebrafish, suggesting that the role of this gene predates the divergence of the fish lineage from that of mammals 420 million years ago ***(4,5)***.

Keratin 8 *(zf-K8)* is a useful marker of zebrafish epidermis in postgastrula to adult stages ***(17)***. Before and during gastrulation, *zf-K8* is expressed in the embryonic enveloping layer, an extraembryonic tissue that covers the developing embryo. K8 intermediate filaments have been studied in *Xenopus laevis* and the mouse, and loss of function studies in these systems indicate that K8 is required for cell movements during gastrulation ***(18–20)***. It will be interesting to learn whether this role is conserved in the zebrafish and whether *zf-K8* has a later role in this system.

The advantage of using the zebrafish to study epidermal development lies in the power of zebrafish genetics. Several large-scale genetic screens have been performed in the zebrafish, and many mutants have been identified containing epidermal defects ***(21,22)***. These lines are likely to contain mutations in known genes required for epidermal

development as well as novel genes involved in this process. Work on these mutants will likely provide insight that is broadly applicable beyond the teleost and will further our understanding of epidermal development in mammals.

This chapter provides several protocols to study development of the zebrafish epidermis and can be applied, with minor modifications, to the adult fish. The protocols are designed to examine gene expression, cell proliferation and apoptosis in the zebrafish. Note that the reagents used here have been used successfully in our laboratory, but other sources are typically acceptable. For additional methods, *see The Zebrafish Book* ***(23)***.

2. Materials

2.1. Useful Epidermal Markers

1. Δ*Np63* is expressed in epidermal cells from gastrulation to 5 d postfertilization and in the adult zebrafish epidermis ***(4,5)***.
2. *Gata2* is expressed in epidermal cells during gastrulation ***(9)***.
3. *Zf-K8* is expressed in the embryonic enveloping layer during gastrula stages and in the epidermis of adults ***(17)***.

2.2. Probe Synthesis

1. Linearized deoxyribonucleic acid (DNA) template.
2. Phenol : $ChCl_3$: isoamyl alcohol (50 : 48 : 2).
3. 100% and 70% ethanol.
4. Diethylpyrocarbonate-treated water.
5. Transcription reaction: (1) transcription buffer (provided with each ribonucleic acid [RNA] polymerase); (2) 10X DIG RNA labeling mix (Roche; cat. no. 1277073); (3) 10X fluorescein RNA labeling mix (Roche; cat. no. 1685619) for two-color *in situ*s; (4) RNase inhibitor (Ambion; cat. no. 2682); (5) T7, T3, or SP6 Polymerase (Roche; cat. no. 0810274, no. 1031163, no. 0881767).
6. DNAse (Roche; cat. no. 0776785).
7. G-50 Sephadex Quick Spin Column (Roche; cat. no. 1274015).
8. 5 *M* ammonium acetate (made RNase free by autoclaving).
9. Isopropanol (kept RNase free after opening a fresh bottle).
10. Hybridization buffer (*see* **Subheading 2.3., item 4**).

2.3. In Situ *Hybridization*

1. 4% paraformaldehyde in phosphate-buffered saline (PBS) pH 7.0–7.5 (make fresh weekly and store at 4°C).
2. PBSTw: PBS/0.1% Tween-20.
3. Proteinase K: stored at 10 mg/mL in PBSTw at –20°C.
4. Hybridization solution (store at –20°C): 50% formamide, 5X SSC, 50 µg/mL heparin, 500 µg/mL tRNA, 0.1% Tween-20, sterile water, adjust pH to 6.0 with 1 *M* citric acid.
5. 20X standard saline citrate (SSC): 3 *M* NaCl, 0.3 *M* sodium citrate, pH to 7.0 with HCl or NaOH depending on initial pH.
6. Blocking solution: PBSTw, 2% heat inactivated goat serum (*see* **Note 1**) (Gibco; cat. no. 16210), 2 mg/mL bovine serum albumin (Sigma; cat. no. A-2153).
7. Alkaline phosphatase-conjugated antidigoxigenin Fab fragments (Roche; cat. no. 1093274) and antifluorescein Fab fragments for two-color *in situ* (Roche; cat. no. 1207741).
8. Coloration buffer: 100 m*M* Tris-HCl, pH 9.5, 50 m*M* $MgCl_2$, 100 m*M* NaCl, 0.1% Tween-20, sterile water.
9. Nitro-blue tetrazolium (Roche; cat. no. 1087479).

10. Bromo-4-chloro-3-indolyl phosphate (Roche; cat. no. 1585002).
11. Glass *in situ* vials (Fisher; cat. no. FS60940D-1).
12. 0.1 *M* glycine, adjust to pH 2.2 with HCl (for two-color *in situ*).
13. Fast Red tablets (Sigma; cat. no. F-4648) for two-color *in situ*.

2.4. Immunocytochemistry

1. PBSTw (PBS/0.1% Tween-20).
2. Proteinase K (stored at 10 mg/mL in PBSTw at –20°C).
3. Blocking solution (*see* **Subheading 2.3., item 6**).
4. Diaminobenzidine tetrahydrochloride (Polysciences, Inc.; cat. no. 04008).
5. Hydrogen peroxide.

2.5. Bromodeoxyuridine (BrdU) Incorporation Assay

1. BrdU (Sigma; cat. no. B-5002).
2. Ringer's solution: 116 m*M* NaCl, 2.9 m*M* KCl, 1.8 m*M* $CaCl_2$, 5 m*M* HEPES, pH 7.2.
3. Dimethyl sulfoxide.
4. 2 *M* HCl.
5. Fluoresceine isothiocynate-conjugated Anti-BrdU antibody (BD Biosciences; cat. no. 347583).

2.6. TUNEL Assay

1. PBSTw (PBS/0.1% Tween-20).
2. 0.1% Triton X-100/0.1% sodium citrate in PBS.
3. Terminal deoxynucleotidyl transferase (Invitrogen; cat. no. 10533065).
4. TdT buffer (Invitrogen; cat. no. Y95102).
5. Digoxigenin-labeled dUTP (Boehringer-Mannheim; cat. no. 1209256).
6. Blocking solution (*see* **Subheading 2.3., item 6**).
7. Alkaline phosphatase-conjugated antidigoxigenin Fab fragments (Roche; cat. no. 1093274).
8. Nitro-blue tetrazolium (Roche; cat. no. 1087479).
9. Bromo-4-chloro-3-indolyl phosphate (Roche; cat. no. 1585002).
10. Coloration buffer (*see* **Subheading 2.3., item 8**).

3. Methods

3.1. In Situ *Hybridization*

3.1.1. Probe Synthesis

1. Linearize 10 µg DNA with appropriate restriction enzyme (*see* **Note 2**). Extract twice with phenol : $ChCl_3$: isoamyl alcohol.
2. Ethanol precipitate and resuspend the DNA in 10 µL DEPC water.
3. Transcription reaction: 2 µL 10X transcription buffer; 2 µL 10X DIG (or fluorescein) RNA labeling mix; 1–2 µg linearized DNA template; 1 µL RNase inhibitor; 1 µL T7, T3, or SP6 polymerase; and DEPC water to 20 µL. Incubate at 37°C for 2 h.
4. Add 1 µL RNase-free DNase and incubate at 37°C for 30 min.
5. Add 80 µL DEPC water.
6. Prepare a G-50 Sephadex Quick Spin Column according to the manufacturer's specifications. Load the RNA prep (100 µL) onto the G-50 Column and spin at 3/4 speed in a clinical centrifuge for 5 min.
7. Add 10 µL 5 *M* ammonium acetate and 110 µL isopropanol.
8. Spin at maximum speed (RCF 14,000*g*, which corresponds to 14,000 rpm in a standard bench top microcentrifuge) for 30 min at 4°C in a microcentrifuge.
9. Wash the pellet with 70% ethanol and spin at maximum speed for 10 min.

10. Remove the supernatant and air-dry the pellet for a few minutes at room temperature.
11. Resuspend the pellet in 40 µL DEPC water. Run 2 µL on a 1% agarose gel to test the quality of the RNA (*see* **Note 3**). Before running the gel, the RNA probe should be heated to 70°C for 2 min to eliminate secondary structure. The probe should run as a single band on the gel.
12. Add 160 µL hybridization buffer and store at –20°C.

3.1.2. In Situ *Hybridization* ***(Fig. 2B)***

All steps should be performed in microcentrifuge tubes using 250–500 µL volumes. Washes do not require agitation and should be performed at room temperature unless otherwise specified.

1. Fix embryos in 4% paraformaldehyde (PFA) for 2 h at room temperature or 4°C overnight. Dechorionate embryos manually using forceps. Embryos older than 18 h should be dechorionated before fixation to prevent tail curling. Embryos can be used immediately or dehydrated in 100% methanol and stored at –20°C for several months.
2. Wash 5 × 5 min in PBSTw.
3. Embryos older than 10 h postfertilization (hpf) should be permeabilized with 10 µg/mL proteinase K in PBSTw as follows: 10 hpf, 1 min; 12 hpf, 2 min; 16 hpf, 4 min; 24 hpf, 5 min; 36 hpf, 8 min; 48 hpf, 11 min.
4. Refix in 4% PFA for 20 min at room temperature. Rinse 5 × 5 min in PBSTw.
5. Prehybridize in hybridization solution (hyb) for 1 h in a 65°C water bath.
6. Add labeled probe to the hybridization solution. Probes vary in quality and therefore should initially be tested at various concentrations (e.g., 1:50 to 1:300).
7. Hybridize 12–36 h (depending on the quality of the probe) in a 65°C water bath.
8. Remove used probe and save. Probes can be reused several times. Wash embryos as follows:
   15-min washes at 65°C:
   a. 75% hyb: 25% 2X SSC
   b. 50% hyb: 50% 2X SSC
   c. 25% hyb: 75% 2X SSC
   d. 2X SSC
   Wash 2 × 30 min in 0.2X SSC at 65°C
   5-min washes at room temperature:
   e. 75% 0.2X SSC: 25% PBSTw
   f. 50% 0.2X SSC: 50% PBSTw
   g. 25% 0.2X SSC: 75% PBSTw
   PBSTw
9. Antibody detection of DIG-labeled probe: Incubate the embryos in blocking solution for 1 h at room temperature.
10. Remove blocking solution and add the alkaline phosphatase-conjugated antidigoxigenin Fab fragments (anti-dig-AP antibody), diluted 1:5000, in blocking solution. Incubate 2 h at room temperature or overnight at 4°C.
11. Wash embryos 5 × 15 min in PBSTw.
12. Coloration reaction: wash embryos 2 × 10 min in coloration buffer.
13. Replace coloration buffer with NBT/BCIP solution (*see* **Note 4**): 45 µL NBT; 45 µL BCIP; and 10 mL coloration buffer.
14. For one-color *in situ*, stop the reaction by rinsing with PBSTw and refix for 20 min. After washing embryos out of fix (4 × 5 min with PBSTw), embryos can be stored in methanol or mounted for photography immediately after transferring into 70% glycerol. Transfer stepwise, with 10-min washes in increasing concentrations of glycerol (75% PBS:25% glycerol, 50% PBS:50% glycerol, 70% glycerol:30% PBS).

15. For two-color *in situ*, rinse embryos with PBSTw followed by 2 × 10 min incubations in 0.1 *M* glycine, pH 2.2.
16. Wash 3 × 5 min PBSTw.
17. Incubate embryos in blocking solution 1 h.
18. Incubate in antifluorescein–alkaline phosphatase-conjugated antibody (diluted 1:10,000 in blocking solution) for 2 h at room temperature.
19. Wash embryos 5 × 15 min in PBSTw.
20. Fast Red staining (*see* **Note 5**): a) wash 2 × 10 min in 0.1 *M* Tris, pH 8.2; b) Make Fast Red solution with Fast Red and buffer tablets according to the manufacturer's specifications; and c) Incubate embryos in Fast Red solution in the dark at room temperature. Staining will take from 1 h to several days, depending on the quality of the probe and the expression level of the transcript.
21. Rinse embryos in PBSTw and fix in 4% PFA for 20 min.

3.2. Immunocytochemistry

1. Fix embryos in 4% paraformaldehyde (*see* **Note 6**), pH 7.0, in PBS for 2 h at room temperature or 4°C overnight.
2. Wash embryos 4 × 5 min in PBSTw. Dechorionate embryos manually. Embryos older than 18 h should be dechorionated before fixation to prevent tail curling.
3. Permeabilize embryos older than 10 h postfertilization in 10 µg/mL proteinase K as described in **Subheading 3.1.2., step 3**.
4. Incubate embryos in blocking solution 1 h at room temperature.
5. Remove the blocking solution and replace with an appropriate dilution of antibody in blocking solution. Incubate for 2 h to overnight. Because antibodies vary in quality, the optimal concentration and incubation for each specific antibody should be determined using wild-type embryos.
6. Wash 3 × 15 min in PBSTw.
7. Secondary antibody: incubate embryos with appropriate dilution of secondary antibody (fluorescently conjugated or horseradish peroxidase conjugated), usually 1:1000 to 1:5000. Incubate 2 h at room temperature.
8. Wash 4 × 15 min in PBSTw.
9. When using a HRP-conjugated secondary antibody, develop in 0.1 mg/mL DAB and 0.1% hydrogen peroxide.
10. Replace DAB solution with PBSTw to stop the reaction and fix for 30 min with 4% PFA. Embryos can be stored in methanol at –20°C.

3.3. BrdU Incorporation Assay (Fig. 2A)

1. Dechorionate embryos manually using forceps.
2. Dilute BrdU to 10 m*M* in Ringer's solution. To 425 µL BrdU solution, add 75 µL dimethyl sulfoxide. Cool in an ice water bath (6°C) for 10 min.
3. Place 5–10 embryos in a microcentrifuge tube and remove the excess medium. Add 100 µL BrdU/DMSO solution and incubate in the 6°C water bath for 30 min.
4. Quickly rinse embryos with Ringer's solution five times. Transfer the embryos to a Petri dish with Ringer's solution and incubate for 1–2 h.
5. Fix embryos in 4% PFA for 2 h at room temperature or overnight at 4°C for antibody staining.
6. Acid treatment: rinse embryos three times in PBS and incubate for 1 h in 2 *M* HCl at 37°C.
7. Rinse in PBSTw and follow the antibody staining protocol using an anti-BrdU antibody diluted 1:50 in blocking solution.

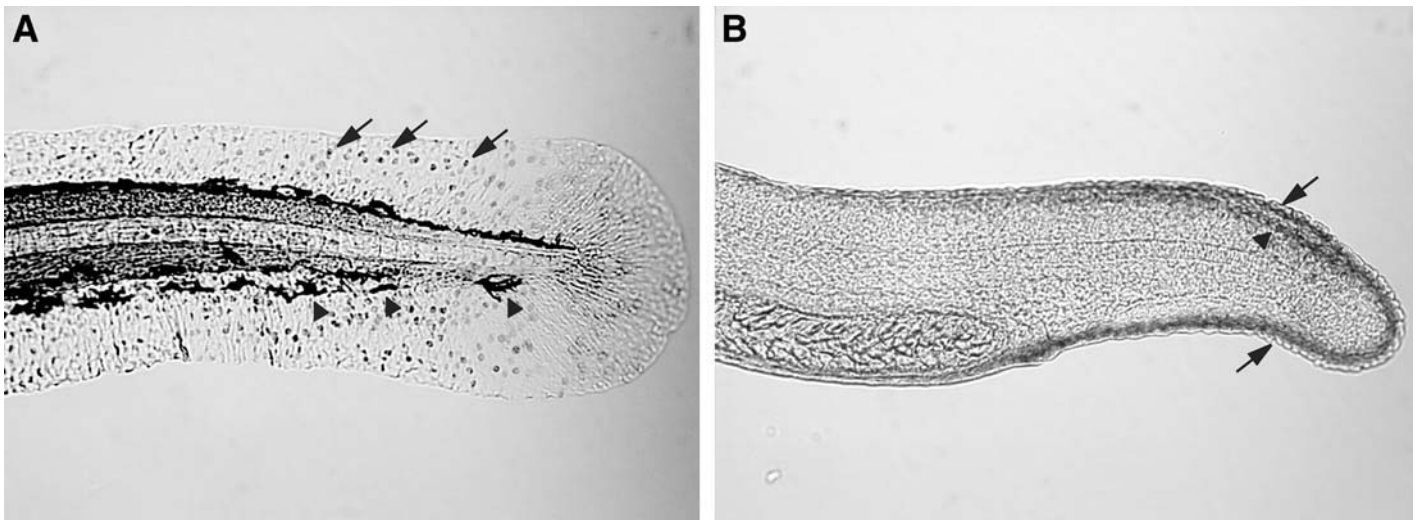


Fig. 2. **A,** BrdU-positive cells in the epidermis of a 48 hpf zebrafish (arrows). Arrowheads indicate melanophores. **B,** *In situ* hybridization for *msxB* at 25 hpf. Expression can be detected in the epidermis of the fin fold (arrows) and the dorsal mesenchyme that underlies the epidermis (arrowhead).

3.4. TUNEL Assay

1. Dechorionate embryos manually and fix in 4% PFA.
2. Wash embryos 4 × 5 min in PBS.
3. Incubate 15 min in 0.1% Triton X-100/0.1% sodium citrate in PBS.
4. Wash 2 × 10 min in TdT buffer.
5. Labeling (for 1 mL): 200 µL 5X TdT buffer; 0.5 µL Dig-labeled dUTP; 10 µL terminal transferase; and 800 µL sterile water. Add labeling solution to embryos and incubate 1 h at 37°C.
6. Wash 4 × 5 min in PBSTw.
7. Incubate 15 min at 85°C to inactivate TdT.
8. Incubate in blocking solution (*see* **Subheading 2.3., item 6**) for 1 h at room temperature.
9. Replace blocking solution with Anti-dig-AP diluted to 1:5000 in blocking solution.
10. Develop using NBT/BCIP as for *in situ* hybridization.

4. Notes

1. Goat serum should be heat inactivated at 65°C for 45 min prior to use.
2. After cutting with restriction enzymes that leave 3′ overhangs, DNA should be treated with T4 DNA polymerase (NEB; cat. no. M0203S) to prevent RNA polymerase from binding to the 3′ overhangs.
3. All components used to check the quality of the probe should be treated with 100 m*M* NaOH/0.5% sodium dodecyl sulfate for 1 h prior to use to eliminate RNase activity.
4. This step can be performed in glass *in situ* vials and should be performed in the dark. Monitor the reaction closely as some probes will react rapidly, whereas others may take several days.
5. For two-color *in situ*, it is recommended that the stronger probe be detected with Fast Red, which is less sensitive than NBT/BCIP.
6. Some antigens are sensitive to paraformaldehyde. For alternative fixation procedures, *see The Zebrafish Book* **(23)**.

Acknowledgments

The *in situ* hybridization protocol was adapted from protocols by C. Moens, C. Thisse, and B. Thisse. We also thank Hyunsook Lee for contributions to the immunocytochemistry and BrdU incorporation protocols.

References

1 Henrikson, R. C. and Matoltsy, A. G. (1967) The fine structure of teleost epidermis. 1. Introduction and filament-containing cells. *J. Ultrastruct. Res.* **21,** 194–212.
2 Hawkes, J. W. (1974) The structure of fish skin. I. General organization. *Cell Tissue Res.* **149,** 147–158.
3 Henrikson, R. C. (1967) Incorporation of tritiated thymidine by teleost epidermal cells. *Experientia* **23,** 357–358.
4 Bakkers, J., Hild, M., Kramer, C., Furutani-Seiki, M., and Hammerschmidt, M. (2002) Zebrafish DeltaNp63 is a direct target of Bmp signaling and encodes a transcriptional repressor blocking neural specification in the ventral ectoderm. *Dev. Cell.* **2,** 617–627.
5 Lee, H. and Kimelman, D. (2002) A dominant-negative form of p63 is required for epidermal proliferation in zebrafish. *Dev. Cell.* **2,** 607–616.
6 Conrad, M., Lemb, K., Schubert, T., and Markl, J. (1998) Biochemical identification and tissue-specific expression patterns of keratins in the zebrafish *Danio rerio*. *Cell Tissue Res.* **293,** 195–205.

7 Chua, K. L. and Lim, T. M. (2000) Type I and type II cytokeratin cDNAs from the zebrafish *(Danio rerio)* and expression patterns during early development. *Differentiation* **66,** 31–41.

8 Kimmel, C. B., Warga, R. M., and Schilling, T. F. (1990) Origin and organization of the zebrafish fate map. *Development* **108,** 581–594.

9 Read, E. M., Rodaway, A. R., Neave, B., Brandon, N., Holder, N., Patient, R. K., et al. (1998) Evidence for non-axial A/P patterning in the nonneural ectoderm of *Xenopus* and zebrafish pregastrula embryos. *Int. J. Dev. Biol.* **42,** 763–774.

10 Yang, A. and McKeon, F. (2000) P63 and P73: P53 mimics, menaces and more. *Nat. Rev. Mol. Cell. Biol.* **1,** 199–207.

11 van Bokhoven, H. and McKeon, F. (2002) Mutations in the p53 homolog p63: allele-specific developmental syndromes in humans. *Trends Mol. Med.* **8,** 133–139.

12 Yang, A., Schweitzer, R., Sun, D., Kaghad, M., Walker, N., Bronson, R. T., et al. (1999) p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. *Nature* **398,** 714–718.

13 Mills, A. A., Zheng, B., Wang, X. J., Vogel, H., Roop, D. R., and Bradley, A. (1999) p63 is a p53 homologue required for limb and epidermal morphogenesis. *Nature* **398,** 708–713.

14 Barrow, L. L., van Bokhoven, H., Daack-Hirsch, S., Andersen, T., van Beersum, S. E., Gorlin, R., et al. (2002) Analysis of the p63 gene in classical EEC syndrome, related syndromes, and non-syndromic orofacial clefts. *J. Med. Genet.* **39,** 559–566.

15 McGrath, J. A., Duijf, P. H., Doetsch, V., Irvine, A. D., de Waal, R., Vanmolkot, K. R., et al. (2001) Hay-Wells syndrome is caused by heterozygous missense mutations in the SAM domain of p63. *Hum. Mol. Genet.* **10,** 221–229.

16 Celli, J., Duijf, P., Hamel, B. C., Bamshad, M., Kramer, B., Smits, A. P., et al. (1999) Heterozygous germline mutations in the p53 homolog p63 are the cause of EEC syndrome. *Cell* **99,** 143–153.

17 Imboden, M., Goblet, C., Korn, H., and Vriz, S. (1997) Cytokeratin 8 is a suitable epidermal marker during zebrafish development. *Cell Biol.* **320,** 689–700.

18 Torpey, N., Wylie, C. C., and Heasman, J. (1992) Function of maternal cytokeratin in *Xenopus* development. *Nature* **357,** 413–415.

19 Heasman, J., Torpey, N., and Wylie, C. (1992) The role of intermediate filaments in early *Xenopus* development studied by antisense depletion of maternal mRNA. *Dev. Suppl.* 119–125.

20 Baribault, H., Price, J., Miyai, K., and Oshima, R. G. (1993) Mid-gestational lethality in mice lacking keratin 8. *Genes Dev.* **7,** 1191–1202.

21 Haffter, P., Granato, M., Brand, M., Mullins, M. C., Hammerschmidt, M., Kane, D. A., et al. (1996) The identification of genes with unique and essential functions in the development of the zebrafish, *Danio rerio*. *Development* **123,** 1–36.

22 Gaiano, N., Amsterdam, A., Kawakami, K., Allende, M., Becker, T., and Hopkins, N. (1996) Insertional mutagenesis and rapid cloning of essential genes in zebrafish. *Nature* **383,** 829–832.

23. Westerfield, M. (ed.) (2000) *The Zebrafish Book.* University of Oregon Press, Eugene, OR.

17

Analysis of E2F Factors During Epidermal Differentiation

Wing Y. Chang and Lina Dagnino

Summary

The multigene E2F family of transcription factors is central in the control of cell cycle progression. The expression and activity of E2F proteins is tightly regulated transcriptionally and posttranslationally as a function of the proliferation and differentiation status of the cell. In this chapter, we review protocols designed to determine E2F mRNA abundance in tissues by *in situ* hybridization techniques. The ability to culture primary epidermal keratinocytes and maintain them as either undifferentiated or terminally differentiated cells allows the biochemical and molecular characterization of changes in E2F expression and activity. Thus, we also discuss in detail methods to analyze E2F protein abundance by immunoblot and their ability to bind DNA in cultured cells using electrophoretic mobility shift assays.

Key Words:

E2F transcription factors; epidermis; keratinocytes; proliferation; differentiation.

1. Introduction

The epidermis is one of the most abundant epithelial cell types, providing an essential protective barrier against the environment. The epidermis is a stratified squamous epithelium continuously replenished by committed stem cells, which can either self-renew or differentiate ***(1)***. The lowermost basal layer contains keratinocyte stem cells and undifferentiated but committed proliferative cells. The uppermost suprabasal layers consist of terminally differentiated, quiescent keratinocytes, responsible for the protective barrier properties of the skin. An essential network that regulates cell proliferation includes the multigene family of E2F transcription factors. E2F activity is essential for expression of genes necessary for deoxyribonucleic acid (DNA) synthesis and S-phase progression. In this chapter, protocols to study the expression and DNA binding of E2F genes in the epidermis and in primary keratinocyte cultures will be described ***(2–4)***. The protocols for isolation of primary cultures are developed for murine keratinocytes, whereas the analysis of E2F proteins is applicable to mouse and human cells and/or tissues.

2. Materials

2.1. DNA Probes for In Situ *Hybridization*

1. Mouse pAlter-E2F1.
2. Mouse pBK-E2F2-D2.
3. Mouse BS-E2F3.
4. Mouse SK-E2F4 (PstI deletion).

From: *Methods in Molecular Biology, vol. 289:*
Epidermal Cells: Methods and Protocols
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

5. Mouse SK-E2F5.
6. DNA purification kit (e.g., Qiagen; cat. no. 12243).

All cDNA plasmids can be prepared and purified with the DNA purification kit. The plasmids are available from L. Dagnino (ldagnino@uwo.ca).

2.2. Antibodies for Immunoblotting

1. Mouse monoclonal anti-E2F1 (NeoMarkers MS-880) or mouse polyclonal anti-E2F1 (Santa Cruz Biotechnology, sc-251).
2. Mouse monoclonal anti-E2F2 (NeoMarkers MS-252 or rabbit Santa Cruz Biotechnology, sc-633).
3. Rabbit monoclonal anti-E2F3 (Santa Cruz Biotechnology, sc-878 and sc-879).
4. Rabbit polyclonal anti-E2F4 (Santa Cruz Biotechnology, sc-866).
5. Rabbit polyclonal anti-E2F5 (Santa Cruz Biotechnology, sc-999).
6. Horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, 115-035-146).
7. Horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, 111-035-144).

2.3. Oligonucleotides for Electrophoretic Mobility Shift Assays (EMSA)

1. E2F DNA-binding element from the mouse dihydrofolate reductase promoter: DHFR-a (5′-CTA GAG CAA TTT CGC GCC AAA CTT G-3′) and DHFR-b (5′-CAT CCA AGT TTG GCG CGA AAT TGC T-3′).
2. DHFR mutant oligonucleotide incapable of binding to E2F: DHFR-c (5′-CTA GAG CAA TTT CGA GCC AAA CTT G-3′) and DHFR-d (5′-CAT CCA AGT TTG GCT CGA AAT TGC T-3′).

2.4. Reagents

2.4.1. In Situ *Hybridization*

1. Diethylpyrocarbonate (DEPC)-treated dH_2O.
2. Phosphate-buffered saline (PBS, made with DEPC-treated dH_2O).
3. Placental RNase inhibitor (RNA guard; Invitrogen; cat. no. 15518-012).
4. 5X NTP (5 m*M* each ATP (Invitrogen; cat. no. 18330-019), CTP (Invitrogen; cat. no. 18331-017), GTP (Invitrogen; cat. no. 18332-015).
5. [^{33}P]-UTP (3000 Ci/mmol, Amersham Pharmacia; cat. no. AH9903).
6. RNase-free DNase I (Invitrogen; cat. no. 18047-019).
7. Yeast tRNA (Invitrogen; cat. no. 15401-011; 10 mg/mL dissolved in DEPC-treated dH_2O).
8. Proteinase K (Invitrogen; cat. no. 25530-015; 20 mg/mL dissolved in DEPC-treated dH_2O, stored at –20°C in 0.5-mL aliquots).
9. 7.5 *M* Ammonium acetate (prepared with DEPC-treated dH_2O).
10. 20% Paraformaldehyde, pH 7.0, dissolved in PBS made with DEPC-treated dH_2O (stored at –20°C in 40-mL aliquots).
11. Triethanolamine hydrochloride (TEA) buffer for acetylation: TEA solution (3.72 g TEA dissolved in 200 mL DEPC-treated dH_2O, plus 0.448 mL 10 *N* NaOH, prepared just prior to use).
12. Acetic anhydride.
13. Hybridization mix: 0.3 *M* NaCl, 20 m*M* Tris-HCl, pH 8.0, 5 m*M* EDTA, 10 m*M* Na–phosphate buffer, pH 8.0, 1X Denhardt's; 10% dextran sulphate, 0.5 mg/mL yeast tRNA, 50% formamide. This solution can be stored at –80°C for up to 8 mo).
14. 20X SSC.

15. Photographic emulsion (Kodak NTB-2 nuclear emulsion; cat. no. 1654433).
16. Kodak D-19 developer and Kodafix no. 146-4080.
17. Kodak Safelight Filter no. 2 with a 15-W bulb.
18. Dessicant.
19. T7 RNA polymerase (50 U/µL, supplied with 5X buffer, Invitrogen; cat. no. 18033-019).
20. SP6 RNA polymerase (15 U/µL, supplied with 5X buffer, Invitrogen; cat. no. 18018-010).
21. T3 RNA polymerase (20 U/µL, supplied with 5X buffer, MBI Fermentas; cat. no. EP0101).
22. Restriction endonucleases that digest plasmid templates only once (supplied with 10X reaction buffer).

2.4.2. Isolation and Culture of Primary Keratinocytes

1. Minimum essential medium without $CaCl_2$ (e.g., EMEM without $CaCl_2$, Cambrex; cat. no. 06-174G).
2. Fetal bovine serum (FBS) pretreated with Chelex 100 chelating resin (200–400 mesh, sodium salt, Bio–Rad Laboratories) to remove Ca^{2+} ions (*see* **Note 1**). Stir the serum with the resin (40 g resin/500 mL serum) at room temperature for 1 h. Filter through paper to remove the resin, and follow by 0.45-µm filter sterilization (*see* **Note 2**).
3. Stock HICTE supplements: hydrocortisone (500 µg/mL dissolved in EtOH, stored at –20°C), insulin (5 mg/mL, dissolved in dH_2O, pH adjusted to 3.0 with HCl, stored at –20°C), cholera toxin (1 mg/mL, dissolved in dH_2O, stored at –20°C), triiodothyronin (T3, 340 µg/mL dissolved in 0.1 *N* NaOH, stored at –20°C), epidermal growth factor (100 µg/mL dissolved in sterile dH_2O. Store at –20°C in single-use aliquots).
4. Penicillin (100 U/mL) and streptomycin (0.1 mg/mL).
5. Keratinocyte growth medium: EMEM without $CaCl_2$, supplemented with 8% chelex-treated FBS, antibiotics (100 U/mL penicillin; 0.1 mg/mL streptomycin), epidermal growth factor (5 ng/mL), hydrocortisone (74 ng/mL), cholera toxin (10^{-10} *M*), insulin (5 µg/mL), and triiodothyronine (6.7 ng/mL).
6. 0.4% Trypan blue stain dissolved in 0.85% NaCl.

2.4.3. EMSA

1. EMSA Lysis buffer: 50 m*M* HEPES, pH 7.7; 250 m*M* KCl; 10% glycerol; 0.1% nonidet P-40; 0.4 m*M* NaF; 0.4 m*M* Na_3VO_4; 0.1 m*M* EDTA. Add dithiothreitol (DTT, 1 m*M*, final) and protease inhibitors just prior to using (0.5 m*M* phenylmethylsulfonyl fluoride, 1 µg/mL each leupeptin, pepstatin, and aprotinin).
2. 5X E2F binding buffer: 20 m*M* HEPES, pH 7.6; 50% glycerol, 2.5 m*M* $MgCl_2$, 40 m*M* KCl, 0.1 m*M* EGTA, 0.5 mg/mL acetylated bovine serum albumin, 0.5 m*M* DTT, 0.4 m*M* spermine.
3. Sonicated and boiled salmon sperm DNA (ssDNA), 3–5 mg/mL.
4. Loading dye: 5 µL 10X TBE, 0.1% Bromophenol blue, 0.1% xylene cyanol, 10% Ficoll.
5. Polyacrylamide gels for electrophoresis: 10.3 mL of acrylamide/bis-acrylamide (29 : 1), 3 mL 10X TBE, 3 mL 50% glycerol, 43.2 mL dH_2O, 0.48 mL 10% ammonium persulfate (freshly made), 31.9 mL TEMED.
6. Electrophoresis buffer: 0.25X TBE.

2.4.4. Immunoblot Analysis

1. N2 Lysis buffer: 20 m*M* HEPES, pH 7.8, 450 m*M* NaCl, 0.4 m*M* EDTA, 25% glycerol, protease inhibitors (0.5 m*M* phenylmethylsufonyl fluoride, 1 µg/mL each aprotinin, leupeptin, and pepstatin; freshly added), 0.5 m*M* DTT.
2. TBST: 100 m*M* Tris-HCl, pH 7.5, 0.9% NaCl, 0.1% Tween-20.

3. 4X Sample buffer: 100 m*M* Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate, 40% glycerol, 40 m*M* DTT, 0.2% Bromophenol blue.
4. 5% BLOTTO: 5% powdered skim milk in TBST.

3. Methods

3.1. In Situ *Hybridization Using Tissue Sections*

3.1.1. Tissue Sections

Good signals are obtained using 8-µm frozen tissue sections. Cryosections should be stored at –80°C until immediately before use (*see* **Note 3**).

3.1.2. Preparation of the Template

1. To linearize the cDNA template assemble the following reaction: 30 µL purified DNA (20–30 µg); 10 µL 10X restriction enzyme buffer; 55 µL dH_2O; and 5 µL restriction endonuclease (*see* **Note 4**).
2. Incubate at 37°C for 1.5 h.
3. Run a 1-µL sample on an agarose gel to verify that digestion of the template is complete. If not, add 2 µL of restriction endonuclease, incubate at 37°C for 1 h, and verify again. It is essential that the template be completely digested prior to proceeding to ribroprobe synthesis.
4. Purify the template by sequential extractions with Tris-HCl-equilibrated phenol, phenol : chloroform/isoamyl alcohol (50 : 45 : 5 v/v/v), and chloroform : isoamyl alcohol.
5. Add 10 µL 3 *M* NaOAc, pH 5.5, vortex, add 250 µL of –20°C EtOH.
6. Precipitate the DNA in dry ice for 30 min or at –20°C overnight.
7. Collect the DNA precipitate by centrifugation (20,800*g*, 4°C, 15 min).
8. Remove the supernate, wash the pellet by gently adding cold (–20°C) 70% EtOH.
9. Centrifuge (20,800*g*, 4°C, 5 min), remove the supernate, and allow the pellet to air-dry for 5 min.
10. Dissolve the DNA in 15 µL DEPC-treated dH_2O.
11. Determine the DNA concentration by measuring $OD_{260/280}$ and adjust to 500 ng/µL.
12. Store at –20°C.

3.1.3. Synthesis and Purification of the Riboprobe

1. For the in vitro transcription reaction, add to a microfuge tube in the following order: 6 µL DEPC-treated dH_2O; 5 µL 5X RNA polymerase buffer; 1 µL RNA guard; 5 µL 5X NTP; 2 µL DNA template (500 ng/µL); 2.5 µL [^{33}P]-UTP; and 1 µL RNA polymerase (e.g., T3, T7, or SP6 RNA polymerase as required).
2. Incubate at 37°C for 1–2 h.
3. Add 1 µL RNase-free DNase I (10 U/µL) and 1 µL RNA guard. Incubate at 37°C for 10 min.
4. Add 63 µL DEPC-treated dH_2O and 10 µL 3 *M* NaOAc.
5. Extract once with phenol/chloroform (*see* **Note 5**) and once with chloroform. Use pure phenol or dH_2O-equilibrated phenol.
6. Sequentially add, mixing after each addition, 36.4 µL 7.5 *M* NH_4OAc, 1 µL yeast tRNA, and 400 µL absolute EtOH.
7. Precipitate the RNA in dry ice for 20 min or at –20°C overnight.
8. Recover the precipitate by centrifugation (20,800*g*, 4°C, 15 min), followed by a rinse with cold 70% EtOH and a second brief centrifugation (20,800*g*, 4°C, 5 min).
9. Remove the supernate, air-dry the pellet for 5 min and resuspend in 50 µL DEPC-treated dH_2O.
10. Determine the specific activity of the riboprobe by liquid scintillation counting of 1 µL of a 10^{-1} dilution of the RNA.

11. Verify the quality of the riboprobe (i.e., presence of a substantial fraction of full-length transcripts) by electrophoretic separation of a sample corresponding to 50,000–100,000 cpm on a DNA sequencing gel. Probes containing ≤250-base fragments generally do not yield very strong signals.
12. Use the probe immediately or store at –80°C for up to 5 d.

3.1.4. Pretreatment of Tissue Sections

1. Allow frozen sections to air-dry and warm to room temperature (30 min).
2. Fix in 4% paraformaldehyde (in PBS) 20 min at room temperature.
3. Wash twice in PBS for 5 min.
4. Incubate 7.5 min with proteinase K (20 µg/mL, final concentration) dissolved in a buffer containing 50 m*M* Tris-HCl, pH 7.5, and 5 m*M* EDTA (*see* **Note 6**).
5. Wash in PBS for 5 min.
6. Fix in 4% paraformaldehyde for 5 min, followed by a 3-min PBS wash.
7. During **steps 4–6**, prepare TEA buffer for acetylation.
8. Add 0.5 mL acetic anhydride to TEA buffer with vigorous mixing. Incubate the slides for 5 min, shaking the slide-holding rack every 45–60 s. Add another 0.5–mL aliquot of acetic anhydride and incubate the slides for 5 additional min, shaking the slide rack every 45–60 s.
9. Rinse with PBS for 5 min, and with saline (0.9% NaCl) for 5 min.
10. Dehydrate the sections by sequential immersion into 30%, 50%, 70%, 85%, 95%, 100%, and 100% EtOH diluted with saline (30 s each, 5–10 min for 70% EtOH).
11. Air-dry sections and use immediately or store at –80°C.

3.1.5. Hybridization of Tissue Sections

1. Denature probe by heating to 100°C for 2 min, followed immediately by incubation in ice for 5 min. Denature enough probe to allow use of 5×10^6 to 2×10^7 cpm/slide. Mix the denatured probe with hybridization solution (100–150 µL/slide).
2. Trace a circle on the glass slide around the tissue to be hybridized with a wax pen (e.g., Dako Pen for immunohistochemistry). Place probe-containing hybridization mix on the tissue, spreading carefully over the entire surface inside the wax tracing. Place slide in a plastic chamber lined with Kimwipes® soaked in 5X standard saline citrate (SSC)/50% formamide, and incubate at 55°C overnight.

3.1.6. Posthybridization Treatment of Sections

1. Set two water baths to 65°C and 37°C. All washing solutions should be prewarmed.
2. Briefly rinse slides by immersion in a 50-mL plastic tube containing 5X SSC. Place the hybridized slides in a holding rack, leaving an empty slot in between (*see* **Note 7**). Wash in 50% formamide/2X SSC at 65°C for 30 min.
3. Wash in RNase buffer (0.5 *M* NaCl/10 m*M* Tris-HCl, pH 8.0/5 m*M* EDTA. Do not use DEPC-treated dH_2O), at 37°C for 10 min. Repeat twice more.
4. Wash in RNase buffer containing 20 µg/mL RNase A, incubating at 37°C for 30 min.
5. Wash in fresh RNase buffer at 37°C for 15 min.
6. Wash with continuous shaking in fresh 50% formamide/2X SSC solution at 65°C for 20 min.
7. Wash in 2X SSC at 37°C for 15 min, followed by 0.1X SSC at 37°C for 15 min.
8. Dehydrate the tissue by rapid immersion in 30%, 60%, 80%, and 95%, EtOH diluted in 0.3 *M* NH_4OAc, followed by two rinses with 100% EtOH.
9. Air-dry slides.

3.1.7. Preparation of Photographic Emulsion

1. Preparation of photographic emulsion: set up the safelights no closer than 4 ft from photographic emulsion. Set a water bath to 42°C. Make sure the water level reaches to the upper

level of the emulsion, and cover any indicator lights on the water bath with aluminum foil. Melt emulsion by placing in the water bath. Make sure all glass- and plasticware used for the emulsion is clean and free of traces of old emulsion.
2. Slowly pour emulsion into a beaker containing an equal volume of 0.6 *M* NaOAc, keeping it at all times at least 4 ft away from the safelight. Mix gently, avoiding the formation of bubbles. Aliquot diluted emulsion in small slide mailers to fill them up. Wrap the slide mailers three times in aluminum foil, and store at 4°C.

3.1.8. Emulsion Coating and Exposure of Tissue Sections

1. Preparation of the dark room: set water bath to 42°C and place an aliquot of diluted emulsion to melt (20–30 min). Prepare the following material: Dessicant (e.g., Dririte), Kimwipes®, aluminium foil, slide box, a cardboard box large enough to hold the slide box, latex gloves, test tube racks to hold slides, forceps, tape, spare clean glass slides.
2. Slowly dip two test glass slides into the emulsion, and place them vertically, using the test tube rack to hold the slides. Bring the test slides outside the dark room to check that the level of emulsion coats the slide, and to ensure there are no air bubbles on the coating emulsion. If there are air bubbles, dip a few more test slides, until no more bubbles are visible. Keep the emulsion at 42°C.
3. Dip each slide slowly, leaving it in the emulsion for 10–15 s, and remove from the emulsion slowly and at a constant speed (about 5 s) to obtain a uniform layer of emulsion coating. Place the slides vertically in the test tube rack. Allow the slides to dry for 2–3 h at room temperature in the dark.
4. Wrap a fair amount of dessicant in Kimwipes®, and place inside slide box, between two blank slides, leaving enough places to hold the emulsion-coated slides. Place the dry emulsion-coated slides in this box. Close the box and wrap three times with foil. Place the slide boxes in a cardboard box containing additional dessicant. Store at 4°C for 2–4 wk.

3.1.9. Development and Counterstaining of Tissue Sections

1. Allow slides to warm to room temperature.
2. Prepare developer: heat 250 mL dH_2O to 52°C and add 19.5 g developer. Stir to dissolve and cool on ice to 16°C.
3. Prepare fixative, diluting Kodafix 1 : 3 (v/v) with dH_2O.
4. Take developer, fixative, staining racks, and staining jars to the dark room.
5. In the dark, load slides on staining racks. Develop by sequential immersion in the following solutions: developer (16°C, 2 min, *see* **Note 8**), water (30 s), fixative (5 min), water (two thorough washes).
6. Counterstain lightly tissue sections with 0.1% Toluidine blue (dissolved in 10 m*M* NaOAc, pH 4.6) or with hematoxillin/eosin. If using hematoxillin, ensure a nonacid preparation is used (e.g., Harris hematoxillin) to avoid dissolving the silver grains.
7. Dehydrate tissue by sequential immersion in the following:

$$H_2O \rightarrow 70\%\ EtOH \rightarrow 100\%\ EtOH\ (3\ min, twice) \rightarrow$$
$$1:1\ EtOH/xylene\ (3\ min) \rightarrow Xylene\ (3\ min, twice).$$

8. Mount using two or three drops of Permount, avoiding formation of air bubbles. If mounting is imperfect, remove cover slip by immersion in xylene, and repeat the procedure.

3.2. Isolation and Culture of Primary Keratinocytes

3.2.1. Tissue Harvesting

1. Although it is possible to establish primary keratinocyte cultures from newborn mice up to 4 d of age, plating efficiency is optimal using 0- to 2-d-old animals. The skin is harvested

from CO_2-euthanized animals immersed in Betadine for 5 min, rinsed twice in 70% EtOH, and kept on ice.
2. Under sterile conditions, limbs, tail and head are amputated.
3. Using a scalpel, a longitudinal incision through the skin is made all along the back of the carcass.
4. Using two forceps, the skin is gently removed, starting at the incision point.
5. The skin is stretched out and floated with the dermis side down on fresh 0.25% trypsin at 4°C for 15–20 h. Ensure that the edges are not folded; otherwise the skin will not float properly. If keratinocytes will be isolated from transgenic mice, the isolated skin can be stored overnight at 4°C in phosphate-buffered saline, while genotyping is conducted, followed by incubation with fresh 0.25% trypsin at 4°C for 15–20 h.

3.2.2. Trypsin Digestion and Isolation of a Keratinocyte Suspension

1. After overnight incubation, replace the trypsin with new 0.25% trypsin, and incubate the tissues at 37°C for 1–1.5 h, depending on the thickness of the skin.
2. After the second trypsin digestion, the skin is placed on a plastic bacterial culture dish, epidermis side down, and the dermis is gently pulled off with the tip of a forceps (*see* **Note 9**). Place the epidermis in a bacterial culture dish containing keratinocyte growth medium. Mince the epidermis with sterile scissors and transfer to a sterile conical tube. Incubate at 37°C with gentle rocking for 15–25 min to release the cells from the cornified envelope. Filter the cell suspension through a 70-µm nylon mesh (e.g., Falcon). Mix a 20-µL aliquot of cells with 20 µL of Trypan blue stain and determine the number of viable cells/mL of suspension using a haemocytometer.

3.2.3. Culture of Keratinocytes

1. Plate the keratinocytes on culture dishes, using $2–3 \times 10^5$ cells/cm^2, to obtain a culture approx 50% confluent the following day (*see* **Note 10**). Culture at 37°C for 24 h.
2. Remove the growth medium and nonadherent cells and replace with fresh culture medium. Thereafter, replace the culture medium every 48 h (*see* **Note 11**).

3.3. Analysis of E2F Factors by Immunoblot

3.3.1. Preparation of Cell Extracts

1. Remove the growth medium and rinse the cultures once with ice-cold PBS. Keep the culture dishes and cells on ice at all times.
2. Add 1 mL of PBS to the culture dish. Harvest the keratinocytes by gently scraping them off the dish with a cell scraper with 1 mL PBS on ice. Transfer the cell suspension to a microfuge tube.
3. Centrifuge the cells (4°C, 500*g*, 5 min for undifferentiated cells; 1580*g* 7–9 min for differentiated cells) and suspend the cell pellet in approximately one pellet volume of N2 lysis buffer.
4. Conduct three cycles of freeze-thawing in liquid N_2.
5. Centrifuge cell debris in a microfuge at 20,800*g* for 10 min at 4°C and transfer supernatant to a new tube. Measure the protein concentration of the lysates (*see* **Note 12**).

3.3.2. Immunoblots

1. Prepare lysate samples. To denature the proteins in the lysates, add one-third volume of 4X sample buffer, boil for 5 min, and microfuge at 20,800*g* for 3 min.
2. Resolve by electrophoresis on 9% denaturing polyacrylamide gels and transfer the proteins to a polyvinyledene fluoride membrane.

3. After rinsing, block the membrane by incubation in 5% BLOTTO for 2 h at 22°C, followed by three 10-min TBST washes.
4. Probe the blots by incubation for 2 h at 22°C with the primary anti-E2F antibody of interest, diluted to 0.4 µg/mL in TBST containing 0.1% bovine serum albumin.
5. Wash the membrane with TBST for 15 min, and repeat this wash twice more.
6. Incubate the membrane with horseradish peroxidase-conjugated secondary antibody diluted 1:5000 in TBST containing 5% BLOTTO, followed by three 15-min washes with TBST.
7. Develop the blot using enhanced chemiluminescence.

3.4. Analysis of E2F Factors by EMSA

3.4.1. Probe Preparation

1. Oligonucleotide annealing: double-stranded (ds) wild-type E2F DNA-binding elements are obtained by annealing the DHFR-a and DHFR-b oligonucleotides. To this end, mix 10 µg of each oligonucleotide in a 1 *M* NaCl solution, adjusting the volume to obtain a final DNA concentration of 1 µg/µL. Prepare a 100-mL boiling water bath. Incubate the DNA in the boiling water for 1 min. Remove the bath from the heat source and continue incubating the DNA until the water has cooled to room temperature.
2. Oligonucleotide labeling: An aliquot of the double-stranded oligonucleotide prepared is diluted to 40 ng/µL. The double-stranded oligonucleotide is designed to have 5′ overhangs that are filled in with Klenow enzyme. Assemble the labeling reaction to contain 40–80 ng double-stranded oligonucleotide, 50 µCi [α^{32}P]dCTP (3000 Ci/mmol aqueous solution), 25 µ*M* each of dATP, dGTP and dTTP, and 4-µ labeling-grade Klenow enzyme. Incubate at 37°C for 10–30 min. Purify the labeled oligonucleotide by filtration through a G-25 Sephadex column (e.g., G-25 Quickspin columns, Roche). Determine the specific activity of the purified probe by liquid scintillation counting of a 1-µL aliquot. The probe can be used immediately or stored at –20°C in an appropriate shielded container. Probes are good for 1–2 wk.

3.4.2. Preparation of Cell Extracts

1. Place the culture dishes on ice. Cells should be kept on ice during the entire procedure.
2. Aspirate the growth medium and rinse the cells with ice-cold calcium-free PBS or with ice-cold KREBS saline.
3. Aspirate PBS.
4. Add 1 mL ice-cold PBS or KREBS/100-mm culture dish.
5. Carefully scrape the cells off the plate, gently pushing them to one corner.
6. Transfer the cell suspension to a microfuge tube, and centrifuge for 2 min at 500*g*, 4°C.
7. Place the tubes in ice, and remove the supernatant without disturbing the cell pellet.
8. Add 2–4 pellet volumes of ice-cold EMSA lysis buffer. Suspend the cells thoroughly, and rock on ice for 30 min.
9. Centrifuge at 20,800*g* for 15 min, 4°C.
10. Transfer the supernatant to a clean tube.
11. Take 1–2-µL aliquots to determine protein concentration. The remainder of the extract can be kept on ice if used immediately, or frozen in single-use aliquots and stored at –70°C until used.

3.4.3. Binding Reactions

Each binding reaction is assembled in a 25-µL total volume containing 5 µL 5X binding buffer, 60 ng/µL ssDNA, cell lysate (10–25 µg protein), 6 ng/µL double-stranded mutant E2F oligonucleotide (*see* **Note 13**), and 20,000 cpm E2F probe. Incubate on ice

for 1 h. If required, add 1 µL of supershifting antibody, and incubate on ice for an additional 45-min interval.

3.4.4. Electrophoresis

Binding reactions are resolved by electrophoresis using nondenaturing polyacrylamide gels. Generally, 5% gels are used. The gels are prerun for 15 min at 4°C. Prior to loading the binding reactions, the wells in the gel are rinsed. An additional sample is prepared and loaded on the gel, containing 20,000 cpm of probe (without cell extract) mixed with 10 µL of loading dye. The gel is run at 4°C, at 200–350 V until the Bromophenol blue has reached the bottom of the gel (2.5–3.5 h).

3.4.5. Processing and Development of Gels

After electrophoresis, the gel is transferred to filter paper, dried, and exposed to X-ray film.

4. Notes

1. Keratinocytes are maintained as undifferentiated cells by culture in medium containing < 0.1 m*M* Ca^{2+}. Thus, it is essential to substantially reduce the Ca^{2+} content from FBS. The final Ca^{2+} concentration of the growth medium supplemented with chelex-treated FBS is approx 0.05 m*M* and can easily be determined by atomic absorption techniques.
2. Serum lots must be screened because they show variability in supporting keratinocyte growth.
3. It is possible to use paraffin sections, which tend to better preserve the morphology of the tissue. However, the sensitivity tends to be substantially reduced, which may hamper detection of the less abundant E2F transcripts (e.g., E2F1).
4. The enzyme selected must digest the template only at one site corresponding to either the beginning or the end of the cDNA of interest, to yield either the sense or the antisense probe.
5. Use pure phenol or dH_2O-equilibrated phenol, not TE-equilibrated phenol.
6. The 7.5-min digestion time with proteinase K is very important, as shorter incubations may result in inadequate probe permeation into the tissue, and longer times may cause tissue losses.
7. Leaving additional spaces between each slide allows for more efficient mixing with the wash solutions, and greatly increases washing efficacy, decreasing the background.
8. The temperature of the developer is critical to avoid overdeveloping the emulsion during the 2-min immersion time.
9. If the trypsin digestion has been adequate, the epidermis will adhere to the dry plastic surface of the bacterial culture dish, allowing easy separation from the dermis. Difficulty separating the epidermis from the dermis indicates incomplete trypsin digestion. In this case, continue incubation at 37°C for a few more minutes.
10. Keratinocytes adhere better to culture dishes manufactured for primary cell culture (e.g., Primaria).
11. Primary keratinocytes will undergo a few population doublings and then senesce. To obtain consistent experimental results, it is best to use cells 2–5 d after plating. To induce terminal differentiation, $CaCl_2$ (0.1–1 m*M*, final) can be added to the culture medium. Cells differentiate by 24 h after Ca^{2+} addition.
12. Generally, the amount of lysates to be analysed depends on the individual E2F factor examined, as well as the cell type. For keratinocyte lysates, 100 µg protein are required for E2F1, E2F2, and E2F3, whereas 50 µg suffice for E2F4 or E2F5 analysis.
13. Double-stranded mutant E2F oligonucleotide is prepared by annealing the DHFR-c and the DHFR-d oligonucleotides, following the procedure outlined in **Subheading 3.3.1.**

References

1 Dotto, G. P. (1999) Signal transduction pathways controlling the switch between keratinocyte growth and differentiation. *Crit. Rev. Oral Biol. Med.* **10,** 442–457.

2. D'Souza, S. J. A., Pajak, A., Balazsi, K., and Dagnino, L. (2001) Ca^{2+} and BMP-6 signalling regulate E2F during epidermal keratinocyte differentiation. *J. Biol. Chem.* **276,** 23,531–23,538.

3 Dagnino, L., Fry, C. J., Bartley, S. M., Farnham, P., Gallie, B. L., and Phillips, R. A. (1997) Expression patterns of the E2F family of transcription factors during mouse nervous system development. *Mech. Dev.* **66,** 13–25.

4. Dagnino, L., Fry, C. J., Bartley, S. M., Farnham, P., Gallie, B. L., and Phillips, R. A. (1997) Expression patterns of the E2F family of transcription factors during murine epithelial development. *Cell Growth Differ.* **8,** 553–563.

18

Analysis of HOX Homeodomain Proteins and Gene Transcripts in the Epidermis

László G. Kömüves and Corey Largman

Summary

HOX homeodomain proteins are thought to be master developmental regulators of tissue patterning during embryogenesis. These DNA binding proteins also have diverse roles in adult cell function, and derangement of *HOX* genes has been associated with several types of cancer. In this chapter we present protocols for the immunohistochemical localization of HOX proteins in the epidermis. We also provide *in situ* hybridization protocols for detection of *HOX* gene mRNA transcripts in the epidermis.

Key Words:

HOX; homeobox; homeodomain; epidermis; skin; immunohistochemistry; *in situ* hybridization; transcription factor; gene regulation.

1. Introduction

The molecular mechanisms that regulate pattern formation in the developing skin are largely unknown. The *HOX* homeobox genes are fundamental regulators of body patterning in the developing *Drosophila* embryo and regulate aspects of skeletal pattern formation during mammalian embryonic development. There are 39 human *HOX* genes, and many of these have been detected as either the mRNA or protein products within the epidermis. The *HOX* homeobox genes encode proteins containing a 60-amino acid homeodomain motif that functions as a deoxyribonucleic acid (DNA)-binding moiety. The HOX proteins often appear to function with DNA binding partner proteins that include the PBX and MEIS homeodomain proteins. In this chapter, we review methods for both HOX proteins and their known partners, as well as a few addition homeodomain proteins, shown to be expressed in epidermis. Because the HOX homeodomain proteins are thought to function as transcription factors, it has been anticipated that they would be localized to the nucleus within the epidermis. However, our laboratory has observed that most HOX as well as other non-HOX homeodomain proteins are often detected in the cytoplasm within the epidermis. Because the non-HOX homeodomain protein PBX moves from cytoplasm to nucleus in response to signaling pathways, it is anticipated that as yet uncharacterized molecular signaling may regulate the subcellular localization of HOX proteins within the epidermis. Thus the spatial analysis of HOX proteins within the layers of the epidermis, as well as their subcellular localization in

From: *Methods in Molecular Biology, vol. 289:*
Epidermal Cells: Methods and Protocols
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

normal and diseased states, may provide information on the biological role of these proteins in skin homeostasis, patterning, and repair. In this chapter, protocols to study expression of *HOX* genes and the protein products will be described in detail. Many of the antibody and *in situ* hybridization reagents described were developed in our laboratory, and are available upon request. In addition, a growing number of antibodies to HOX proteins are commercially available.

1.1. Immunohistochemical Localization of HOX Proteins

1.1.1. Protocols for HOX Protein Visualization

We have used a number of closely related protocols, all of which share the common feature of a tyramide amplification step, to visualize HOX proteins in developing and adult human and murine epidermis ***(1–3)***. Consistent with the putative role of HOX proteins as transcription factors, their expression levels and/or stability is sufficiently low that detection in the skin without tyramide enhancement has not been possible for the various HOX proteins examined to date. For successful detection, the primary antibody directed against the HOX protein, normally raised in rabbits, but sometimes in guinea pigs, chickens, or rats, is detected with a peroxidase-coupled bridging antibody Fab fragment of the appropriate specificity for detection of the primary IgG. Signal amplification is achieved by either direct deposition of fluorescently labeled tyramide or by biotinylated tyramide deposition, followed by a streptavidin-coupled peroxidase or alkaline phosphatase to catalyze the deposition of colorimetric or fluorimetric substrates. Normally the second peroxidase catalyzed reaction is accomplished with one of several commercially available ABC-peroxidase or ABC-phosphatase reaction systems. Tissues are counterstained with one of a number of different reagents to reveal tissue morphology for light microscopy or nuclear localization for fluorescent labeling experiments. Where relevant, we note blocking, detection, bridging, or amplification reagents that we have successfully used as alternatives to the basic protocol. By listing these alternative reagents we wish to illustrate that there is no single required protocol, and that a variety of different reagent combinations have been successfully employed, based within the constant theme of tyramide amplification, to detect HOX proteins in the skin (*see* **Note 1**).

1.1.2. Antibodies for HOX Proteins

Because of the high levels of keratins in commercial animal feeds (which result in nonspecific reactivity of antibodies to epidermis), we affinity purify all antibodies used to detect proteins in the skin to reduce the substantial amount of nonspecific signal detected using whole antisera. Antibodies produced against protein polypeptides are normally purified on the immobilized protein, whereas antibodies raised against small peptide antigens are often purified against the immobilized peptide or against the whole protein. Unless otherwise noted, all primary antibodies described in this chapter have been affinity purified. The 39 mammalian HOX proteins share the relatively well conserved 60-amino acid homeodomain. In addition, the HOX proteins can be divided into 13 paralog groups that show variable and sometimes substantial areas of sequence homology outside of the homeodomain (*see* **Note 2**). Thus, the choice of a suitable antigen that will produce antisera specific for a given HOX protein can be difficult. The investigator should be cognizant of this situation and consider whether the antibodies

are capable of specific recognition of the desired target. For instance, to specifically detect HOXB13 using antibodies produced against the entire 215 amino acid protein excluding the homeodomain, in addition to positive affinity purification on immobilized HOXB13 protein, it was necessary to negatively affinity purify against HOXC13 and HOXD13 to remove crossreactive antibodies. In addition to the HOX homeodomain proteins, there are numerous non-HOX homeodomain-containing proteins. We have listed a subset of these proteins that have been shown to be expressed in epidermis. Several of these, such as PBX1, 2, and 3, exhibit substantial degrees of amino acid sequence homology.

1.1.1.1. HOX Homeodomain Proteins

Items 1–7 are all available from C. Largman; largman@cgl.ucsf.edu.

1. HOXB4: rabbit anti-peptide.
2. HOXB6: rabbit anti-peptide.
3. HOXB6: rabbit anti-protein.
4. HOXA9: chicken anti-protein
5. HOXA9: rabbit anti-peptide.
6. HOXB13: rabbit anti-peptide.
7. HOXB13: rabbit anti-protein.
8. Affinity purified antibodies available from Santa Cruz Biotechnology (scbt@scbt.com): HOXA1 (N-20, C-20); HOXA2 (N-20, P-20); HOXA5 (N-20); HOXA7 (N-18, G-20); HOXA9 (N-20, A-20); HOXA10 (N-20, A-20); HOXB1 (N-20, S-20); HOXB2 (P-20); HOXB3 (S-20, C-20); HOXB4 (N-18, A-15); HOXB6 (S-20, G-20); HOXD1 (L20, N20); (*see* **Note 3**).
9. Several monoclonal and some polyclonal antibodies to HOX proteins, only a few of which are affinity purified, are available from Covance (ab.products@crpinc.com).
10. A polyclonal nonaffinity-purified antibody to a portion of the HOXA9 protein is available from Upstate Biotechnology (info@upstate.com).

1.1.1.2. Non-HOX Homeodomain Proteins

Items 1–5 are all available from C. Largman; largman@cgl.ucsf.edu.

1. MEIS1: guinea pig anti-protein.
2. MSX1: rabbit anti-peptide.
3. MSX2: rabbit anti-peptide.
4. PBX2: rabbit anti-peptide.
5. PBX2: rabbit anti-protein.
6. Affinity purified antibodies available from Santa Cruz Biotechnology (scbt@scbt.com); antibodies marked with an "x" have been successfully used with epidermal tissue PBX1 (P-20) (x); PBX2 (G-20) (x); PBX3 (D-17) (x); MSX-1 (N-20, E-20, H-85); MSX-2 (T-20, H-70, N-20); MEIS1 (N-17, C-17); MEIS2 (N-17); (*see* **Note 3**).

1.2. In Situ *Hybridization Detection of HOX Gene Transcripts*

The protocol given below describes the localization of endogenous *HOX* mRNAs using digoxigenin (DIG)-labeled RNA probes on paraffin sections. The binding of the DIG-labeled antisense RNA probes is detected with an anti-DIG antibody (Fab fragment)–horseradish peroxidase (HRP) conjugate. To detect rare *HOX* transcripts, it is necessary to use a biotinylated-tyramide amplification step, followed by secondary

amplification with streptavidin peroxidase-conjugated HRP. Signals are visualized with DAB chromogen substrate and H_2O_2, and tissues are counterstained with hematoxylin or other suitable counterstain. Endogenous peroxidase activity could result in false-positive staining; therefore, negative controls should always be included. In addition, traditionally DIG-labeled sense controls derived from transcription of the cognate strand of the template DNA are hybridized with the tissues as a second control for non-specific staining. However, new data suggest that substantial portions of the human genome are transcribed in both directions ***(4)***. This observation is consistent with the fact that we have observed that some sense *HOX* gene transcripts give specific signal patterns in the epidermis. We have used the criteria that in these situations, if a sense transcript from a different *HOX* gene, of similar length and GC composition as the gene of interest, gives negligible background, then the signal detected with the antisense probe is real. Thus for instance, a sense *HOXA5* probe was used to control for nonspecific signal when detecting *HOXA4* ***(5)***.

1.2.1. Riboprobes for In Situ *Hybridization*

Although riboprobes containing the homeobox region have been used by a number of different laboratories to probe gene expression in various tissues, we have designed probes that exclude the homeobox region to eliminate the possibility of nonspecific cross-hybridization of this very highly conserved region with non-targeted *HOX* gene transcripts. To produce antisense probes or sense control RNA fragments, cDNAs of interest must be cloned into a suitable vector, such as Bluescript (Stratagene, La Jolla, CA) or other commercially available vectors containing T3, T7, or SP6 RNA polymerase binding sites. Cloned cDNA fragments from which to produce nonhomeobox probes are marked with "x" and are available from C. Largman (largman@cgl.ucsf.edu):

HOX Gene	Human	Murine	Previously Used for *in situ* detection of epidermal expression
A1	x	x	
A2	x	x	
A3	x		
A4	x	x	x
A5	x		x
A6	x		
A7	x	x	x
A9	x	x	
A10	x	x	
A11	x		
A13	x		
B1	x		
B2		x	
B3	x		
B4	x		x
B5	x		
B6	x	x	x
B7	x	x	x
B8	x	x	
B9		x	
B13	x	x	

HOX Gene	Human	Murine	Previously Used for *in situ* detection of epidermal expression
C4	x	x	x
C5	x		
C6	x		
C7	x		
C8	x		
C9	x		
C10	x		
C11	x		
C12	x		
C13	x		
D1	x	x	
D3	x		
D4	x	x	
D8	x		
D9	x	x	
D10		x	
D13	x		

2. Materials

2.1. Immunohistochemistry

1. Antigen unmasking solution (Vector, H3300) or Target Retrieval Solution (Dako; cat. no. S1700).
2. Hydrogen peroxide (Fisher, H325-500), store at 4°C.
3. Sodium borohydride (S9125).
4. Levamisole (Sigma, L9756).
5. HRP-conjugated secondary antibody: (1) HRP-conjugated, affinity-purified donkey anti-guinea pig IgG $F(ab')_2$ fragment (Jackson Lab, cat. no. 711-035-152); (2) HRP-conjugated, affinity-purified goat anti-rabbit IgG $F(ab')_2$ fragment (Jackson Lab, cat. no. 111-036-0030); (3) other appropriate HRP-conjugated IgG $F(ab')_2$ fragments.
6. Biotinylated tyramide solution (catalyzed signal amplification kit (Dako, K1500).
7. ABC *Elite* peroxidase kit (Vector; cat. no. PK6100).
8. Color development reagents: (1) DAB (diaminobenzidine) kit (Vector, SK4100); *Note:* **Suspected carcinogen!** (2) NBT/BCIP + stop buffer (Chemicon, E-S007); (3) TSA Cyanine 3 Amplification System (Perkin–Elmer Life Sciences, NEL704A); (4) TSA Cyanine Fluorescein Amplification System (Perkin–Elmer Life Sciences, NEL701A).
9. Counterstains: (1) DAPI (Vectashield Mounting Medium with DAPI, Vector, H-1500); (2) methyl green (Vector, H-3402); (3) nuclear fast red (Vector, H-3403); (4) sytox green (Molecular Probes, S-7020); (5) hematoxylin (Fisher, CS400-4D)/Eosin (Ricca Chemicals, 2850-16).
10. Blocking buffer reagents: (1) Bovine serum albumin (BSA, Fraction V; Sigma, A7906) and (2) fish skin gelatin (Sigma, G7765).
11. "Superfrost Plus" slides (Fisher, 12-550-15).
12. PAP pen (Zymed, 00-8888).
13. Mounting medium (Vector, H-1500).
14. TTBS buffer and wash solution: 10 m*M* Tris-HCl, pH 7.6, 500 m*M* NaCl, 0.1% Tween-20 in double-distilled H_2O (ddH_2O).
15. Blocking buffer 1: 4% BSA (4 g); 0.5% fish skin gelatin (5 mL of 10% stock); to 100 mL in TTBS buffer.

16. Alternative blocking buffer: 2% normal goat serum (Jackson; cat. no. 005-000-121) or 2% normal donkey serum (Jackson; cat. no. 017-000-121) to 100 mL in blocking buffer 1.
17. Hydrogen peroxide solution: (3%, prepare fresh). Add 15 mL of stock 30% H_2O_2 (stored at 4°C, Fisher H325-500) to 150 mL ddH_2O.
18. Sodium borohydride solution: 5% $NaBH_4$ in ddH_2O.
19. ABC Elite reagent (Vector PK6100): streptavidin reagent (three drops per bottle) and biotin–peroxidase (three drops per bottle B) to 100 mL in blocking buffer or catalyzed Signal Amplification System. Peroxidase (Dako kit; cat. no. K1500).
20. Sytox green counterstain solution: Sytox green (10 n*M* final 500,000-fold dilution of 5 m*M*) stock with ddH_2O.

2.2. In Situ *Hybridization*

Note: All reagents and solutions are RNase free and are prepared using DEPC (diethylpyrocarbonate)-treated, double-distilled water in either new plasticware or glassware that has been acid washed and baked at 300°C overnight. All procedures are performed wearing gloves in a designated RNase-free area.

1. DAB (diaminobenzidine) kit (Vector, SK4100). *Note:* **Suspected carcinogen!**
2. Dextran sulfate (Sigma, D6001).
3. DEPC (Sigma, D5758).
4. Digoxigenin RNA labeling mix (Roche, 1277073).
5. Ethylenediamine tetraacetic acid (EDTA; Sigma, ED255).
6. Ficoll (Sigma, F4375).
7. Formamide (Sigma, F9037).
8. Laurel sulfate, sodium dodecyl sulfate (SDS; Sigma, L4509).
9. Polyvinylpyrrolidine (Sigma, PVP360).
10. Proteinase K (Roche, 1-413-783).
11. RNase inhibitor (Roche, 799027).
12. S4 DNA (single-stranded sonicated salmon testes DNA; Sigma, D7656).
13. SP6 RNA polymerase (Stratagene, 600151).
14. Sodium acetate (Sigma, S9513).
15. Sodium citrate (Sigma, C7254).
16. Sodium pyrophosphate (Sigma, S9515).
17. T3 RNA polymerase (Stratagene, 600111).
18. T7 RNA polymerase (Stratagene, 600123).
19. Triethanolamine (Sigma, T1377).
20. Triton X-100 (Sigma, T8787).
21. Yeast tRNA (Boehringer-Mannheim, 109495).
22. Biotinylated tyramide solution (catalyzed signal amplification kit (Dako, K1500; *see* **Note 4**).
23. ABC *Elite* peroxidase kit (Vector; cat. no. PK6100).
24. DEPC–ddH_2O: Add 0.5 mL diethylpyrocarbonate (Sigma, D5758) (0.1%) to 500 mL double-distilled water in baked glass bottles (4–6 h at 180°C); autoclave 30 min to remove excess DEPC. *Note:* Do not make Tris solutions and then treat with DEPC; make up Tris buffers in DEPC-treated and autoclaved water.
25. Tris–EDTA buffer: 100 m*M* Tris-HCl, 50 m*M* EDTA, pH 8.0, in DEPC–ddH_2O.
26. Proteinase K solution: Add 10 µL proteinase K stock (20 µg/mL) to 200 mL Tris–EDTA buffer.
27. Triethanolamine solution (0.1 *M*): Dilute 40 mL of 0.5 *M* TEA with DEPC–ddH_2O to 200 mL.
28. 100X Denhardt's: Ficoll (10 g); polyvinylpyrrolidine (10 g); BSA (10 g); to 500 mL with DEPC ddH_2O.

29. S4 DNA solution: S4 DNA (50 mg); to 5 mL with DEPC–ddH_2O (heat by microwave to dissolve DNA).
30. 20X standard saline citrate (SSC) solution: NaCl (175.3 g), Na citrate (88.2 g), 1 *N* NaOH (to pH 7.0), to 1 L with DEPC–ddH_2O.
31. Prehybridization solution (4X SSC, 40% formamide): Add 80 mL deionized formamide to 80 mL DEPC–ddH_2O, followed by 40 mL 20X SSC.
32. Hybridization solution (contains tRNA) (in order of addition): deionized formamide 2.5 mL, DEPC–ddH_2O (200 µL), 100X Denhardt's (500 µL), 10% SDS (250 µL), 1 *M* Tris, pH 7.4 (50 µL), 5% sodium pyrophosphate (500 µL), yeast tRNA (5 mg/mL; 500 µL), 20X SSC (500 µL).
33. Alternative hybridization solution (contains S4 DNA and dextran sulfate) (in order of addition): NaCl (0.58 g), DEPC–ddH_2O (0.75 mL), 1 *M* Tris-HCl (pH 7.4; 0.5 mL), 50X Denhardt's (containing 5% SDS + 0.5% sodium pyrophosphate; 2 mL), formamide (5 mL), 50% Dextran sulfate solution, (1 mL); S4 DNA solution (10 mg/mL; 0.2 mL), to 10 mL with DEPC–ddH_2O (dissolve NaCl in DEPC–ddH_2O plus Tris-HCl solution at 65°C, addition of Denhardt's solution produces a cloudy solution, addition of formamide makes clarifies the solution, add other reagents and sufficient DEPC–ddH_2O to make 10 mL of final volume).
34. RNase A solution: 200 µL RNase A (stock concentration: 20 mg/mL) is diluted in 200 mL Tris–EDTA buffer.
35. TTBS buffer: 10 m*M* Tris-HCl, pH 7.6, 500 m*M* NaCl, 0.1% Tween-20 in DEPC–ddH_2O.
36. Blocking buffer: 1% nonfat dried milk (or 4% BSA). 0.5% fish skin gelatin, 0.01% Tween-20) in DEPC–ddH_2O.
37. Alternative blocking buffer: 5% heat-inactivated normal horse serum, and 2% BSA in TTBS.
38. Anti-DIG-peroxidase antibody solution: 1:500 dilution in blocking buffer.
39. Multibiotinylated tyramide amplification reagent.
40. ABC *Elite* Peroxidase kit.
41. DAB substrate solution: dissolve one tablet of DAB in 10 mL TBS 30 min prior to use. To complete DAB substrate solution, add one drop of H_2O_2 per 2 mL DAB solution.

3. Methods

3.1. Immunohistochemical Detection of HOX Proteins

Slides holding tissue sections are carried through the entire procedure in plastic slide racks and immersed in specified solutions in appropriate glass or plastic containers (Tissue-Tek). The protocol includes the following steps: (1) tissue and section preparation; (2) antigen retrieval by microwaving; (3) elimination of endogenous enzyme activity/blocking autofluorescence; (4) immunolabeling; (5) signal amplification; and (6) localization of peroxidase activity.

3.1.1. Tissue and Section Preparation

1. Tissues are fixed with 4% paraformaldehyde in PBS for overnight at 4°C and embedded in paraffin using standard procedures.
2. Cut 5- to 7-µm-thick sections and collect the sections on "Superfrost Plus" slides. Clean microtome with absolute alcohol between blocks to avoid cross-contamination. Use ddH_2O in the flotation bath and clean the surface of the water lens paper before start of a new block. Label slides with a solvent-resistant marker (regular markers wash off during dewaxing).
3. Dry sections overnight in a 55°C incubator.
4. Microwave sections at full power in 1-min steps until the paraffin melts around the tissue (usually three cycles of 1 min).

5. Remove rack from microwave and allow to cool **(hot!)**.
6. Deparaffinize and rehydrate sections: Xylenes I > Xylenes II > 100% alcohol I > 100% alcohol II > 70% alcohol > ddH_2O, 10 min each solution (*see* **Note 5**).

3.1.2. Antigen Retrieval by Microwaving

1. Fill a microwavable plastic container with 150 mL Target Retrieval Solution or Antigen Retrieval Solution. Place slide rack into container, partially cover the container, and place in a microwave oven.
2. Fill another plastic container with water as a heat sink.
3. Microwave at full power for 5 min (one container), 7 min (two containers), or 10 min (three containers).
4. Replace evaporated solution with ddH_2O and microwave the sections for a second time.
5. Remove containers **(hot!)**, replace the evaporated water with ddH_2O; cover the container and cool 20 min.
6. Rinse slides three times with ddH_2O.

3.1.3. Elimination of Endogenous Peroxidase or Alkaline Phosphatase Activity/Blocking Autofluorescence

1. Inactivate endogenous peroxidase activity or alkaline phosphatase activity with 3% H_2O_2 or 0.05% levamisole, respectively, for 5 min.
2. Rinse slides three times with ddH_2O.
3. If pertinent, block nonspecific autofluorescence in 5% Na borohydride for 15–30 min.
4. Rinse slides three times with ddH_2O.

3.1.4. Immunolabeling

All washes are performed on a horizontal shaker at room temperature, unless otherwise noted. Primary and secondary antibody reagents are stored in small aliquots at –20°C and thawed once. Thawed antibodies are stored at 4°C and used for several weeks.

1. Immunolabeling is performed while the slides are kept in large tissue culture plates. Cover the bottom of the plates with Parafilm.
2. Draw a square around the sections with a PAP pen, and cover sections with blocking buffer, and incubate 15 min. Do not allow tissue sections to dry.
3. Incubate with primary antibody for 1–2 h at room temperature or overnight at 4°C. Dilute primary antibody in blocking buffer. Use about 200 µL of antibody/$inch^2$, estimating the amount of diluted antibody needed. The optimal dilution of the antibody should be determined in preliminary experiments. Use antibody at the highest possible dilution, i.e., the lowest possible concentration.
4. Wash with TTBS buffer two times for 5 min.

3.1.5. Signal Amplification

1. Incubate with peroxidase-conjugated second antibody for 30 min. Determine dilution experimentally, starting with a 1 : 250 dilution.
2. Wash with TTBS buffer two times for 5 min.
3. Incubate with biotinylated tyramide for 5–15 min.
4. Wash with TTBS buffer two times for 5 min.
5. Incubate with ABC peroxidase reagent, following ABC kit instructions (Vector), for 30 min.
6. Wash with TTBS buffer two times for 5 min.

3.1.6. Localization of Peroxidase Activity (Colorimetric Method)

1. Prepare substrate (DAB) buffer. Wear gloves and because DAB is light-sensitive, do not expose to bright light.
2. Apply substrate solution to the slides. Monitor reaction under microscope. Every 15–20 min replace substrate solution over sections. Signal should start to develop in 5–10 min. Do not continue staining for more than 30 min.
3. Terminate reaction by washing with ddH_2O in a slide rack 3×5 min. Sections can be left in ddH_2O overnight.

3.1.7. Localization of Peroxidase Activity (Fluorimetric Method)

1. Incubate with fluorochrome-labeled tyramide for 15 min. Fluorochrome-labeled tyramide is diluted in "Amplification Diluent," supplied with the "TSA direct" kit. Cyanine 3-labeled tyramide is diluted 1 : 100; Fluorescein-labeled tyramide is diluted 1:75 in "Amplification Diluent."
2. Wash with TTBS buffer two times for 5 min.

3.1.8. Counterstaining, Dehydration, and Mounting

1. Counterstain with hematoxylin or other suitable stain (Nuclear Fast Red, Sytox Green, or DAPI), for 5–10 min and wash in running tap water for 5 min (*see* **Note 6**).
2. Dehydrate sections: 70% alcohol > 100% alcohol I > 100% alcohol II > Xylenes I > Xylenes II > Xylenes III, 5 min each (*see* **Note 5**).
3. Cover slip sections and let the mounting media set overnight (*see* **Note 7**).

3.2. Localization of HOX mRNA by In Situ Hybridization

3.2.1. Synthesis of RNA Probes

To prepare RNA probes, it is necessary to have a cDNA or genomic fragment of the HOX gene of interest cloned into a doubled-stranded cloning vector containing appropriate viral RNA polymerase initiation sites, designed to facilitate synthesis of the requisite sense or anti-sense RNA strand. We have typically used HOX DNA fragments cloned into Bluescript vector (containing T3 and T7 promoter sites; Stratagene); pCMV (containing T3 and T7 promoter sites; Stratagene); pGEM vector (containing SP6 and T7 promoter sites; (Promega); or sp64 vector (containing SP6 and T7 promoter sites; Promega).

3.2.2. Localization of mRNA in Paraffin-Embedded Epidermis Sections

3.2.2.1. Major Steps of the Procedure

1. Tissue and section preparation.
2. Pretreatment of sections.
3. Prehybridization.
4. Probe hybridization.
5. Posthybridization washes.
6. Detection of biotin-labeled RNA/RNA hybrids.

3.2.2.2. Tissue and Section Preparation

1. Tissues are fixed with 4% paraformaldehyde and embedded in paraffin.
2. Cut 5- to 15-μm-thick sections and collect the sections on positively charged slides.

3. Clean the microtome with abs. alcohol to remove residual RNase contamination before sectioning.
4. Use DEPC–ddH_2O in the flotation bath.
5. Label slides with solvent-resistant marker because regular markers will wash off during dewaxing!
6. Dry the sections overnight in 55°C incubator.
7. Deparaffinize and rehydrate sections:
8. Histosolve I, Histosolve II, Histosolve III, 100% alcohol I, 100% alcohol II, 100% alcohol III, 90% alcohol, 70% alcohol, and 50% alcohol, for 5 min each.

3.2.2.3. Pretreatment of Sections

1. Rinse slides in DEPC–ddH_2O twice for 5 min.
2. Rinse slides in 1X PBS diluted in DEPC–ddH_2O twice for 5 min.
3. Permeabilize the sections with 0.3% Triton X-100 solution in PBS for 15 min at room temperature.
4. Remove proteins from target mRNA with proteinase K treatment in water bath at 37°C for 30 min.
5. Transfer rack(s) with slides into a fresh container and wash with DEPC–ddH_2O twice 5 min each.
6. The change of the containers is important to avoid RNase contamination from the water bath.
7. Wash sections with 0.1 *M* TEA buffer twice 5 min each.
8. Acetylate sections with acetic anhydride in 0.1 *M* TEA buffer.
9. Add 500 µL acetic anhydride to 200 mL 0.1 *M* TEA buffer while stirring for 5 min.
10. Add a second aliquot of acetic anhydride for 5 min.

3.2.2.4. Prehybridization

1. Prehybridize sections in 4X SSC, containing 40% formamide in water bath, at 37°C, for 1 h.
2. Remove rack(s) from container and dry sections at room temperature.
3. Cover the rack with paper towel to protect from dust and contamination.
4. The sections are ready for use in a few hours, or can be stored overnight, or even several days before hybridization.

3.2.2.5. Hybridization

1. Remove the slides from the rack and wipe off the remaining of the prehybridization solution.
2. Draw a square around the sections with a PAP pen and let it dry.
3. Prepare the hybridization buffer.
4. Resuspend probe stocks, then spin them down.
5. Calculate the total volume of probes needed and dilute the probes in the hybridization solution (probe stock + hybridization buffer = probe solution).
6. Vortex the probe buffer gently, and spin it.
7. Make sure to use siliconized, RNase-free tubes and sterile pipet tips.
8. Use about 100 µL probe/$inch^2$ and estimate the amount of probe solution needed.
9. Denature the probes at 68°C, in block heater for 10 min, then immediately chill them on ice.
10. Apply the probe buffer (40–100 µL) over the tissue and cover it with parafilm cover slip.
11. Clean the scissors and forceps with absolute ethanol, and keep the cover slips RNase-free!
12. Make sure to use the appropriate size cover slips.
13. Make sure that there are no air bubbles under the cover slip.
14. Add DEPC–ddH_2O to the wells (1 mL/well) of the SlideMoat incubator.
15. Put the slides into the incubator, and hybridize overnight.

16. The optimal temperature of the hybridization depends on the probe in use and should be determined in preliminary experiments. Usually the hybridization temperature is between 40°C and 55°C.

3.2.2.6. Posthybridization Washes

1. Notice that the protocol from now on does not require RNase-free reagents any more.
2. Fill a container with 2X SSC and use a horizontal slide rack.
3. Remove slides from the incubator and peel off Parafilm cover slips from the slides and transfer the slides to slide rack.
4. Low stringency wash in 2X SSC at room temperature, on a horizontal shaker, 2 × 15 min.
5. Low stringency wash in 2X SSC at 37°C, 2 × 15 min.
6. Remove nonhybridized probes with RNase A (20 µg/mL final concentration) in water bath with shaking at 37°C 30 min.
7. Following RNase A digestion, increase the temperature in the water bath to 45°C.
8. High stringency wash in 0.1X SSC, in water bath with shaking at 45°C for 2 × 30 min.

3.2.2.7. Detection of DIG-Labeled RNA/RNA Hybrids

1. Wash with TTBS on a horizontal shaker for 5 min.
2. Incubate sections with blocking buffer for 15 min.
3. Incubate sections with anti-DIG-peroxidase antibody on a horizontal shaker for 1–2 min.
4. Wash with TTBS for 3 × 5 min.
5. Incubate with Multibiotinylated Tyramide Amplification Reagent for 15 min.
6. Wash with TTBS for 2 × 5 min.
7. Incubate with streptavidin-peroxidase reagent for 15 min.
8. Wash with TTBS for 5 min.
9. Wash with TBS for 2 × 5 min.

3.2.2.8. Color Development (*see* **Note 8**)

1. With extreme care, add DAB substrate to each tissue for an accurate 5–30 min (depending on conditions) development.
2. At the end of the color development wash with ddH_2O several times.
3. Dehydrate sections quickly: (1) 70% alcohol, dip rack 20 times; (2) 100% alcohol 1, dip rack 20 times and leave for 5 min; (3) 100% alcohol 2, dip rack 20 times and leave for 5 min; (4) histosolve 1, dip rack 20 times and leave for 5 min; (5) histosolve 2, dip rack 20 times and leave for 5 min; (6) dehydration is conducted under the fume hood; and (7) cover slip sections using Shandon mounting media and let the mounting media set overnight.

4. Notes

1. There are at least three potential sources of false-positive signals obtained with the recently described procedures: (1) endogenous peroxidase activity within the tissue; (2) nonspecific autofluorescence; and (3) nonspecific binding of the ABC-peroxidase reagent to endogenous avidin-binding components (biotin, mast cells). Endogenous peroxidase can be abolished by treatment with hydrogen peroxide, while autofluorescence can be diminished by sodium borohydride treatment. In our hands, using paraffin-embedded epidermal tissue sections, nonspecific, endogenous avidin-binding was not a serious concern. Moreover, the immunohistochemical reagents were specifically formulated to suppress nonspecific interactions between the tissues and reagents. For this purpose, we used a tris-buffer (pH 7.6) slightly more alkaline than usual, higher ionic strength (500 m*M* NaCl), and 0.1% Tween-20. Moreover a set of controls were always used to

ensure the specificity of the immunoreactions, including (1) isotype-matched control IgG in lieu of the first antibody; (2) omission of the first antibody from the detection sequence; and (3) preimmune serum, diluted to contain identical IgG concentration to the first antibody. In addition, because tyramide-mediated signal enhancement results in an enormous increase in sensitivity, the optimal dilution of the primary antibody becomes a critical step in successful discrimination of HOX protein associated signals from tyramide enhanced background color development. Finally, signal detection with digital or confocal microscopy requires a careful selection of detection conditions (gain and contrast adjustments, digital image modifications, and so on) in order to correctly represent signal localization.

2. As described in **Subheading 1.1.2.**, the 39 HOX proteins share variable degrees of conserved amino acid sequence homology. This can be a danger when attempting to assess expression of a particular HOX protein. However, most HOX proteins show very high levels of homology between murine and human sequences. This fact can often allow the investigator to use a single antibody to detect HOX protein expression in both human and murine epidermis. Most commercially available antibodies to HOX and non-HOX proteins have been designed to minimize the degree of crossreactivity between related HOX proteins and to maximize cross-reactivity for human and murine proteins. However, careful examination of amino acid sequence homology is recommended to ensure reliable, specific identification of target proteins.
3. In our hands some of the antibodies against HOX proteins from Santa Cruz work well for immunohistochemistry, whereas others do not. This is not unanticipated for antisera directed against peptide antigens that may be inaccessible in the tissue. All of the antisera to PBX proteins from this source appear to work for immunohistochemical localization.
4. Although in our published studies we used tyramide-amplification reagents manufactured by DAKO and NEN (now Perkin–Elmer), recently Molecular Probes has begun offering a wide range of fluorochrome-labeled tyramide reagents. We have been using these novel-labeled tyramide reagents with great success, even in double- and triple-labeling studies.
5. Deparaffinization and rehydration are performed in a chemical hood. Dip rack holding slides 20 times and leave immersed for 10 min in each solution. Use reagent grade alcohol for rehydration.
6. Weak nuclear signals can be obscured by Hematoxylin staining. Therefore it may be preferable to either stain very lightly, omit counterstaining, or use an alternative stain. Be certain to use an appropriate nuclear stain. Do not use Sytox Green with FITC or other green fluorochromes.
7. It is convenient to introduce DAPI nuclear stain in the mounting medium.
8. Alternatives to peroxidase-based detection systems. Note that other enzymatic activities, such as alkaline phosphatase; and alternative substrates, such as NBT-BCIP, can be used at the final color localization step (*see* **Subheading 3.2.2.8.**).

References

1. Kömüves, L. G., Shen, W.-F., Kwong, A., Stelnicki, E., Rozenfeld, S., Oda, Y., et al. (2001) Changes in HOXB6 homeodomain protein structure and localization during human epidermal development and differentiation. *Dev. Dyn.* **218,** 636–647.
2. Kömüves, L. G., Michael, E., Arbeit, J. M., Ma, X-K., Kwong, A., Stelnicki, E., et al. (2002) The HOXB4 homeodomain protein is expressed in developing epidermis and skin disorders and modulates keratinocyte proliferation. *Dev. Dyn.* **224,** 58–68.
3. Kömüves, L. G., Ma, X. K., Stelnicki E., Rozenfeld, S., Oda, Y., and Largman, C. (2003) The HOXB13 homeodomain protein is cytoplasmic throughout fetal skin development. *Dev Dyn.* **227,** 192–202.

4 Rinn, J. L., Euskirchen, G., Bertone, P., Martone, R., Luscombe, N. M., Hartman, S., et al. (2003) The transcriptional activity of human chromosome 22. *Genes Dev.* **17,** 529–540.
5. Stelnicki, E. J., Kömüves, L., Kwong, A. O., Holmes, D., Klein, P., Rozenfeld, S., et al. (1998) HOX homeobox genes exhibit spatial and temporal changes in expression during human skin development. *J. Invest. Dermatol.* **110,** 110–115.

19

Apoptosis in the Epidermis

Kiyofumi Yamanishi, Chun-Shen Shen, and Hitoshi Mizutani

Summary

Keratinization is a specialized form of apoptosis that produces the stratum corneum concomitant with keratinocyte cell death. Apoptosis of keratinocytes occurs not only during normal keratinization but also in response to various intracellular or extracellular death stimuli, such as genetic defects or UVB. In general, apoptosis is characterized by chromatin condensation, membrane blebbing, cell shrinkage, formation of apoptotic bodies, and deoxyribonucleic acid fragmentation. The nick-end labeling technique is a method to detect DNA fragmentation and is useful to assess the presence of apoptotic cells in tissues or cells. In this chapter, a practical procedure for nick-end labeling using paraffin-embedded sections of skin specimens is described.

Key Words:

Apoptosis; keratinocytes; epidermis; nick-end labeling; TUNEL; keratinization.

1. Introduction

The terminal differentiation of epidermal keratinocytes is a specialized form of apoptosis. In the final stage of the process, keratinocytes die and become cornified cells. The stratum corneum is composed of cornified cells and serves as a barrier against physical, chemical, and biological invasion. During normal epidermal keratinization, keratinocyte nuclei seem to suddenly disappear in the transition from the granular layer to the stratum corneum. Apoptosis is characterized by chromatin condensation, membrane blebbing, cell shrinkage, the formation of apoptotic bodies, and deoxyribonucleic acid (DNA) fragmentation (reviewed in **ref.** ***1***). DNA fragmentation has been only rarely detected in the transition from the stratum granulosum to the stratum corneum ***(2,3)***. Apoptosis of keratinocytes occurs not only during normal keratinization but also in response to various intracellular or extracellular death stimuli, such as genetic defects ***(4)*** or UVB ***(5)***. To examine DNA fragmentation in tissue sections, the nick-end labeling technique is useful for paraffin-embedded pathological sections. This chapter describes a standard method that is routinely used in our laboratory to examine DNA fragmentation in the epidermis ***(6)***. The nick-end labeling method includes three steps: (1) proteinase digestion to increase the accessibility of substrates and terminal deoxynucleotidyl transferase (TdT) enzymes; (2) TdT-catalyzed labeling of fragmented DNA termini with biotin-16-dUTP; and (3) detection of incorporated biotin-16-dUMP with peroxidase reaction.

From: *Methods in Molecular Biology, vol. 289:*
Epidermal Cells: Methods and Protocols
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

2. Materials

2.1. Chemicals

1. Tris(hydroxymethyl)aminomethane (Tris; Wako Pure Chemical Industries, Ltd., Osaka, Japan; cat. no. 204-07885).
2. Sodium cacodylate trihydrate (Wako Pure Chemical Industries, Ltd., Osaka, Japan; cat. no. 194-04852).
3. Dithiothreitol (Wako Pure Chemical Industries, Ltd., Osaka, Japan; cat. no. 041-08971).
4. Cobalt chloride hexahydrate ($CoCl_2$•$6H_2O$) (Wako Pure Chemical Industries, Ltd., Osaka, Japan; cat. no. 036-03682).
5. Polyoxyethylene (20) sorbitan monolaurate (Tween-20) (Wako Pure Chemical Industries, Ltd., Osaka, Japan; cat. no. 163-11512).
6. Bovine serum albumin (BSA; Sigma Chemicals Co., St Louis, MO; cat. no. A-7888).
7. Hydrogen peroxide solution (30%) (H_2O_2 solution; Wako Pure Chemical Industries, Ltd., Osaka, Japan; cat. no. 086-07445).
8. Proteinase K (Roche Diagnostics GmbH, Mannheim, Germany; cat. no. 1413783).
9. Terminal deoxynucleotidyl transferase (TdT; TaKaRa Bio Inc, Shiga, Japan; cat. no. 2230A).
10. Biotin-16-dUTP: 1 *M* biotin-16-dUTP (Roche Diagnostics GmbH, Mannheim, Germany; cat. no. 1093070).
11. Extravidine®-Peroxidase (Sigma Chemicals Co., St. Louis, MO; cat. no. E2886).
12. 3,3′Diaminobezidine tetrahydrochloride tablet (DAB•4 HCl 10 mg/tablet) (Wako Pure Chemical Industries, Ltd., Osaka, Japan; cat. no. 049-22831).
13. Hematoxylin solution: Mayer's hemalum solution (Merck, Darmstadt, Germany; cat. no. 109249).
14. Crystal/mount (Biomeda, Foster City, CA; cat. no. MO2).

2.2. Reagents

1. DN buffer: 30 m*M* Tris-HCl, pH 7.2, 140 m*M* sodium cacodylate, 4 m*M* $MgCl_2$, 0.1 m*M* dithiothreitol (*see* **Note 1**). The solution is stable at least for 1 mo at –20°C.
2. TdT buffer: 30 m*M* Tris-HCl, pH 7.2, 140 m*M* sodium cacodylate, 1 m*M* $CoCl_2$. The solution is stable at least for a few months at –20°C.
3. TB buffer: 300 m*M* NaCl, 30 m*M* sodium citrate. After autoclaving, the solution is stable for a few months at room temperature.
4. Proteinase K Solution: dilute proteinase K to 20 µg/mL with 20% glycerol. Store the solution at –20°C.
5. TdT reaction mixture: 0.1 U/µL TdT, 10 µ*M* biotin-16-dUTP in TdT buffer. Dilute 1 m*M* biotin-16-dUTP to 10 µ*M* and TdT to 0.1 U/µL with TdT buffer. Prepare just before use.
6. Phosphate-buffered saline (PBS)–Tween-20 solution: 0.05% Tween-20 in sterile Ca^{2+}-, Mg^{2+}-free PBS. Store the solution at room temperature.
7. BSA solution: prepare 2% BSA in sterile PBS. Prepare just before use.
8. ExtrAvidine-peroxidase solution: Dilute Extravidine®-Peroxidase 1:500 with dH_2O. Prepare just before use.
9. DAB solution: add one DAB tablet to 50 mL of 0.05 *M* Tris-HCl, pH 7.6, and dissolve well, then add 10 µL of 30% H_2O_2. Prepare just before use.

2.3. Other Materials

1. APS-coated glass slides: Superfrost® APS-coated (Matsunami Glass, Ltd., Kishiwada, Japan; cat. no. S8441).
2. PAP Pen: DAKO Pen (DakoCytomation, Glostrup, Denmark; cat. no. S2002).
3. Incubation chamber (Cosmo Bio Co., Ltd. Tokyo, Japan; cat. no. 20CG).

3. Methods

3.1. Preparation of Tissues

Fix tissues in 4% buffered formaldehyde overnight and embedd in paraffin. Mount 4- to 6-µm-thick sections on APS-coated glass slides.

3.2. Deparaffinization

1. Xylene, 3 × 5 min.
2. Acetone, 5 min.
3. Surround sections with a PAP Pen, and air-dry.
4. Rehydrate slides in a graded ethanol series: 100% ethanol, 3 min; 90% ethanol, 3 min; 80% ethanol, 3 min; and 70% ethanol, 3 min.
5. Rinse sections with dH_2O, 3 × 5 min.

3.3. TdT-Mediated dUTP-Biotin Nick-End Labeling (TUNEL) Reaction

1. Treat slides with 20 µg/mL proteinase K solution for 15 min.
2. Rinse with dH_2O, 3 × 5 min.
3. Immerse slides in 2% H_2O_2 for 5 min.
4. Rinse with dH_2O, 3 × 5 min *see* **Note 2**).
5. Immerse slides in TdT buffer for 5 min.
6. Add TdT reaction mixture to slides and incubate at 37°C for 60 min in a humidified sealed box (*see* **Notes 3** and **4**).
7. Stop the reaction by immersing the slides in TB buffer for 5 min.
8. Rinse with dH_2O, 3 × 5 min.
9. Immerse slides in PBS–Tween-20 solution for 5 min.
10. Add 100 µL BSA solution to each slide and incubate for 10 min.
11. Rinse with dH_2O, 3 × 5 min.
12. Immerse slides in PBS for 5 min.
13. Add 100 µL extravidine-peroxidase solution to each section and incubate them at 37°C for 30 min.
14. Rinse with dH_2O, 3 × 5 min.
15. Immerse slides in PBS for 5 min.

3.4. Color Development and Mounting

1. Add DAB solution to slides and observe color development with a microscope (*see* **Note 5**).
2. When appropriate color development has been achieved, rinse slides in tap water and immerse them in dH_2O for 1 min.
3. Stain slides with hematoxylin solution for 2 s.
4. Rinse slides in tap water and immerse them in dH_2O for 1 min.
5. Mount slides using an appropriate amount of crystal/mount (*see* **Note 6**). Allow the sections to sit overnight at room temperature.
6. Examine staining by light microscopy **(Fig. 1)**.

4. Notes

1. Sodium cacodylate is highly toxic, and appropriate handling and disposal are necessary for safety.
2. A PBS-immersed filter paper is laid on the bottom of the incubation chamber.
3. For a positive control, treat the DNA with DNase between **Subheading 3.3., steps 4** and **5**, as follows: Immerse the slides in DN buffer for 5 min. Treat sections with DNase I solution (5000 U/mL in DN buffer) for 10 min. Rinse with dH_2O, 3 × 5 min.

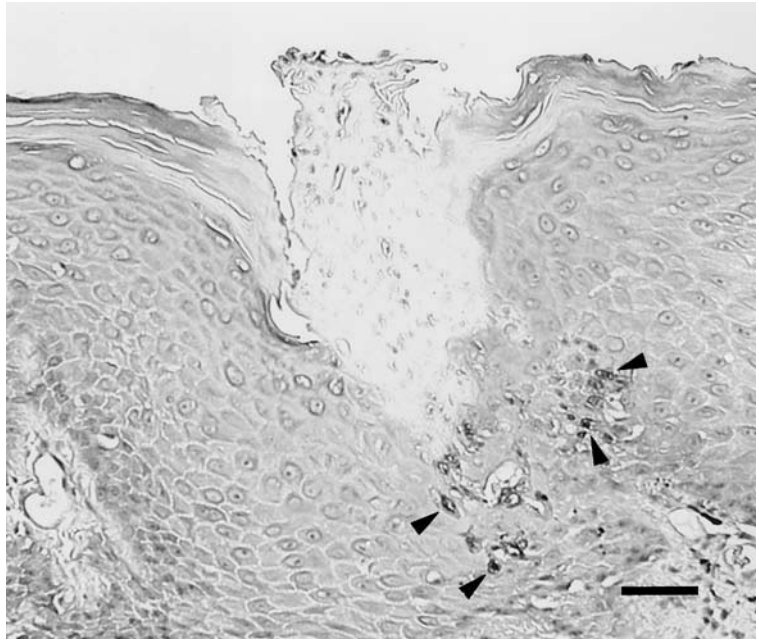


Fig. 1. Detection of apoptosis in the epidermis by TUNEL. Arrowheads indicate TUNEL-positive, apoptotic epidermal cells in porokeratosis. Bar, 50 µm.

4. For a negative control, the TUNEL reaction without the TdT enzyme should be performed in parallel.
5. Nuclei of old paraffin-embedded tissues may be nonspecifically positive for TUNEL staining, because of spontaneous nicking in the DNA. In such cases, all nuclei may be positive.
6. To avoid remaining of air bubbles around tissue sections, don't use a cover glass with crystal/mount.

References

1. Kerr, J. F. R. and Harmon, B. V. (1991) Definition and incidence of apoptosis: an historical perspective, in *Apoptosis: The Molecular Basis of Cell Death* (Tomei, L. D. and Cope, F. O., eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 5–29.
2 McCall, C. A. and Cohen, J. J. (1991) Programmed cell death in terminally differentiating keratinocytes: role of endogenous endonuclease. *J. Invest. Dermatol.* **97,** 111–114.
3 Gandarillas, A., Goldsmith, L. A., Gschmeissner, S., Leigh, I. M., and Watt, F. M. (1999) Evidence that apoptosis and terminal differentiation of epidermal keratinocytes are distinct processes. *Exp. Dermatol.* **8,** 71–79.
4 Bongiorno, M. R. and Arico, M. (2002) The behaviour of Bcl-2, Bax and Bcl-x in Darier's disease. *Br. J. Dermatol.* **147,** 696–700.
5 Aragane, Y., Kulms, D., Metze, D., Wilkes, G., Poppelmann, B., Luger, T. A., et al. (1998) Ultraviolet light induces apoptosis via direct activation of CD95 (Fas/APO-1) independently of its ligand CD95L. *J. Cell. Biol.* **140,** 171–182.
6. Shen, C. S., Tabata, K., Matsuki, M., Goto, T., Yokochi, T., and Yamanishi, K. (2002) Premature apoptosis of keratinocytes and the dysregulation of keratinization in porokeratosis. *Br. J. Dermatol.* **147,** 498–502.

20

Fate of Desmosomal Proteins in Apoptotic Epidermal Cells

Jörg Weiske and Otmar Huber

Summary

In epidermal cells desmosomes represent major sites of basolateral cell-cell contacts that play an important role for epidermal homeostasis. Excess or damaged cells are often removed by apoptosis. To release apoptotic cells from the tissue, intercellular contacts have to be broken. Here detailed protocols to analyze desmosomal proteins in apoptotic cells are described.

Key Words:

Apoptosis; desmoglein; desmocollin; desmoplakin; plakophilin; intermediate filament.

1. Introduction

Apoptosis is a highly conserved biological process that is required for the destruction of excess and damaged cells during development and in the homeostasis of multicellular organisms. Inappropriate or impaired apoptosis has been implicated in the development of many human diseases, including cancer ***(1–3)***. Many observations point to an important role of apoptosis for epidermal biology ***(4)***.

Apoptotic cell death induces dramatic molecular changes in cell morphology, such as membrane blebbing, cytoplasmic/nuclear condensation, and loss of cell-cell contacts. Most of the morphological changes are caused by a set of cysteine proteases that are activated in apoptotic cells. These proteases are members of a large protein family known as the caspases. Caspases are highly conserved through evolution and specifically cleave substrate proteins C-terminal to aspartate residues ***(5)***. Morphological changes observed during apoptosis in part result from effects on cell-cell contacts. In this respect, components of the cadherin–catenin adhesion complex in epithelial adherens junctions have been shown to be targeted by caspases ***(6,7)***. Desmosomes represent a second type of cadherin-mediated cell-cell contacts especially prominent in epidermal cells that are anchored to the intermediate filament system. The molecular pathology of human skin disorders and the characterization of animal models in which desmosomal components have been genetically altered revealed the critical role of desmosomes for tissue formation and maintenance. Desmosomes are composed of multiple components that form a complex network of proteins belonging to three families, the desmosomal cadherins (desmocollin 1–3 and desmogleins 1–4), the Armadillo

From: *Methods in Molecular Biology, vol. 289:*
Epidermal Cells: Methods and Protocols
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

repeat proteins (plakoglobin and plakophilins 1–3), and the plakin protein family members (desmoplakin, periplakin, envoplakin, and plectin). The molecular structure of the desmosomal–protein complex is summarized in recent comprehensive reviews ***(8–11)***. In this chapter, detailed protocols to analyze the fate of desmosomal proteins in apoptotic epidermal cells will be described.

2. Materials

2.1. Cells

HaCat; a spontaneously immortalized, nontumorigenic keratinocyte cell line (request from Dr. Norbert Fusenig: n.fusenig@dkfz-heidleberg.de) (*see* **Note 1**).

2.2. Cell Culture Plastic and Glassware

1. 100 × 20-mm tissue culture dishes (Falcon/Becton-Dickinson; cat. no. 3003).
2. 35-mm tissue culture dishes (Falcon/Becton-Dickinson; cat. no. 3001).
3. Multiwell™ six-well tissue culture plates (Falcon/Becton-Dickinson; cat. no. 3046).
4. Nunc Cryo Tube™ vials (Nunc; cat. no. 377267).
5. Serological pipets (Falcon/Becton-Dickinson).
6. 15-mL tubes (Nunc; cat. no. 366079).
7. 50-mL tubes (Nunc; cat. no. 373687).
8. Cell lifter (Costar®; cat. no. 3008).
9. Improved Neubauer chamber (VWR international, cat no. 631F1110).
10. Nicool LM10 freezing machine (VWR international, cat no. 478C3003); (*see* **Note 2**).

2.3. Media and Reagents

1. Dulbecco's phosphate-buffered saline (PBS) with Ca^{2+} and Mg^{2+} (PBS+; PAA Laboratories GmbH; cat. no. H15-001 or GibcoBRL; cat. no. 14040-091).
2. Dulbecco's PBS without Ca^{2+} and Mg^{2+} (PBS–; PAA Laboratories GmbH; cat. no. H15-002 or GibcoBRL; cat. no. 14190-094).
3. Dulbecco's modified Eagle's medium (PAA Laboratories GmbH; cat. no. E15-810 or GibcoBRL; cat. no. 31966).
4. Penicillin-streptomycin (GibcoBRL; cat. no. 15140-122).
5. Dimethyl sulfoxide (DMSO) Hybri Max® (Sigma; cat. no. D2650).
6. Fetal calf serum (Biochrom AG Seromed®; cat. no. S0115) (*see* **Note 3**).
7. Trypsin/ethylene diamine tetraacetic acid (EDTA) (0.25% [w/v] trypsin, 1 m*M* EDTA) (GibcoBRL; cat. no. 25200-056).

2.4. Antibodies

1. Anti-desmoglein-3 (clone 62), directed against the desmoglein-3 cytoplasmic domain (BD Transduction Laboratories; cat. no. D28120).
2. Anti-desmoglein-3 (clone 5H10), directed against the desmoglein-3 extracellular domain (request from Dr. Masayuki Amagai; amagai@sc.itc.keio.ac.jp).
3. Anti-desmocollin-3 (clone Dsc3-U114; Progen; cat. no. 65193).
4. Anti-desmoplakin 1 and 2 (clone 2.15; Progen; cat. no. 61003).
5. Anti-plakophilin-1 (clone 670), directed against the Arm-repeat domain (request from Dr. Mechthild Hatzfeld; mechthild.hatzfeld@medizin.uni-halle.de).
6. Anti-cytokeratin pan (Progen; cat. no. 10550).
7. Peroxidase-conjugated AffiniPure $F(ab')_2$ fragment goat anti-mouse IgG (H+L) (Dianova; cat. no. 115-036-062).

8. Peroxidase-conjugated AffiniPure F(ab′)$_2$ fragment goat anti-rabbit IgG (H+L) (Dianova; cat. no. 111-036-045).
9. Alexa Fluor® 594 goat anti-rabbit IgG (H+L) conjugate (Molecular Probes; cat. no. A-11012).
10. Alexa Fluor® 488 goat anti-mouse IgG (H+L) conjugate (Molecular Probes; cat. no. A-11029).
11. Anti-poly-(ADP-ribose)-polymerase (PARP) (Roche Applied Science; cat. no. 1835238).
12. M-30 cyto-death (Roche Applied Science; cat. no. 2140322).

2.5. Induction of Apoptosis

1. Staurosporine (Sigma; cat. no. S5921); stock solution: 0.5 m*M* in dimethyl sulfoxide (DMSO), stable at –20°C for up to 2 yr.
2. Camptothecin (Sigma; cat. no. C9911); stock solution: 2 mg/mL in DMSO, stable at –20°C for up to 2 yr.
3. Tumor necrosis factor-α (Biomol; cat. no. 50435); stock solution: 10 µg/mL in PBS with 5% bovine serum albumin, stable at –20°C for up to 2 yr.
4. Anti-CD95 antibody (clone CH-11) (Immunotech; cat. no. 1504); stock solution: 500 µg/mL in PBS/glycerol, The purified liquid form should be stored at –20°C until the expiration date stated on the vial label.
5. Caspase-3-inhibitor-2 (Z-DEVD-FMK; Calbiochem®; cat. no. 264155); stock solution: 50 m*M* in DMSO. Aliquot and store at –20°C for up to 1 yr.
6. Matrix metalloproteinase-inhibitor-1 (MMP inhibitor I) (Calbiochem®; cat. no. 444250); stock solution: 100 m*M* in DMSO. Aliquot and store at –20°C for up to 1 yr.

2.6. Solubilization of Desmosomal Proteins

1. BCA protein assay system (Pierce; cat. no. 23225).
2. Urea (VWR international; cat. no. 108488).
3. Complete™-EDTA protease inhibitor mixture (Roche Applied Science; cat. no. 1697498).
4. Lysis buffer: 10 m*M* imidazole, pH 6.8; 100 m*M* KCl, 300 m*M* sucrose, 2 m*M* $MgCl_2$, 10 m*M* EGTA, 1 m*M* NaF, 1 m*M* Na_2MoO_4, 1 m*M* $NaVO_3$, 0.2% (v/v) Triton X-100, Complete™-EDTA protease inhibitor mixture. Store at 4°C and use within 1 wk.

2.7. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

1. Rotiphorese® gel 30, 30% (w/v) acrylamide/0.8% (w/v) bisacrylamide (Roth; cat. no. 3029.1).
2. Ammoniumperoxodisulfate (Roth; cat. no. 9592.3).
3. TEMED (Roth; cat. no. 2367.3).
4. 1.5 *M* Tris-HCl, pH 8.8.
5. 0.5 *M* Tris-HCl, pH 6.8.
6. 10% (w/v) SDS.
7. 4X SDS sample buffer: 0.1 *M* Tris-HCl, pH 6.8; 8% (w/v) SDS, 40% (w/v) glycerol, 20% (w/v) 2-mercaptoethanol, and 0.4 mg/mL Bromophenol blue (Merck; cat. no. 111746). Aliquot and store at –20°C. Working solution may be stored for several months at room temperature (*see* **Note 4**).
8. Prestained™ Benchmark Protein Ladder (e.g., GibcoBRL; cat. no. 10748-010).
9. 2-Propanol.
10. Electrophoresis buffer: 24.8 m*M* Tris, 192 m*M* glycine, 0.1% (w/v) SDS; store at room temperature.
11. Electrophoresis apparatus (e.g., C.B.S. Dual Vertical Mini Gel), 1.5-mm spacers, glass plates and combs.
12. Constant current power supply (e.g., Biometra Power Pac P25; Bio-Rad Power Pac 200).

2.8. Semidry Electroblotting

1. PolyScreen® polyvinylidene difluoride (PVDF) transfer membrane (Perkin–Elmer Life Sciences; cat. no. NEF1002).
2. Whatman 3 MM paper (Whatman® International Ltd.; cat. no. 1003-917).
3. Anode buffer 1: 300 m*M* Tris-HCl, pH 9.4; store at room temperature.
4. Anode buffer 2: 30 m*M* Tris-HCl, pH 9.4; store at room temperature.
5. Cathode buffer: 25 m*M* Tris-HCl, pH 9.6, 40 m*M* ε-amino-*N*-caproic acid, 0.1% (w/v) SDS; store at room temperature.
6. Semidry-transfer blotting apparatus (e.g., Bio-Rad Trans Blot SD Semidry-Transfer Cell; cat. no. 170-3940).
7. Constant current power supply (*see* **Subheading 2.6.**).

2.9. Immunodetection

1. TST buffer: 10 m*M* Tris-HCl, pH 7.5; 150 m*M* NaCl, 0.1% (v/v) Tween-20; store at room temperature.
2. Lumi-Light Western blotting substrate (Roche Applied Science; cat. no. 2015200).
3. Biomax MR films (Eastman Kodak Company; cat. no. 8941114).

2.10. Immunoprecipitation

1. Protein A Sepharose® CL-4B (Amersham Biosciences; cat. no. 17-0963-03). 1 : 1 slurry in CSK buffer, 0.2% (w/v) bovine serum albumin; store at 4°C.
2. CSK buffer: 10 m*M* imidazole, pH 6.8; 50 m*M* NaCl; 300 m*M* sucrose; 3 m*M* $MgCl_2$; and 0.5% (v/v) Triton X-100; store at 4°C.
3. 2X SDS sample buffer: 65 m*M* Tris-HCl, pH 6.8; 3% (w/v) SDS; 30% (w/v) glycerol; 5% (v/v) 2-mercaptoethanol; and 0.4 mg/mL Bromophenol blue. Aliquot and store at –20°C. Working solution may be stored for several months at room temperature (*see* **Note 4**).

2.11. Immunofluorescence Microscopy

1. 0.1% (w/v) gelatin (Sigma; cat. no. G2500) in PBS–; autoclave solution and store at room temperature.
2. Methanol; store at –20°C.
3. Cover slides 18 × 18 or 22 × 22 mm; before use, autoclave cover slides or immerse in ethanol and sterilize in a flame.
4. Microscope slides (76 × 26 mm).
5. 3.7 % (w/v) paraformaldeyde (Merck; cat. no. 818715). Heat 3.7 g paraformaldehyde in about 80 mL of PBS– to 80°C under constant stirring until it is completely disolved. Add 10 µL 1 *M* $CaCl_2$ and 10 µL 1 *M* $MgCl_2$. Adjust to pH 7.4 and top volume to 100 mL with PBS–. Aliquot and store at –20°C for up to 6 mo. Use new aliquots for every experiment (*see* **Note 5**).
6. Block solution: PBS+, 0.1% (v/v) goat serum.
7. PBS+/glycine: PBS+, 25 m*M* glycine.
8. ProTaqs Mount Fluor (BIOCYC; cat. no. 401603095).
9. Goat serum (Sigma; cat. no. G6767).
10. Axioplan fluorescence microscope (Zeiss).
11. Confocal microscope LSM510 (Zeiss).

2.12. Recombinant Expression and Purification of GST-Tagged Cytoplasmatic Domain of Desmoglein-3

1. *Escherichia coli* XL1-blue competent cells.
2. pGEX4T-1-hDsg3$_{cyto}$; prokaryotic expression vector encoding a GST-human desmoglein-3 cytoplasmatic domain fusion protein.
3. Water bath.
4. Isopropyl-β-D-thiogalactopyranoside (IPTG) (AppliChem; cat. no. A1008.0025); stock solution: 1 *M* IPTG in H_2O, store at –20°C after sterile filtration.
5. Sonicator (e.g., Sonifier Cell Disrupter B12, Branson Power Company Danbury).
6. Vertical electrophoresis apparatus (e.g., SE600 Series, Hoefer), 1.5-mm spacers, and combs.
7. Visking dialysis membrane, cut off 14 kDa (Roth; cat. no. 1780.1).
8. Coomassie® Brilliant Blue R250 (Merck; cat. no. 102085).
9. Glutathione-agarose (Sigma; cat. no. G4510).
10. Glutathione, reduced form (Sigma; cat. no. G6013).
11. Purified, active recombinant human caspase-3 (BD PharMingen; cat. no. 66281V).
12. Acrylamide (Roth; cat. no. 7906.2).
13. *N*, *N*′-methylene-bis-acrylamide (bisacrylamide) (Roth; cat. no. 7867.1).
14. Blotting membranes Fluorotrans (PVDF membrane; 0.2 µm; Fluka; cat. no. 15290).
15. Whatman 3MM paper (Whatman® International Ltd.; cat. no. 1003-917).
16. Semidry-transfer blot (*see* **Subheading 2.7.**).
17. Constant current power supply (*see* **Subheading 2.6.**).
18. TEMED (Roth; cat. no. 2367.3).
19. Poly-Prep® chromatography columns (Bio-Rad Laboratories; cat. no. 731-1550).
20. 1.5 *M* Tris-HCl, pH 8.8.
21. 0.5 *M* Tris-HCl, pH 6.8.
22. 10% (w/v) SDS.
23. 4X SDS sample buffer: 0.1 *M* Tris-HCl, pH 6.8, 8% (w/v) SDS, 40% (w/v) glycerol, 20% (w/v) 2-mercaptoethanol, and 0.4 mg/mL Bromophenol blue. Aliquot and store at –20°C. Working solution may be stored for several months at room temperature (*see* **Note 4**).
24. Prestained™ Benchmark Protein Ladder (e.g., GibcoBRL; cat. no. 10748-010).
25. 2-Propanol.
26. Electrophoresis buffer: 24.8 m*M* Tris, 192 m*M* glycine, 0.1% (w/v) SDS; store at room temperature.
27. Blotting buffers for subsequent Edman sequencing (prepare fresh prior to use):
    Anode buffer: 50 m*M* boric acid/NaOH-buffer, pH 9.0.
    Cathode buffer: 50 m*M* boric acid/NaOH-buffer pH 9.0, 0.1% (w/v) SDS, 10% (v/v) methanol.
    PVDF buffer: 50 m*M* boric acid/NaOH-buffer, pH 9.0, 10% (v/v) methanol.
28. 2X Caspase-buffer: 40 m*M* Pipes, pH 7.2, 200 m*M* NaCl, 20 m*M* dithiothreitol, 2 m*M* EDTA, 0.2% (w/v) CHAPS, 20% (w/v) sucrose, store at 4°C.
29. BenchMark™ Protein Ladder (e.g., Gibco-BRL; cat. no. 10747-012).
30. LB-agar: LB-medium: 1% (w/v) Bacto-tryptone, 5% (w/v) Bacto-yeast extract, 86 m*M* NaCl, pH 7.5.
31. LB–Amp plate: 1.5% (w/v) agar in LB medium, 50 µg/mL ampicillin.
32. Lysis buffer: PBS+, Complete™-EDTA protease inhibitor, prepare fresh prior to use.
33. Wash buffer: PBS+, 0.5% (v/v) Triton X-100, prepare fresh prior to use.
34. Elution buffer: PBS+, 20 m*M* glutathione, prepare fresh prior to use.

3. Methods

3.1. Cell Culture

HaCat cells were cultivated in DMEM complemented with 10% (v/v) fetal bovine serum in the presence of 100 U/mL penicillin and 100 µg/mL streptomycin.

3.1.1. Thawing of Cells

1. Remove cells from liquid nitrogen store and quickly thaw them in a 37°C water bath.
2. Transfer cell suspension into a sterile 50-mL conical tube containing 20 mL of prewarmed cell culture medium.
3. Spin cells at 310*g* for 5 min at room temperature. Remove medium and gently resuspend the cell pellet in 10 mL medium.
4. Plate cell suspension into a 100-mm cell culture dish and incubate at 37°C in a 5% CO_2 atmosphere. Change medium every day until cells are confluent.

3.1.2. Passage of HaCat Cells

1. Remove medium and wash cells twice with 10 mL prewarmed PBS–.
2. Add 5 mL trypsin–EDTA solution to the culture dish.
3. Incubate at 37°C for 5–10 min and monitor the detachment of cells under the microscope.
4. To generate a single-cell suspension remaining cell aggregates can be broken by gentle pipetting up and down. Wash cells as described in **Subheading 3.1.1.**
5. Count the cells in an improved Neubauer chamber and add 0.5–1 × 10^6 cells to 10 mL culture medium and plate on a 100-mm culture dish. Change the medium every 24 h.

3.1.3. Cell Freezing

1. Prepare freezing medium: 10% (v/v) DMSO, 20% (v/v) fetal calf serum, 70% (v/v) cell culture medium.
2. Prepare single cell suspension as described in **Subheading 3.1.2.**
3. Pellet all cells of a 100-mm culture dish by centrifugation at 310*g* for 5 min at room temperature. Gently resuspend cells in 1 mL freezing medium and transfer to a cryo vial.
4. Freeze cells in a Nicool LM10 freezing machine (30 min, level 3; 10 min, level 10).
5. Immediately transfer cryovials to a liquid nitrogen tank for storage.

3.2. Western Blot Analysis of Desmosomal Proteins in Apoptotic Epidermal Cells

This section describes the induction of apoptosis in HaCat cells and the analysis of apoptotic cleavage products of desmosomal proteins by Western blotting.

3.2.1. Induction of Apoptosis

1. Plate 1 × 10^6 cells into 35-mm cell culture dishes and cultivate for 20 h.
2. Remove medium and wash cells with 10 mL prewarmed PBS+.
3. Induce apoptosis by addition of 2 mL fresh medium supplemented with 4 µL staurosporine or 2 µL camptothecin (*see* **Note 6**).
4. For inhibitor studies, preincubate the cells, for example, with 2 mL of medium supplemented with 50 µ*M* caspase inhibitor Z-DEVD-FMK and/or 100 µ*M* matrix metalloproteinase inhibitor MMPI-1 for 30 min before induction of apoptosis by addition 4 µL staurosporine or 2 µL camptothecin.
5. Add DMSO alone as control, the final DMSO concentration must not exceed 0.5% (v/v).

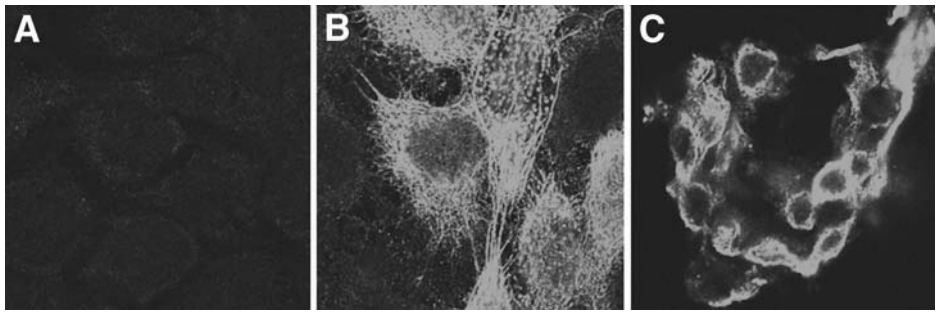


Fig. 1. Staining of HaCat cell with the anti-M30 cyto-death antibody detecting apoptotic keratin 18 cleavage fragments. In confocal images at time point 0 h **(A)** no staining is detectable, whereas 3 h **(B)** and 6 h **(C)** after induction of apopotsis with staurosporine, a dramatic increase in keratin 18 cleavage fragments is detectable concomitant with a change in cell morphology.

6. Successful induction of apoptosis can be proved by Western blotting of cell lysates with anti-PARP antibodies (*see* **Subheadings 3.2.2.–3.2.5.** and **Note 7**) or immunofluorescence analysis with the anti-M30 cyto-death antibody (*see* **Subheading 3.4.5.** and **Fig. 1**).

3.2.2. Solubilization of Desmosomal Proteins

1. Cells are harvested at different time points after induction of apoptosis (e.g., 0, 3, 6, 9, 12, 24 h) to analyze the kinetics of apoptotic cleavage of desmosomal proteins.
2. Collect detached cells from the culture medium by centrifugation at 310*g* for 10 min. Remove cell culture medium and store at –20°C.
3. Gently wash adherent cells twice with PBS+ and incubate with 150 µL ice-cold lysis-buffer for 10 min at 4°C.
4. Scrape cells from the culture dish with a cell lifter and pool with cells pelleted from the cell culture supernatant. After centrifugation (20,800*g*, 10 min, 4°C) remove the supernatant (cell lysate) and store at –20°C.
5. To solubilize desmoplakin 1 and 2, add an equal volume of 8 *M* urea to the pooled cells in lysis buffer and incubate for 24 h at 4°C under constant rotation. Remove insoluble material by centrifugation (20,800*g*, 10 min, 4°C) and use the supernant for Western blot analysis.
6. Determine the protein concentration of the lysates with the BCA protein assay according to the manufacturer's instructions.
7. Mix equal amounts of the lysates with 4X SDS sample buffer.
8. Heat the samples for 5 min at 96°C and spin for 2 min at 20,800*g*.
9. Load 50 µg into each well of the SDS polyacrylamide gel (80 × 85 × 1.5 mm). Use a prestained molecular weight marker as reference.

3.2.3. SDS–PAGE

1. Assemble the glass plates according to the manufacturer's instructions. Use 1.5-mm spacers.
2. Prepare the resolving gel solution as listed in **Table 1** and add TEMED last to start polymerization (*see* **Note 8**). Immediately cast the resolving gel solution into the mold and leave approx 25 mm for the stacking gel.
3. Cover the resolving gel with 2-propanol. Be careful not to intermix the 2-propanol and gel solution.
4. Allow the gel to polymerize for 30 min.
5. Discard the 2-propanol and wash the top of the polymerized gel several times with deionized H_2O to completely remove the 2-propanol. Remove any remaining fluid with a paper towel.

Table 1
Composition of the Resolving Gel

Proteins	Desmoplakin	Plakophilin-1, Desmocollin	Desmoglein-3
Percentage of resolving gel	5%	7.5%	10%
1.5 *M* Tris-HCl, pH 8.8	2.5 mL	2.5 mL	2.5 mL
H_2O	5.63 mL	4.8 mL	3.97 mL
Rotiphorese® gel 30	1.67 mL	2.5 mL	3.33 mL
10% (w/v) SDS	0.1 mL	0.1 mL	0.1 mL
10% (w/v) APS	0.1 mL	0.1 mL	0.1 mL
TEMED	0.01 mL	0.01 mL	0.01 mL

Numbers are given for one minigel (spacer, 1.5 mm). Different percentages of gels are recommended for the analysis of the different desmosomal proteins and their cleavage fragments.

Table 2
Composition of Stacking Gel Solution

Percentage of stacking gel	4.5%
0.5 *M* Tris-HCl, pH 6.8	0.83 mL
H_2O	1.93 mL
Rotiphorese® gel 30	0.5 mL
10% (w/v) SDS	0.034 mL
10% (w/v) APS	0.034 mL
TEMED	0.005 mL

Numbers are given for one minigel (spacer, 1.5 mm).

6. Prepare the stacking gel solution as listed in **Table 2**. Start polymerization by adding TEMED.
7. Immediately cast the stacking gel solution and insert the 1.5-mm comb. **Avoid introducing air bubbles.**
8. Allow the stacking gel to polymerize for 30 min.
9. Remove the comb and wash the wells immediately with deionized H_2O to remove any unpolymerized acrylamide.
10. Mount the gel plates in the electrophoresis chamber and add the electrophoresis buffer. Remove bubbles that are trapped at the bottom of the gel between the glass plates.
11. Load 30 µL of the samples (50 µg) into the wells.
12. Start electrophoresis with a constant current of 10 mA/gel. After the samples have concentrated in the stacking gel, increase the power to 20 mA/gel. Stop electrophoresis just before the Bromophenol blue front runs out of the gel.
13. Remove the gel from glass plates and assemble an immunoblot as described in **Subheading 3.2.4.**

3.2.4. Electroblotting

Here we describe the transfer the proteins onto PVDF membranes (0.45-µm pore size) by semidry blotting (*see* **Note 9**).

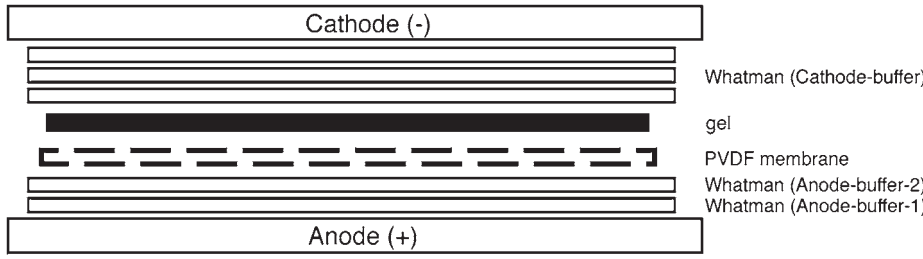


Fig. 2. Schematic presentation of a semidry Western blotting sandwich.

1. Wet the PVDF membrane with methanol for at least 2 min and incubate in anode buffer 2 for at least 5 min.
2. Soak a sheet of 3MM Whatman paper in anode buffer 1, a second sheet in anode buffer 2, and three sheets in cathode buffer for at least 1 min.
3. Assemble the blot as shown in **Fig. 2**. **Avoid air bubbles!** (*See* **Note 10**.)
4. Perform the transfer at a constant current of 4.5 mA/cm^2. Transfer times for the different desmosomal proteins are given in **Table 3**.
5. Stop transfer and rinse membrane in TST buffer.

3.2.5. Immunodetection

All steps are performed at room temperature.

1. Block the PVDF membrane with TST buffer for 1 h.
2. Incubate the PVDF membrane with diluted primary antibody (*see* **Table 4**) for 1 h in a gently shaking tray.
3. Remove the antibody solution and wash the membrane three times with 10–20 mL of TST buffer for 5 min (*see* **Note 11**).
4. Incubate the PVDF membrane for 30 min with a peroxidase-conjugated secondary antibody diluted 1 : 10,000 in TST buffer.
5. Wash the membrane three times as described in **step 3**.
6. For chemoluminescence detection remove excess buffer by rolling a clean glass pipet over the PVDF membrane and subsequently incubate membrane with 2 mL Lumi-Light Western blotting substrate for 2 min. Remove excess fluid as above and wrap the PVDF membrane in plastic wrap.
7. Signals are detected by exposure of the membrane to an X-ray film for 1–10 min depending on the signal intensity.

3.3. Immunoprecipitation

This section describes an immunoprecipitation method to analyze the shedded extracellular domain fragment of desmoglein-3.

1. Collect the cell culture supernants at different time points (e.g., 0, 3, 6, 9, 12, and 24 h) after induction of apoptosis and centrifuge for 10 min, 20,800*g* at 4°C.
2. Preclear 1 mL of the supernatant by preincubation with 30 µL of Protein A Sepharose slurry (1 : 1 in CSK buffer, 0.2% [w/v] BSA) for 30 min at 4°C under constant agitation.
3. Remove Protein A Sepharose beads and insoluble material by centrifugation for 10 min, 20,800*g* at 4°C.
4. Add 40 µL of the anti-desmoglein-3 (5H10) antibody to the cell culture supernatant and incubate for 30 min at 4°C under constant agitation.

Table 3
Transfer Times for the Desmosomal Proteins

Protein	Time (min)
Desmoglein-3	30
Desmocollin	25
Desmoplakin	45
Plakophilin-1	20

Table 4
Antibody Dilutions in TST Buffer

Antibody	Dilution
Anti-desmoglein-3, intracellular domain (clone 62)	0.25 µg/mL
Anti-desmoglein-3, extracellular domain (clone 5H10)	1:200
Anti-desmocollin (clone Dsc3-U114)	1:200
Anti-desmoplakin 1 and 2 (clone 2.15)	0.2 µg/mL
Anti-plakophilin-1 (clone 670)	1:5000

5. Add 40 µL of 1:1 slurry of the Protein A-Sepharose and incubate for a further hour.
6. Sediment the Protein A-Sepharose beads and wash five times with CSK buffer by centrifugation at 2700*g*, 1 min at 4°C.
7. Elute immunocomplexes with 20 µL 2X SDS sample buffer, boil the samples for 5 min and spin for 2 min at 20,800*g*.
8. Run samples on a 7.5% SDS polyacrylamide gel (spacer, 0.75) and perform Western blot analysis as described in **Subheadings 3.2.3.–3.2.5.**

3.4. Immunofluorescence

This section describes indirect immunfluorescence protocols to detect and localize desmosomal proteins and their fragments and keratin fragments in apoptotic cells.

3.4.1. Plating of Cells on Cover Slips

1. Put sterile cover slips in six-well dishes.
2. Add 2 mL of a sterile 0.1% (w/v) gelatin solution to cover the slides.
3. Incubate the cover slips for 20 min at room temperature.
4. Remove the gelatin solution completely and wash the cover slips with 3 mL of PBS+ twice.
5. Prepare a single-cell suspension from a confluent cell layer as described in **Subheading 3.1.2.** and transfer cells to the gelatin-coated cover slips at a density of 0.5–1 × 10^6 cells per well.
6. Grow cells for 18 h.
7. Induce apoptosis by adding 1 µ*M* staurosporine.
8. After 6 h, remove the medium and wash twice with PBS+ (prewarmed to 37°C). **Avoid pipetting buffer directly onto the cells because the cells are only loosely attached.**

Because the protocols for staining of individual desmosomal proteins include distinct but important differences, especially in fixation procedures, they are described in separate sections. (**Note:** Apoptotic cells only loosely attach to the cover slides and therefore it is critical to add and remove buffers or antibodies solutions extremely carefully to avoid detachment of cells.) All fixation, washing and staining procedures are performed in Multiwell™ six-well tissue culture plates.

3.4.2. Immunofluorescence Staining of Desmoglein-3 Cytoplasmatic Domains

1. Permeabilize the cells by incubation in 0.5% (v/v) Triton X-100 for 20 min on ice.
2. Gently wash twice with 3 mL PBS+. Take care not to detach cells.
3. Fix cells in 3.7% (w/v) paraformaldehyde for 20 min at room temperature.
4. Remove paraformaldehyde and gently wash twice with PBS+.
5. To block excess paraformaldeyhde, incubate with PBS+/glycine for 5 min and wash twice with PBS+.
6. Block unspecific binding with block solution for 30 min at room temperature.
7. Dilute anti-desmoglein-3 (clone62) antibody to 1 µg/mL in block solution (*see* **Note 12**).
8. Add 200 µL of primary antibody solution to the cover slides and incubate for 30 min at room temperature (*see* **Note 13**).
9. Remove primary antibody and gently wash three times with 3 mL block solution.
10. Dilute Alexa Fluor488™ goat anti-mouse IgG (secondary antibody) 1 : 500 in block solution (*see* **Note 12**).
11. Add 200 µL of secondary antibody solution and incubate for 30 min at room temperature. Perform all following steps in the dark.
12. Remove the antibody and gently wash three times with block solution.
13. Carefully remove excess fluid with Kimwipes® from one edge of the cover slide. Air-dry the cover slide for 1 min, but avoid drying completely.
14. Pipet 35 µL of Pro Taqs Mount Fluor onto a clean microscope slide.
15. Invert the cover slip and gently put the cover slip onto the drop of mounting medium by immersing one edge of the cover slip into the drop and gently lowering it. Avoid air bubbles.
16. Dry for 2 h at room temperature in the dark.
17. Analyze for successful staining of the cells in an immunofluorescence microscope. Final analysis should be performed in a confocal microscope.

3.4.3. Double Staining of the Desmoglein-3 Extracellular Domain and Cytokeratin

1. Fix the cells in cold methanol (precooled at –20°C) for 10 min at –20°C.
2. Remove the methanol and wash three times with PBS+. Take care not to detach cells.
3. Block nonspecific binding with block solution for 30 min at room temperature.
4. Dilute anti-desmoglein-3 (clone 5H10) antibody 1 : 100 in block solution (*see* **Note 12**).
5. Add 200 µL of primary antibody solution to the cells and incubate for 30 min at room temperature (*see* **Note 13**).
6. Remove anti-Desmoglein-3 antibody and wash three times with 3 mL block solution.
7. Dilute anti-pan-cytokeratin antibody 1 : 100 in block solution and add 200 µL on the cover slide (*see* **Note 12**).
8. Incubate for 30 min at room temperature.
9. Remove the anti-cytokeratin pan antibody and wash three times with block solution.
10. Dilute both Alexa Fluor488™ goat anti-mouse IgG and Alexa Fluor594™ goat anti-rabbit IgG 1 : 500 in block solution (*see* **Note 12**).
11. Add 200 µL of secondary antibody mixture and incubate for 30 min at room temperature. Perform all following steps in the dark.
12. Remove the antibody and gently wash three times with block solution.

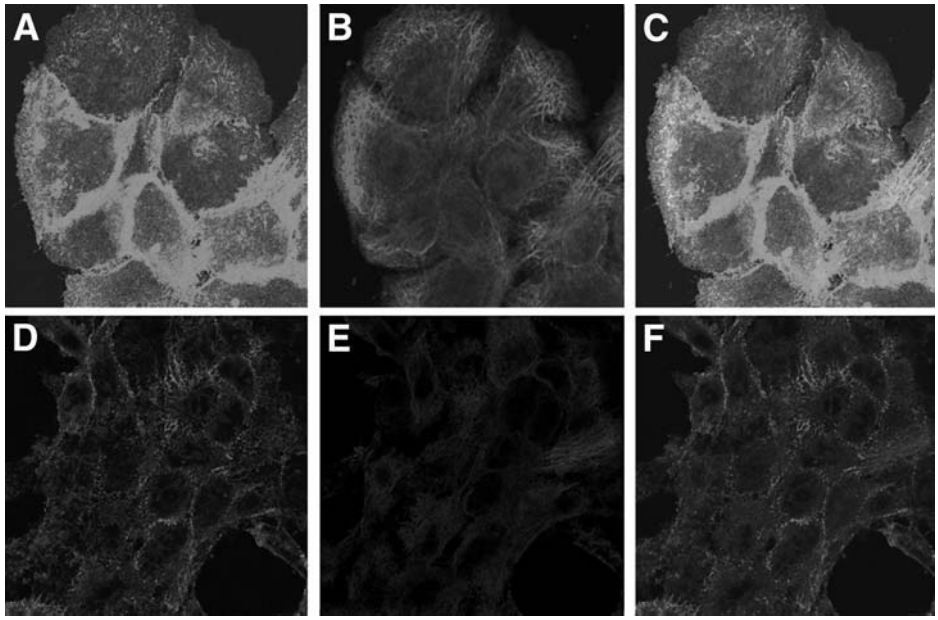


Fig. 3. Confocal immunofluorescence micrographs of HaCat cells double stained with anti-desmoglein-3 (clone 5H10) antibody **(A**,**D)** and anti-cytokeratin pan antibody **(B**,**E)**. Merged images are shown in panels **(C**,**F)**. Panels **(A–C)** represent cells at time point 0 h and panels **(D–F)** show cells 6 h after induction of apoptosis with staurosporine. See color insert following p. 238.

13. Carefully remove excess fluid with Kimwipes® from one edge of the cover slip. Air-dry the cover slip for 1 min, but avoid to drying completely.
14. Place 35 µL of Pro Taqs Mount Fluor onto a clean microscope slide.
15. Invert the cover slip and gently put the cover slip onto the drop of mounting medium by immersing one edge of the cover slip into the drop and gently lowering it. Avoid air bubbles.
16. Dry for 2 h at room temperature in the dark.
17. Analyze for successful staining of the cells in an immunofluorescence microscope. Final analysis should be performed in a confocal microscope (*see* **Fig. 3**).

3.4.4. Staining of Desmoplakin

1. Fix the cells in ice cold methanol for 10 min at –20°C.
2. Gently wash twice with 3 mL PBS+. Take care not to detach cells.
3. Block nonspecific binding with block solution for 30 min at room temperature.
4. Dilute anti-desmoplakin 1 and 2 antibody to a concentration of 1 µg/mL in block solution (*see* **Note 12**).
5. Add 200 µL of primary antibody solution to the cells and incubate for 30 min at room temperature (*see* **Note 13**).
6. Remove primary antibody and wash three times with 3 mL block solution.
7. Dilute Alexa Fluor488™ goat anti-mouse IgG 1 : 500 in block solution (*see* **Note 12**).
8. Add 200 µL of secondary antibody solution and incubate for 30 min at room temperature. Perform all following steps in the dark.
9. Remove antibody and wash three times with block solution.
10. Carefully remove excess fluid with Kimwipes® from one edge of the cover slip. Air-dry the cover slip for 1 min, but avoid to drying completely.

11. Pipet 35 µL Pro Taqs Mount Fluor onto a clean microscope slide.
12. Invert the cover slip and gently put the cover slide onto the drop of mounting medium by immersing one edge of the cover slip into the drop and gently lowering it. Avoid air bubbles.
13. Dry for 2 h at room temperature in the dark.
14. Analyze for successful staining of the cells in an immunofluorescence microscope. Final analysis should be performed in a confocal microscope.

3.4.5. Staining of Plakophilin-1

1. Permeabilize the cells by incubation in 0.5% (v/v) Triton X-100 for 20 min on ice.
2. Wash twice very careful with 3 mL of PBS+.
3. Fix the cells in 3.7% paraformaldehyde for 20 min at room temperature.
4. Aspirate the paraformaldehyde and wash twice with PBS+.
5. Incubate with PBS/glycine for 5 min and wash twice with PBS+.
6. Block nonspecific binding with block solution for 30 min at room temperature.
7. Dilute anti-plakophilin-1 (670) antibody 1 : 250 in block solution (*see* **Note 12**).
8. Add 200 µL of primary antibody solution on the cover slips and incubate for 30 min at room temperature (*see* **Note 13**).
9. Remove primary antibody and wash three times with 3 mL block solution.
10. Dilute Alexa Fluor594™ goat anti-rabbit IgG 1 : 500 in block solution (*see* **Note 12**).
11. Add 200 µL of secondary antibody conjugated to Fluor594 and incubate for 30 min at room temperature. Perform all following steps in the dark.
12. Remove antibodies and wash three times with block solution.
13. Carefully remove excess fluid with Kimwipes® from one edge of the cover slip. Air-dry the cover slip for 1 min, but avoid drying completely.
14. Place 35 µL Pro Taqs Mount Fluor onto a clean microscope slide.
15. Invert the cover slip and gently put the cover slip onto the drop of mounting medium by immersing one edge of the cover slip into the drop and gently lowering it. Avoid air bubbles.
16. Dry for 2 h at room temperature in the dark.
17. Analyze for successful staining of the cells in an immunofluorescence microscope. Final analysis should be performed in a confocal microscope.

3.4.6. M30 Cyto-Death Staining

1. Fix the cells in ice cold methanol for 10 min at –20°C.
2. Gently wash twice with 3 mL PBS+. Take care not to detach cells.
3. Block nonspecific binding with block solution for 30 min at room temperature.
4. Dilute anti-M30 cyto-death antibody 1 : 100 in block solution (*see* **Note 12**).
5. Add 200 µL of primary antibody solution to the cells and incubate for 30 min at room temperature (*see* **Note 13**).
6. Remove primary antibody and wash three times with 3 mL block solution.
7. Dilute Alexa Fluor488™ goat anti-mouse IgG 1 : 500 in block solution (*see* **Note 12**).
8. Add 200 µL of secondary antibody solution and incubate for 30 min at room temperature. Perform all following steps in the dark.
9. Remove antibody and wash three times with block solution.
10. Carefully remove excess fluid with Kimwipes® from one edge of the cover slip. Air-dry the cover slip for 1 min, but avoid drying completely.
11. Place 35 µL Pro Taqs Mount Fluor onto a clean microscope slide.
12. Invert the cover slip and gently put the cover slip onto the drop of mounting medium by immersing one edge of the cover slip into the drop and gently lowering it. Avoid air bubbles.
13. Dry for 2 h at room temperature in the dark.
14. Analyze for successful staining of the cells in an immunofluorescence microscope. Final analysis should be performed in a confocal microscope.

3.5. In Vitro Caspase Cleavage of Desmoglein-3

This section describes an in vitro method to identify caspase cleavage sites in substrate proteins. The substrate proteins or domains of the substrate proteins are expressed by recombinant protein expression technology. To facilitate purification, it is recommended to express the substrate proteins as fusion proteins ***(12)***.

3.5.1. Recombinant Expression and Purification of GST-Dsg3$_{cyto}$

3.5.1.1. Expression of GST-Dsg3$_{cyto}$

1. Inoculate 50 mL LB/Amp medium with a colony of bacteria transformed with pGEX4T1-Dsg3$_{cyto}$ and grow overnight by shaking at 37°C (*see* **Note 14**).
2. Inoculate 300 mL LB/Amp supplemented with 1% (w/v) glucose with 10 mL of overnight culture in a 2-L Erlenmayer flask. Grow to an OD_{578} 0.5–0.7 at 30°C on a rotatory shaker (180 rpm).
3. Induce expression of GST-Dsg3$_{cyto}$ by additon of 1 m*M* IPTG.
4. After 1 h, harvest bacteria by centrifugation at 6000*g* for 10 min at 4°C and discard the supernatant. **Perform all the following steps at 4°C.**
5. Wash bacterial pellet in 20 mL of cold PBS+ and centrifuge at 6000*g* for 10 min at 4°C. Discard supernatant and remove the residual liquid by putting up the tubes in an inverted position.
6. Resuspend bacteria in 5 mL cold lysis buffer.
7. Transfer the suspension to a 50-mL tube in an ice bath and lyse bacteria by ultrasonic treatment. Sonicate five times 20 s each at an output control of 6 and a constant duty cycle of 40%. To avoid overheating include 30-s pauses between the pulses.
8. Control complete lysis under the microscope.
9. Centrifuge the lysate at 20,000*g* for 30 min at 4°C to remove insoluble debris.
10. Transfer the supernatant (bacterial lysate) to a fresh 15-mL tube and keep on ice.

3.5.1.2. Purification of GST-Dsg3$_{cyto}$

1. Fill 2 mL of a 1 : 1 slurry of glutathione-agarose beads into a Poly-Prep chromatography column (8 × 40 mm) and equilibrate the final gel bed (1 mL) with 10 mL cold PBS+. Avoid letting the column runs dry.
2. Gently add the bacterial lysate (*see* **Subheading 3.5.1.2., step 10**) to the column without resuspending the gel bed and run the column by gravity flow.
3. Wash the column with 10 mL wash buffer and subsequently with 20 mL PBS+.
4. Elute bound protein with 5 mL elution buffer in 0.5-mL fractions.
5. Analyze the fractions by SDS–PAGE (10%) (*see* **Subheading 3.2.3.**) and stain gel with Coomassie blue.
6. Pool fractions containing high concentrations of protein and dialyze against 10 m*M* Tris-HCl, pH 8.0, for 24 h. Change dialysis buffer twice to completely remove glutathione.
7. Shock-freeze protein in liquid nitrogen and store at –20°C.

3.5.2. Caspase-3 Cleavage

1. Determine the concentration of the GST-fusion protein with the BCA protein assay.
2. Set up a cleavage reaction:
   105 µL 2X caspase cleavage-buffer
   + *x* µL GST-fusion protein (5–10 µg)
   + *x* µL recombinant caspase-3 (100 ng)
   Add water to a final volume of 210 µL.

Table 5
Composition of the Preparative Gel

	Resolving gel solution (14%)	Stacking gel solution (4.5%)
7.5 g acrylamide and 0.2 g bis-acrylamide in 25 mL H_2O	18.4 mL	2.5 mL
1.5 *M* Tris-HCl, pH 8.8	10 mL	–
0.5 *M* Tris-HCl, pH 6.8	–	4.2 mL
H_2O	10.8 mL	7.8 mL
10% (w/v) SDS	0.4 mL	0.168 mL
10% (w/v) APS	0.4 mL	0.168 mL
TEMED	0.01 mL	0.01 mL

3. Incubate at 37°C for 4–6 h.
4. Add 70 µL 4X SDS sample buffer, boil the probe and spin for 2 min at 20,800*g*.

3.5.3. Preparative SDS–PAGE and Electroblotting

1. Assemble the glass plates according to the manufacture's instructions. Use a gel with dimensions of approx 160 × 140 × 1.5 mm.
2. Prepare a 14% acrylamide solution as shown in **Table 5** (do not add TEMED), prepare all solutions directly before use. Filter (0.45 µm) and degas solutions (*see* **Note 8**).
3. Start polymerization by adding 10 µL TEMED and pour gels quickly. Allow to polymerize for at least 4 h.
4. Load 260 µL of the cleavage reactions into the wells of the gel. Run gel at a constant current of 7 mA/gel (best overnight). Stop electrophoresis before the Bromophenol blue dye front runs out of the gel.
5. Transfer the cleavage products to a PVDF membrane (0.2-µm pore size) by semidry blotting.
6. Wet the PVDF membrane with methanol for 2 min and incubate for 5 min in PVDF buffer.
7. Assemble the blot sandwich **(avoid air bubbles)**: three sheets of 3MM Whatman paper soaked in anode buffer; PVDF membrane; polyacrylamide gel; and three sheets of 3MM Whatman paper soaked in cathode buffer.
8. Perform the electrotransfer with a constant current of 4.5 mA/cm^2 for 1 h.
9. Detect the cleavage products on the PVDF membrane by Coomassie staining (**Subheading 3.5.4.**).

3.5.4. Coomassie Staining of Proteins on PVDF Membrane

1. Prepare the staining solution by dissolving 50 mg Coomassie Brilliant Blue R250 in 250 mL methanol. Add 250 mL H_2O and filter the solution to remove any undissolved stain particles. Prepare freshly.
2. Immerse the membrane in staining solution and place on a rotating platform for 1 h.
3. Destain the membrane in 50% (v/v) methanol + 10% (v/v) acetic acid solution until the bands are clearly visible. Repeatedly change destaining solution.
4. Rinse the membrane with H_2O when nearly no background of stain is visible on the membrane and subsequently air-dry membrane.
5. Excise the stained bands of the cleavage product(s) and store at –20°C.
6. Sequence the cleavage product(s) by Edman degradation.

4. Notes

1. The methods described in this section can be applied for epithelial cells in general.
2. Freezing of cells with the Nicool LM10 cell freezing machine is very simple and highly reproducible with excellent survival rates. This is of special importance when critical cell lines including ES cells or primary cells (e.g., keratinocytes) have to be frozen. Up to now, no other freezing procedure we have used was more efficient. If such a system is not available cells can be frozen alternatively, for example, with the Nalgene® Cryo freezing container (Nalgene; cat. no. 115650).
3. Fetal bovine serum was heat inactivated at 56°C for 30 min and stored at 4°C.
4. 2-Mercaptoethanol has a very foul odor, and high concentrations are extremely destructive to skin and eyes, so prepare 2X or 4X SDS sample buffer in a chemical fume hood and wear gloves.
5. Paraformaldehyde is toxic, wear appropriate gloves and prepare the 3.7% (w/v) paraformaldehyde solution in a chemical fume hood.
6. Alternatively induce apoptosis by addition of 2 mL fresh medium supplemented with 20 µL of tumor necrosis factor-α or 0.8 µL of anti-CD95 antibody and incubate for 48 h.
7. PARP is a nuclear enzyme involved in DNA repair that is activated in response to DNA damage. PARP is cleaved by caspases in an early step during apoptosis and is considered to be a characteristic hallmarks of apoptosis. Cleavage of PARP results in specific 89-kDa and 24-kDa fragments that can be detected with antibodies. For Western blot analysis of cell lysates, separate total protein lysates on 7.5% SDS polyacrylamide gels. Transfer the protein onto membranes by semidry blotting for 20 min. For immunodetection, dilute the anti-PARP antibody 1 : 1000 in TST buffer.
8. Acrylamide is a potent neurotoxin and is absorbed through the skin (effects are cumulative), so be careful and wear gloves.
9. Alternatively proteins can be transferred by tank blotting. Instead of PVDF membranes, nitrocellulose membranes can be used. Nitrocellulose membranes, however, are more sensitive to tearing.
10. Air bubbles that might have been trapped between the layers during assembly of the blot sandwich can be removed by gently rolling a pipet or a 15-mL tube over the sandwich.
11. Most antibodies can be reused at least three to five times.
12. To remove insoluble material or precipitates from the antibody solutions it is recommended to centrifuge at high speed (20,800*g*, 1 min) prior to use.
13. To reduce the amount of antibody required for staining pipet 100 µL of antibody solution onto a sheet of parafilm and put cover slide in an inverted position onto the drop. To avoid drying generate a humid atmosphere by adding some drops of water at the corner of the parafilm sheet and use a lid. Avoid mixing of the water with the antibody solutions.
14. Highest expression rates are obtained with freshly transformed bacteria.

Acknowledgments

We thank Torsten Schöneberg for help in confocal immunofluorescence microscopy. We thank the Deutsche Forschungsgemeinschaft, the VW-Foundation and the Sonnenfeld-Foundation for funding.

References

1 Krammer, P. H. (2000) CD95's deadly mission in the immune system. *Nature* **407,** 789–795.
2. Ferri, K. F. and Kroemer, G. (2001) Organelle-specific initiation of cell death pathways. *Nat. Cell Biol.* **3,** E-255–E-263.
3 Meier, P., Finch, A., and Evan, G. (2000) Apoptosis in development. *Nature* **407,** 796–801.

4 Hathaway, T. R. and Kuechle, M. K. (2002) Apoptosis and cutaneous biology. *Adv. Dermatol.* **18,** 287–311.
5 Earnshaw, W. C., Martins, L. M., and Kaufmann, S. H. (1999) Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Annu. Rev. Biochem.* **68,** 383–424.
6 Steinhusen, U., Weiske, J., Badock, V., Tauber, R., Bommert, K., and Huber, O. (2001) Cleavage and shedding of E-cadherin after induction of apoptosis. *J. Biol. Chem.* **276,** 4972–4980.
7 Weiske, J., Schoeneberg, T., Schroeder, W., Hatzfeld, M., Tauber, R., and Huber, O. (2001) The fate of desmosomal proteins in apototic cells. *J. Biol. Chem.* **276,** 41,175–41,181.
8 Garrod, D. R., Merritt, A. J., and Nie, Z. (2002) Desmosomal cadherins. *Curr. Opin. Cell Biol.* **14,** 537–545.
9 Garrod, D. R., Merritt, A. J., and Nie, Z. (2002) Desmosomal adhesion: structural basis, molecular mechanism and regulation. *Mol. Membr. Biol.* **19,** 81–94.
10 Green, K. J. and Gaudry, C. A. (2000) Are desmosomes more than tethers for intermediate filaments? *Nat. Rev. Mol. Cell Biol.* **1,** 208–216.
11 Huber, O. (2003) Structure and function of desmosomal proteins and their role in development and disease. *Cell Mol. Life Sci.* **60,** 1872–1890.
12. Huber, O. and Huber-Wunderlich, M. (2000) Recombinant proteins, in *Journal of chromatography library* **61,** *Protein liquid chromatography* (Kastner, M., ed.), Elsevier Science B. V., Amsterdam, The Netherlands, pp. 557–586.

21

Analysis of Connexin 43 Expression on Keratinocytes Using Flow Cytometry

Maja Matic, Christopher Pullis, Marc G. Golightly, and Sanford R. Simon

Summary

Single-cell suspensions of primary keratinocytes comprise a heterogeneous cell population that consists of basal cells (stem cells, transient amplifying cells, and post-mitotic basal cells) and suprabasal cells at different stages of differentiation. Quantitative data for the differential expression of epitopes on single cells can be obtained using a flow cytometer. Simultaneous analysis of two intracellular epitopes, keratin 14 and connexin 43, using flow cytometry after keratinocyte isolation, fixation, permeabilization, and fluorescent immunolabeling is described. Three subsets of cells could be distinguished: stem cells (basal cells [keratin 14 positive] that lack connexin 43 expression); suprabasal cells (connexin 43-positive, keratin 14-negative cells); and basal cells (keratin 14 positive) that express connexin 43. The last population of keratinocytes includes both transient amplifying cells and postmitotic basal cells. The scatter characteristics of each cell population are also described.

Key Words:

Epidermis; keratinocytes; stem cells; gap junctions; connexins; fluorescence activated cell sorting; immunocytochemistry.

1. Introduction

Epithelial cells of the epidermis are coupled by gap junctions, or clusters of intercellular aqueous channels composed of connexin molecules. The connexins are encoded by a multigene family ***(1–3)***. The expression of 10 connexins has been reported in the human epidermis ***(4,5)***, and mutations in some connexin genes have been linked to human disorders of the skin ***(6)***. Recently, it has been shown that keratinocytes, which lack the expression of connexin 43 (Cx43), exhibit many characteristics attributed to stem cells, including the relative number of cells within the basal layer, cell size, and granularity ***(7,8)***.

In this chapter we describe the isolation of neonatal foreskin keratinocytes (*see* **Subheadings 3.1.** and **3.2.**); fixation and permeabilization of keratinocytes (*see* **Subheading 3.3.**); and immunocytochemistry using antibodies against Cx43 and K14 (*see* **Subheading 3.4.**). Flow cytometer instrument settings, and controls required for the analysis of keratinocytes via a two-color method using indirect fluorescent techniques are outlined in **Subheading 3.5.** Finally, the gating strategies used to analyze relative size and granularity of distinct cell populations that differ in K14 and Cx43 expression are described in **Subheading 3.6.**

From: *Methods in Molecular Biology, vol. 289:*
Epidermal Cells: Methods and Protocols
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

In the early 1970s, flow cytometers were initially used for blood cell counts. Today, the most sophisticated instruments use three or more lasers and multiple fluorescence detectors, which has expanded the capabilities beyond what is generally needed for most clinical and research applications. However, these advances have enabled extremely detailed analysis of cell surface and intracellular physical and antigenic properties, such as relative size and granularity (complexity), as well by quantitative multiple antigens (both cell surface and intracellular) as determined fluorescent intensity of the probe being used. The more complex flow cytometers (fluorescent-activated cell sorter) can physically separate and purify a heterogeneous population by any one or combinations of the parameters listed previously.

To facilitate acquisition and data analysis in core facility laboratories where epidermal cells are not routinely analyzed, a step-by-step procedure of flow cytometric analysis of keratinocytes is described.

2. Materials

2.1. Equipment

1. Flow cytometer: FACScan (Becton Dickinson, Mountainview, CA).
2. Cell Quest software (BD Biosciences).
3. Inverted light microscope (Nikon).
4. Micro centrifuge, 4°C (Marathon Micro H, Fisher Scientific, Pittsburgh, PA).
5. Incubator 37°C.
6. Shaker (titer plate shaker, Lab-line instruments, Inc., Melrose Park, IL), or equivalent.
7. Scissors and forceps (autoclaved) (Fisher Scientific, Pittsburgh, PA).
8. 1.5-mL Eppendorf microcentrifuge tubes (USA Scientific, Ocala, FL; cat. no. 1615-5500).
9. 15-mL Falcon Blue Max Polypropylene conical tubes (Beckton Dickinson, Franklin Lakes, NJ; cat. no. 352096).
10. Disposable sterile Pasteur pipets.
11. 100 × 20 mm and 60 × 15 mm disposable round-bottom sterile plasticware (Becton Dickinson; cat. no. 353003 and 353004, respectively).
12. Sterile 35-µm strainer tubes (Becton Dickinson; cat. no. 2235).
13. 0.22-µm filters (Millipore, Bedford, MA; cat. no. 5CGPU01RE).
14. Hemocytometer.

2.2. Keratinocyte Isolation

1. Dispase II (Boeringer Mannheim, Indianapolis, IN; cat. no. 165859).
2. Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY; cat. no. 11995).
3. Phosphate-buffered saline (PBS; HyClone, Logan; cat. no. SH30028.02).
4. Fetal bovine serum (HyClone; cat. no. SH30070.03).
5. Penicillin G (Sigma, St. Louis, MO; cat. no. p-3032).
6. Streptomycin sulfate (Sigma; cat. no. S-6501).
7. Trypsin (ICN Biomedicals, Irvine, CA; cat. no. I03140).
8. Ethylenediamine tetraacetic acid (EDTA; Boeringer Mannheim, Indianopolis, IN; cat. no. 808270).
9. Ham's F12 medium (Gibco; cat. no. 11765).

2.3. Immunocytochemistry

1. Bovine serum albumin (BSA; Sigma; cat. no. A-3059).
2. HistoChoice (Amresco, Solon, OH; cat. no. H120).

3. Triton X-100 (Sigma; cat. no. T-9284).
4. Mouse monoclonal antibody to connexin 43 (Chemicon, Temecula, CA; cat. no. MAB3068), IgG1 isotype aliquoted and stored at –20°C.
5. Mouse monoclonal antibody against cytokeratin 14 (Sigma, St. Louis, MO; cat. no. C8791), IgM isotype aliquoted and stored at –70°C.
6. Allophycocyanin rat anti-mouse IgG1 monoclonal antibody (BD biosciences; cat. no. 550874) stored at 4°C in the dark.
7. Fluorescein-conjugated goat anti-mouse IgM antibody (Sigma; cat. no. F-9259) stored in the dark at 4°C.

2.4. Solutions

1. Dispase (10X) 25 mg/mL in DMEM, sterile-filtered, kept at –20°C in 2-mL aliquots (for at least 3 mo).
2. Triton X-100: 0.03% in PBS kept at 4°C.
3. EDTA: 0.01% in sterile PBS.
4. Trypsin: 0.05% in sterile PBS, kept at –20°C in 2-mL aliquots.
5. Fetal bovine serum (FBS): 5% in sterile PBS (PBS/FBS).
6. DMEM/F12, 3 : 1 with 100 U/mL penicillin and 100 µg/mL streptomycin (DF/PS).
7. PBS with 100 U/mL penicillin and 100 µg/mL streptomycin (PBS/PS).
8. BSA: 5% in PBS.

3. Methods

3.1. Epithelial Sheath Preparation

1. Collect neonatal foreskins (*see* **Note 1**) within 2 h after routine circumcisions in 15-mL polypropylene tubes containing 2–3 mL of DF/PS. Remove subcutaneous fat using forceps and scissors.
2. Wash the tissue by dipping it consecutively in three round-bottom dishes filled with PBS/PS.
3. Cut the tissue in 5 × 5-mm pieces and incubate overnight at 4°C in a round bottom dish containing 1X Dispase solution in DF/PS.
4. Remove epithelial sheaths from the underlying dermis with forceps (*see* **Note 2**) and place them in 60 × 15-mm round bottom dishes containing EDTA solution.

3.2. Obtaining a Single-Cell Suspension

1. Add 2 mL trypsin solution (0.05% in PBS) and agitate the plates for 5–10 min at 37°C using a shaker (*see* **Notes 3** and **4**).
2. Collect the solution using a sterile Pasteur pipet in a 15-mL polypropylene tube and neutralize trypsin with 10% FBS.
3. Wash the sheaths once with PBS containing 5% (PBS/FBS) and add the solution to the tube.
4. Distribute the content into Eppendorf tubes and centrifuge at 4°C at 410*g* for 4 min (*see* **Note 5**).
5. Count the cells (*see* **Note 6**).

3.3. Fixation and Permeabilization of Keratinocyres

1. Use 10^6 cells per reaction (*see* **Note 7**).
2. Resuspend cells in 1 mL HistoChoice and incubate for 30 min at room temperature using a Pasteur pipet (*see* **Notes 8** and **9**).
3. Wash the cells twice in PBS/FBS.
4. Permeabilize the cells with 0.03% Triton X-100 for 5 min at 4°C.
5. Wash cells three times in PBS/FBS.

3.4. Immunocytochemistry

1. Incubate sample with a monoclonal anti-Cx43 antibody (dilution 1:300 in PBS), and an IgM monoclonal anti-keratin 14 (K14) antibody (dilution 1:200 in PBS) for 1 h at room temperature (*see* **Note 10**). Incubate single positive controls with only one of each of the primary antibodies. The secondary antibody control should be incubated in PBS/FBS.
2. Wash the cells twice in PBS/FBS.
3. Incubate sample and secondary antibody control with rat anti-mouse IgG1 antibody conjugated to allophycocyamin (dilution 1:66), and fluorescein-conjugated goat anti-mouse IgM antibody (dilution 1:300) respectively (*see* **Note 10**). Single positive controls are incubated with only one corresponding secondary antibody.
4. Wash cells three times in PBS/FBS.

3.5. Flow Cytometry Instrument Settings and Controls

1. Pass each sample through 35-μm strainer tube (*see* **Note 11**).
2. Set forward scatter to E-1 linear setting and adjust voltage and gain on photomultiplier tubes (*see* **Note 12**).
3. Use standard flow cytometric gating techniques to exclude cellular debris and select cells for fluorescence analysis (*see* **Fig. 1A**).
4. Collect at least 10,000 events for each analysis.
5. Use secondary antibody control to compensate fluorescence; adjust voltages on photomultiplier tubes (*see* **Note 13** and **Fig. 1B**).
6. Compensate fluorescence using single positive controls (*see* **Note 14**).
7. Mix single positive controls, run the sample, and adjust compensation (*see* **Note 15** and **Fig. 1C**).

3.6. Correlation of Light Scatter Characteristics With Fluorescence Intensities

1. Acquire sample (*see* **Fig. 2A**).
2. Set three separate gates corresponding to R2, R3, and R4 and a virtual gate for each of the three gates (*see* **Notes 16** and **17**).
3. Back gate R2, R3, and R4 (*see* **Fig. 2B–D**). **Note 18** pertains to **Fig. 2D**.

4. Notes

1. To obtain keratinocytes with uniform scatter patterns, foreskins of similar color should be collected. Keratinocytes with different pigment levels may differ in their side scatter characteristics.
2. The epithelial sheath removed from the dermis should be placed in a round bottom dish in a single layer containing 2 mL of EDTA (0.01% in PBS) with the basal layer facing the solution. Sheaths will tend to orient themselves with the basal layer facing the solution because the keratinized surface is hydrophobic. After all the sheaths are removed and placed in EDTA, a cold solution of trypsin (0.05%) is added.
3. The higher yield of cells is obtained by using round-bottom dishes for trypsinization instead of 15-mL tubes, which could alternatively be used. When using tubes, epithelial sheaths overlap each other, causing keratinocytes released by trypsin to get trapped within undigested, sticky epithelial layers.
4. Trypsinization for 5–10 min mainly digests the basal layer. Longer incubation in trypsin/EDTA solution may release an increased number of keratinocytes from suprabasal layers. Longer incubation may cause decrease of basal keratinocyte viability.
5. Centrifugation at 4°C at 410*g* for 4 min is used to collect the cells following each wash.
6. Usually, a yield of more than 1×10^6 cells per foreskin is obtained.

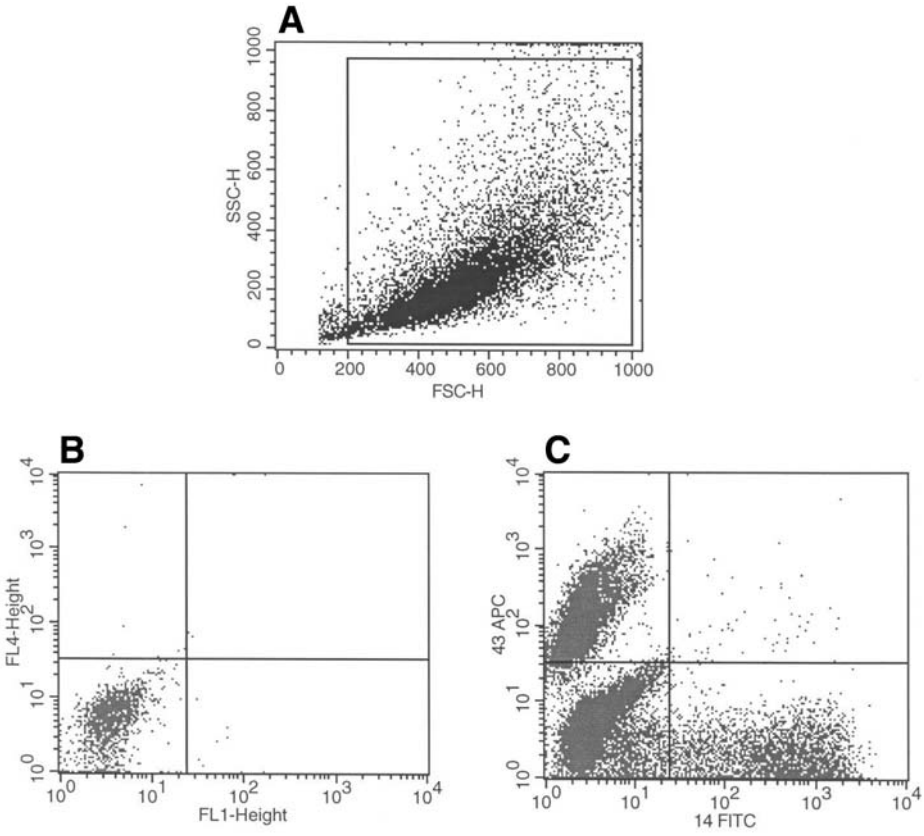


Fig. 1. Flow cytometry analysis. Scatter plot in arbitrary units on a linear scale showing forward and side scatter **(A)**. Each dot represents the forward and side scatter values for a single cell. A rectangular gate is used to exclude cell debris and select cells for dual fluorescent analysis. Control for secondary antibodies falls into lower left quadrant **(B)**. A scatter plot of the mixture of two single positive controls, Cx43-APC and K14-FITC **(C)**.

7. Three samples are needed: (1) secondary antibody control (cells stained only with secondary antibodies); (2) the single positive control for Cx43; and (3) the single positive control for K14.
8. HistoChoice preserves the cell morphology and is nontoxic.
9. Make sure that this step is performed quickly and thoroughly, which will prevent fixing cell aggregates.
10. After dilution, add 5% of the BSA solution and spin antibodies for 1 min at 410*g* to precipitate–immune complexes.
11. This prevents clogging of the instrument with cell aggregates.
12. Linear setting E-1 is chosen for keratinocyte because of their relatively large size, in contrast to linear setting E00 used for most leukocyte cell analysis.
13. Fluorescence intensities for the secondary antibody control should fall in the lower left quadrant **(Fig. 1B)**.
14. Fluorescence intensity for the single positive control (Cx43-APC) should fall in the upper left quadrant (a negative population is also seen). Fluorescence intensity for single positive control (K14-FITC) should fall in the lower right quadrant (a negative population is also seen).

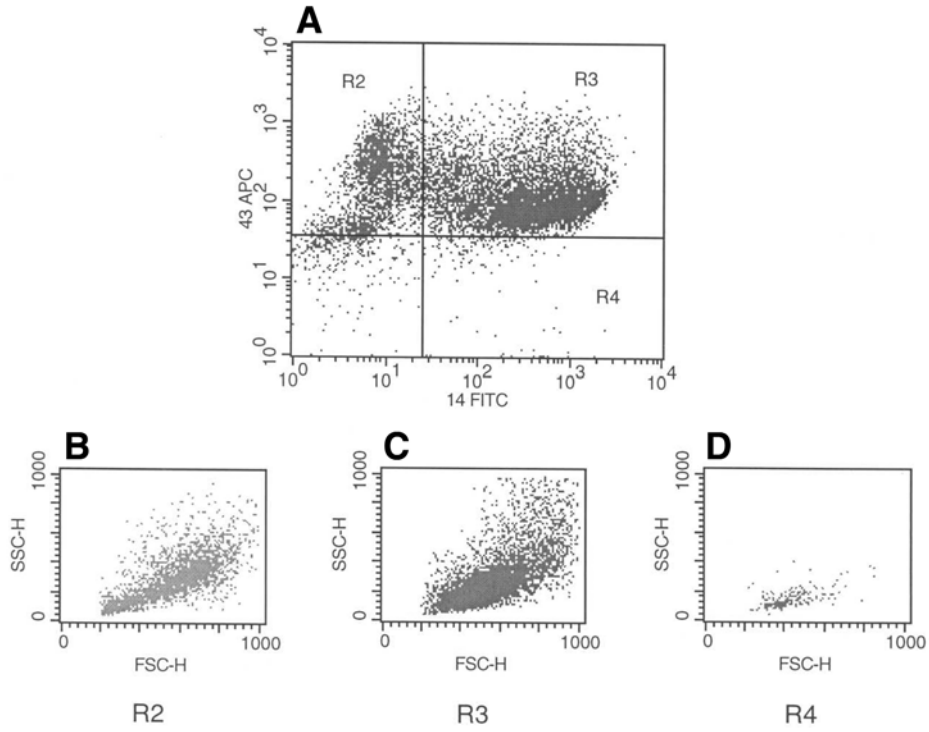


Fig. 2. Flow cytometry analysis. Scatter plot showing fluorescent intensity of keratinocytes double labeled with antibody against Cx43 (APC), *y* axis, and antibody against K14 (FITC), *x* axis, measured in arbitrary units on a log scale **(A)**. Quadrants are established using secondary control, and single-color positive controls (*see* **Fig. 1B–D**). Suprabasal cells (Cx43+, K14–) are located in the upper left quadrant, R2. Presumptive stem cells (Cx43–, K14+) are located in the lower right quadrant, R4, whereas the rest of the basal cells (Cx43+, K14+) are located in the upper right quadrant, R3. Negative cells are located in the lower left quadrant. Scatter plots showing forward and side scatter values **(B–D)** for suprabasal cells **(B)**; basal cells excluding presumptive stem cells **(C)**; and presumptive stem cells **(D)**.

15. This combined sample should give a green only population (lower right quadrant), a red only population (upper left quadrant), and a negative population (lower left quadrant). This is necessary for proper fluorescent compensation to correct for any bleed-over between the two fluorescent photomultiplier tubes.
16. The fluorescence dot plot can be divided into four quadrants: Cx43 negative and K14 negative (lower left), Cx43 positive and K14 negative (upper left), Cx43 positive and K14 positive (upper right), and Cx43 negative and K14 positive (lower right).
17. Virtual gates or logical gates are combined gates. In this case a virtual gate is used to analyze granularity of the cells in a particular quadrant (for example R2, **Fig. 2A**) and consists of two gates: gate "quadrant R2" and the gate quadrant in **Fig. 1A**. The later gate has to be included to exclude cells outside the quadrant in **Fig. 1A**, which are excluded from the initial analyses. The gates are set using software menu "gate list."
18. The Cx43 negative, K14 positive population besides being small in number, is also relatively small in size and has low complexity *(7,8)*.

Acknowledgments

This work was supported by the USPHS/NIH/NIAMS grant no. K01AR02079 to M.M.

References

1 Beyer, E. C., Paul, D. L., and Goodenough, D. A. (1990) Connexin family of gap junction proteins. *J. Membr. Biol.* **116,** 187–194.
2. Wilecke, K., Hennemann, H., Dahl, E., Jungbluth, S., and Heynkes, R. (1991) The diversity of connexin genes encoding gap junction proteins. *Eur. J. Cell Biol.* **56,** 1–7.
3 Kumar, N. M. and Gilula, N. B. (1992) Molecular biology and genetics of gap junction channels. *Semin. Cell Biol.* **3,** 3–16.
4 Di, W.-L., Rugg, E. L., Leigh, I. M., and Kelsell, D. P. (2001) Multiple epidermal connexins are expressed in different keratinocyte subpopulations including connexin 31. *J. Invest. Dermatol.* **117,** 958–964.
5 Salomon, D., Masgrau, E., Vischer, S., Ullrich, S., Dupont, E., Sappino, P., et al. (1994) Topography of mammalian connexins in human skin. *J. Invest. Dermatol.* **103,** 240–247.
6 Richard, G. (2001) Human connexin disorders of the skin. *Cell Commun. Adhes.* **8,** 401–407.
7 Matic, M., Evans W. H., Brink, P. R., and Simon, M. (2002) Epidermal stem cells do not communicate through gap junctions. *J. Invest. Dermatol.* **118,** 110–116.
8. Matic, M. and Simon, M. (2003) Label retaining cells (presumptive stem cells) of mice vibrissae do not express gap junction protein connexin 43. *J. Invest. Dermatol., Symp. Proc.* **8,** 92–95.

22

MMP-9 and TIMP-1 Assays in Keratinocyte Cultures

Takashi Kobayashi

Summary

Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) are known to play important roles in the metabolism of epidermal tissue. Among them, MMP-9 and its relatively specific inhibitor, TIMP-1, have been reported to be involved in a variety of pathophysiological conditions. To detect MMP-9 and TIMP-1 in conditioned medium from keratinocytes in culture, I describe methods of gelatin-zymography and reverse zymography (which are used both qualitatively and quantitatively) and of Western blotting analysis using antibodies specific for each molecule. These methods are useful tools for elucidating the pathophysiology of skin conditions and for seeking new drugs.

Key Words:

Keratinocyte; MMP; TIMP; zymography; Western blot.

1. Introduction

Keratinocytes comprise the majority of epidermal cells. They are located at the interface of the whole body and are considered to play a fundamental role in maintaining the homeostasis of our body. Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) are known to play important but contrasting roles in tissue metabolism ***(1,2)***. Among them, MMP-9 has been reported to be involved in a variety of pathophysiological conditions in the field of dermatology ***(3–9)***. Therefore, methods to detect MMP-9 and its relatively specific inhibitor, TIMP-1, in keratinocytes would be useful tools for understanding the pathophysiology of skin diseases and could also be used to monitor models of diseases, including the quest for new drugs. The chapter describes methods of gelatin–zymography and reverse zymography, which are used together with Western blotting analysis to detect MMP-9 and TIMP-1 in conditioned medium from keratinocytes in culture.

Because MMP-9 and MMP-2 are known to be gelatinases ***(1,2,10)***, the gelatin–zymography method has been used to detect these enzymes not only qualitatively but also quantitatively ***(11–16)***. In contrast, reverse zymography has been developed to identify TIMPs ***(16,17)***. These are methods that assess their enzymatic properties together with inhibitory activities. In addition, Western blotting analysis is a useful tool to detect antigenic specificity for each molecule.

From: *Methods in Molecular Biology, vol. 289:*
Epidermal Cells: Methods and Protocols
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

2. Materials

All solutions used for gelatin–zymography and reverse zymography are made with double-distilled or Milli Q-purified water, except for the destaining solution of 30% methanol, 10% acetic acid.

2.1. Gelatin Zymography

1. 2.5% gelatin (w/vs; *see* **Note 1**).
2. Conditioned medium (serum-free) from keratinocytes in culture (*see* **Notes 2** and **3**).
3. 1X running gel buffer: 1.5 *M* Tris-HCl, pH 8.8.
4. 1X stacking gel buffer: 0.5 *M* Tris-HCl, pH 6.8.
5. 30% acrylamide (w/v)-1% *N*′,*N*′-methylene-bis-acrylamide (w/v) in water (*see* **Note 4**).
6. 10% sodium dodecyl sulfate (SDS).
7. *N*,*N*,*N*′,*N*′-methylenebisacrylamide (TEMED).
8. 5% ammonium persulfate (w/v).
9. 5X sample loading buffer: 0.25 *M* Tris-HCl, pH 7.4, 25 m*M* $CaCl_2$, 5% SDS, 0.02% Bromophenol blue, 30% glycerol.
10. Electrophoresis equipment; minislab apparatus and power supply (*see* **Note 5**).
11. Electrophoresis electrode buffer: 15 m*M* Tris, 0.192 *M* glycine, 0.1% SDS (14.35 g glycine, 1.75 g Tris, and 1 g SDS in 1 L water).
12. 2.5% triton X-100 (Polyethylene glycol mono-*p*-isooctylphenyl ether).
13. Enzymatic reaction buffer: 0.05 *M* Tris-HCl, pH 7.4, 20 m*M* NaCl, 5 m*M* $CaCl_2$, 0.02% sodium azide.
14. 0.1% amido black 10B.
15. 30% methanol, 10% acetic acid.

2.2. Reverse Zymography

All materials from **steps 1** to **15** in **Subheading 2.1.** are required. In addition, human recombinant MMP-9 (cat. no. 2911, TECHNE Corporation, Minneapolis, MN) is required (*see* **Note 6**).

2.3. Western Blotting Analysis

All materials from **steps 2** to **10** listed for **Subheading 2.1.** are required for electrophoresis. Additional materials as described below are also required.

1. PVDF (polyvinylidene difluoride) membranes (Immobilon™, Millipore, Boston, MA; cat. no. IPVH 304 F0).
2. Methanol.
3. Blotting buffer: 0.025 *M* Tris, 0.192 *M* glycine, 20% methanol (to make this, mix 14.4 g glycine, 3 g Tris, and 200 mL ethanol in 1 L water.
4. Electroblotting apparatus (for example, Trans-Blot Cell and Systems, Bio-Rad, Hercules, CA).
5. Parafilm (American National Can, Menasha, WI).
6. Blocking buffer: 4% bovine serum albumin, 0.05% Tween-20 in phosphate-buffered saline.
7. Primary antibodies: monoclonal antibodies raised against MMP-9 or TIMP-1 (purified IgG fraction after Protein A Sepharose column, Bio-Rad; **refs. *4*** and ***18***) or monoclonal anti-TIMP-2 antibody (Daiichi Fine Chemical, Toyama, Japan; cat. no. F-70).
8. Wash buffer: 0.05% Tween-20 in phosphate-buffered saline.
9. Secondary antibody: Horseradish peroxidase linked anti-mouse Ig antibody (Amersham Biosciences, Little Chalfont Buckinghamshire, UK; cat. no. NA931V).

10. Detection reagents and equipments: ECL plus Western blotting detection reagents (Amersham Biosciences; cat. no. RPN2132) and X-ray film with cassette.

3. Methods

3.1. Gelatin–Zymography

The gelatin–zymography procedure is made up of three stages: 1) electrophoresis, 2) enzymatic reaction, and 3) staining and destaining of gels.

1. Prepare a running gel, 7.5% acrylamide-*bis*-acrylamide gel containing 0.5% gelatin. Mix 2.5 mL running gel buffer, 2.5 mL 30% acrylamide, 2 mL 2.5% gelatin, 2.7 mL water, 100 µL 10% SDS, 10 µL TEMED, and 190 µL 10% ammonium persulfate. Aspirate the mixture, for example, using a 20-mL syringe (*see* **Notes 7** and **8**). Gently pour the gel between the glass plates. Then, gently overlay acrylamide solution with water and allow it to polymerize.
2. Prepare a stacking gel, 3% acrylamide-*bis*-acrylamide gel. Mix 1 mL of stacking gel buffer, 400 µL 30% acrylamide, 2.4 mL water, 40 µL 10% SDS, 4 µL TEMED, and 156 µL of 10% ammonium persulfate. Mix gently and pour over the polymerized running gel after removing the overlaid water, add a suitable comb, and allow to polymerize.
3. Dilute the conditioned culture medium from keratinocytes with the sample loading buffer (1/4 vol of the conditioned medium) and mix (*see* **Note 9**). Do not boil.
4. Run the gel in electrophoresis electrode buffer at 80 V (constant voltage; *see* **Note 10**).
5. After running the gel, remove the stacking gel, separate the plates and mark the gel (i.e., trim a corner) to maintain the orientation. Incubate the gel in the 2.5% Triton X-100 for 60 min with shaking (*see* **Note 11**).
6. Transfer the gel into the enzymatic reaction buffer and incubate it at 35°C (*see* **Notes 12–14**).
7. Stain the gel with 0.1% amido Black 10B with shaking (*see* **Note 15**).
8. Destain the gel with 30% methanol/10% acetic acid with shaking (*see* **Note 16**).
9. Observe the region of gelatin degradation as clear bands against a background of stained gelatin (**Fig. 1A**; *see* **Notes 17** and **18**).

3.2. Reverse Zymography

Reverse zymography is based on the the same principle as gelatin zymography and is made up of three stages: 1) electrophoresis, 2) enzymatic reaction, and 3) staining and destaining of gels.

1. Prepare a running gel, 12% acrylamide-*bis*-acrylamide gel containing 0.25% gelatin. Mix 2.5 mL running gel buffer, 4 mL 30% acrylamide, 1 mL 2.5% gelatin, 2.2 mL water, 100 µL 10% SDS, 20 µL of 10 µg/mL human recombinant MMP-9, 10 µL TEMED, and 170 µL 10% ammonium persulfate (*see* **Notes 7**, **8**, and **19**). The following steps for electrophoresis are the same as in **2** to **4** in **Subheading 3.1.**
2. After running the gel, incubate it three times in 2.5% TritonX-100 for 30 min each time with shaking (90 min in total; *see* **Note 11**).
3. Transfer the gel into the enzymatic reaction buffer and incubate it at 35°C with shaking (*see* **Note 20**).
4. Stain and destain the gel with 0.1% amido black 10B and with 30% methanol/10% acetic acid, respectively (*see* **Notes 15** and **16**).
5. Observe the region of nondegraded bands against a clear background of gelatin degradation (*see* **Note 17**).

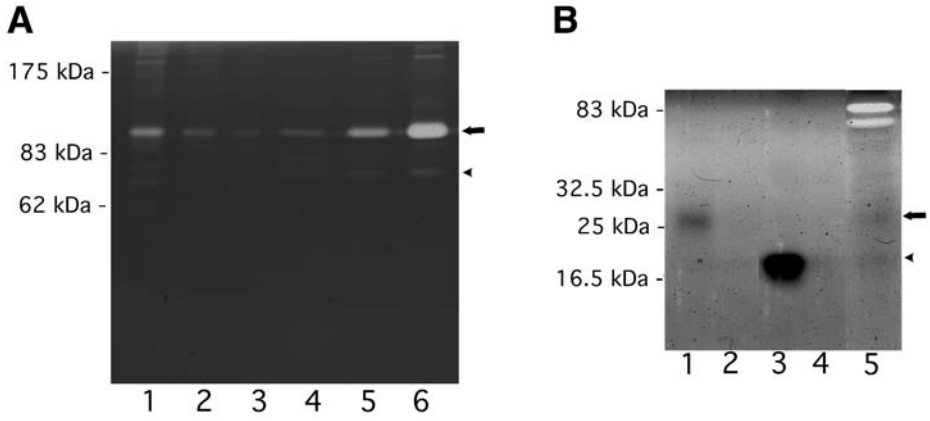


Fig. 1. Gelatin zymography (using a 7.5% acrylamide gel) **(A)** and reverse zymography (using a 12% acrylamide gel) **(B)**. **A**, 1.5 ng (lane 1), 500 pg (lane 2), or 170 pg (lane 3) of human recombinant MMP-9 and aliquots of conditioned culture media of keratinocytes stimulated with high Ca^{2+} concentration (lane 5) or by the additon of transforming growth factor (TGF)-β1 (lane 6), or without stimulation (lane 4). High Ca^{2+} induced MMP-9 secretion selectively, whereas TGF-β1 stimulated MMP-9 and MMP-2 secretion. The arrow and arrowhead show the bands of MMP-9 and MMP-2, respectively. **B**, 13 ng (lane 1) or 4.3 ng (lane 2) of human recombinant TIMP-1 (Daiichi Fine Chemical) or 13 ng (lane 3) or 4.3 ng (lane 4) of human recombinant TIMP-2 or the conditioned medium (lane 5) from human keratinocytes in culture. The bands showing gelatinolytic activities of MMP-9 and MMP-2 are also observed. The arrow and arrowhead show the bands of TIMP-1 and TIMP-2, respectively.

3.3. Western Blotting Analysis

Western blotting analysis composes four stages, 1) electrophoresis, 2) electroblotting, 3) antibody–antigen binding reaction, and 4) detection of signals (*see* **Fig. 2B**; and **Note 21**).

1. Prepare a 7.5% acrylamide-*bis*-acrylamide running gel, which is made using 4.7 mL water instead 2 mL 2.5% gelatin/2.7 mL water as described previously for gelatin zymography to detect MMP-9, or a 15% acrylamide-*bis*-acrylamide gel, which is made up of 5 mL 30% acrylamide and 2.2 mL water instead of 2.5 mL 30% acrylamide, 2 mL 2.5% gelatin, and 2.7 mL water as described in **Subheading 3.1.** (*see* **Notes 2** and **4**).
2. Perform electrophoresis as detailed in **steps 2** to **4** in **Subheading 3.1.**
3. Soak a PVDF membrane in methanol for 60 s.
4. Prewet the PVDF membrane, filter paper, and foam in blotting buffer at 4°C.
5. After electrophoresis, carefully assemble the gel and the PVDF membrane as a blotting sandwich in the apparatus, as shown in **Fig. 2**; with the gel to the anode side (black side in the Bio-Rad apparatus), and the PVDF membrane to the cathode side (clear/white side in the Bio-Rad apparatus; *see* **Note 22**).
6. Transfer proteins to the PVDF membrane in the transfer apparatus at 80 V for 1 h. Use an ice block to keep cold or transfer at 4°C. Stir during the transfer.
7. Attach parafilm to an incubation dish.
8. After transfer, cut the membrane to the exact size of the gel and set it (protein side up) on the parafilm.
9. Soak the membrane in blocking buffer by mounting approx 1 mL blocking buffer per 10 cm^2 membrane using surface tension for 30 min at 4°C (*see* **Note 23**).

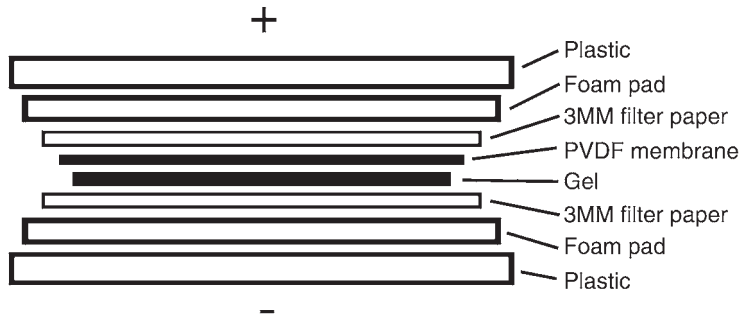


Fig. 2. Schematic representation of the blotting sandwich assembled for Western blotting analysis. + and – indicate cathode and anode, respectively.

10. Replace the blocking buffer with the first antibody solution (5 µg/mL) diluted in blocking buffer and incubate it overnight at 4°C (*see* **Note 23**).
11. Wash the membrane with washing buffer for 10 min with shaking and repeat this three times (four times total).
12. Set up a new parafilm attached to an incubation dish.
13. Soak the membrane in the second antibody solution diluted in washing buffer (10,000X for the Amersham second antibody) and incubate it for 3 h at 4°C (*see* **Note 23**).
14. Wash the membrane with washing buffer for 10 min with shaking, and repeat this three times (four times total).
15. Detect the peroxidase reaction by ECL plus reagent using X-ray film with cassette **(Fig. 3)**.

4. Notes

1. Gelatin (i.e., EIA Grade Reagent, Bio-Rad) is hard to dissolve in water at room temperature. It will more easily dissolve in a water bath at approx 40°C.
2. It is important to use serum-free medium, because serum itself contains MMP-2 and MMP-9 ***(19)***. In the case of HaCat cells or murine keratinocytes, which can be cultured in medium with serum, change to medium without serum to collect conditioned culture medium for identifying gelatinase from those cells.
3. In my experience, fresh conditioned culture medium is relatively stable at 4°C up to 48 h for the assays described previously. Please avoid storing it at 4°C for more than 2 d or at temperatures higher than 4°C. Frozen samples are often more stable. Repeated freeze and thaw cycles, however, can cause degradation of proteins together with their aggregation.
4. Because the acrylamide monomer is a neurotoxin, handle with care. For example, wear a mask and wear gloves when handling acrylamide powder and its solutions.
5. A running gel of 10 mL and a stacking gel of 4 mL in total are used for two sets of mini-slab plates (8.5 cm × 6 cm and 1-mm thickness).
6. Instead of human recombinant MMP-9, conditioned culture medium from TPA-induced human HT-1080 cells or MMP-9-purified using a gelatin-Sepharose column can be used ***(16,20)***.
7. It is important to aspirate the air from the running gel solution just before pouring it to avoid the formation of bubbles in the polymerized gel, which can cause distortion during electrophoresis. I usually use a 20-mL syringe for a 10-mL running gel solution and remove bubbles by closing the opening with parafilm and applying a negative pressure with the syringe of about 5–10 mL until the air bubbles caused by the low pressure in the syringe disappear.
8. The time required for the gel to polymerize will differ depending on the temperature and will take longer at cold temperature. If the amount of ammonium persulfate is increased,

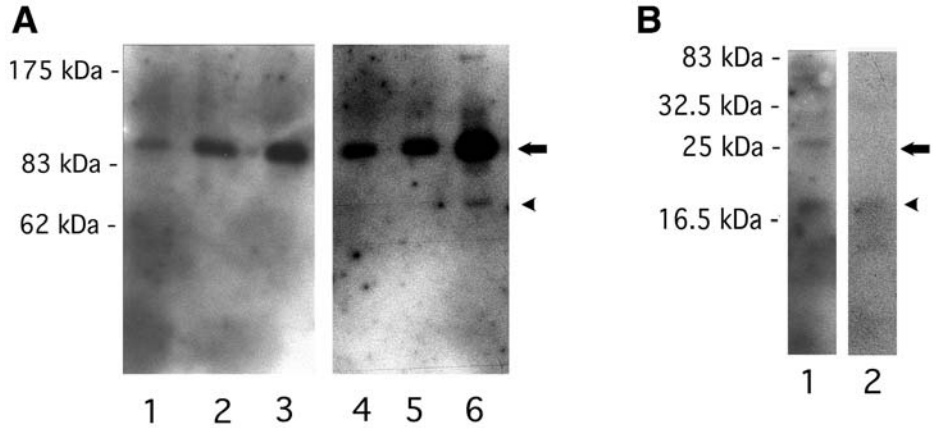


Fig. 3. Western blotting analysis patterns for detecting MMP-9 using a 7.5% acrylamide gel **(A)** and TIMP-1 using a 15% acrylamide gel **(B)**. **A**, Aliquots of conditioned culture media from keratinocytes stimulated with high Ca^{2+} concentrations (lanes 2 and 5) or the addition of TGF-β1 (lanes 3 and 6), or without stimulation (lanes 1 and 4). Samples were applied and analyzed using monoclonal antibodies raised against human MMP-9, one of which is not cross-reactive to MMP-2 (lanes 1, 2, and 3) whereas the other is cross-reactive to MMP-2 (lanes 4, 5, and 6; **ref.** ***18***). The MMP-2 band induced by TGF-β1 was apparently detected by the cross-reactivity. The arrow and arrowhead show the bands of MMP-9 and MMP-2, respectively. **B**, Aliquots of the conditioned media were applied and analyzed using two types of monoclonal antibodies, one of which is raised against human TIMP-1 (lane 1; **ref.** ***4***) and the other is an anti-TIMP-2 antibody (lane 2) without crossreactivity to TIMP-1. The arrow and arrowhead show the bands of TIMP-1 and TIMP-2, respectively.

the time required will be shorter. Conversely, reduce the amount of ammonium persulfate to slow down polymerization in a hot environment.

9. After diluting the sample with sample buffer, the enzyme will be gradually inactivated by SDS. To avoid this, the use of fresh sample after the addition of sample buffer is recommended for gelatin-zymography. The addition of dithiothreitol or 2-mercaptoethanol leads to the inactivation of enzymes and should be avoided.
10. High voltage can cause heating of gels, which can inactivate the enzymatic reaction. To avoid this, less than 100 V is desirable. I recommend performing the electrophoresis at 4°C in an extremely hot environment.
11. To remove the SDS in the gel, incubation in Triton X-100 is important. More than 100 mL of 2.5% Triton X-100 per gel (approx 5 mL of running gel) for each wash is recommended.
12. The reaction time for conditioned culture medium will differ depending on the concentration of the enzyme. For example, 10–15 µL in 100 µL of conditioned culture medium collected from subconfluent keratinocytes for 24 h in one well of a 48-well plate will be enough to observe an MMP-9 band in an overnight reaction.
13. Gelatin-zymography is known to be very sensitive and more enzymatic reaction is expected as the incubation time is longer. However, I have often experienced a limitation in the sensitivity after 5 d of incubation. It is considered that an extremely long incubation time can allow diffusion of enzymes from the gel.
14. The gelatin zymography method is useful not only for detecting metalloproteinases, but also sometimes for identifying other species of enzymes such as serine proteases. To do this or to confirm the gelatinolytic activity by MMPs, 10 m*M* EDTA should be added to the reaction buffer and to the Triton X-100 wash to inhibit the metalloproteinases.

15. Amido black 10B can be reused several times. The average staining time for the first use will be 10 min or so, but It will take longer as the solution is reused. Instead of amido black 10B, Coomassie brilliant blue R-250 can also be used. Detecting fluorescein isothiocyanate-labeled-gelatin with an ultraviolet transilluminator has been used to monitor the reaction of gelatinolytic activity as real-time zymography and reverse zymography ***(16)***.
16. A moderate destaining time will be more than overnight.
17. The gel will shrink in the destaining solution. After destaining, gels can be stored in tap water with 0.02% sodium azide for several days and the resolution will be relatively improved after storage in this way. I usually scan the image of each gel using a scanner attached to a personal computer. After scanning, quantitative analysis for the intensity of the band can be performed using NIH image software ***(14,15)***.
18. As the passage of human keratinocytes in culture is repeated several times, MMP-2 secretion into the conditioned medium will decrease ***(13)***.
19. The gel containing MMP-9 will be gradually inactivated by SDS. Therefore, electrophoresis should be started within a couple of hours after the gel is ready for use.
20. The reaction time for conditioned culture medium takes much longer in reverse zymography than in the gelatin-zymography described. For example, application of 10–15 µL in a 240-µL sample, which is concentrated by filter (Ultrafree, Millipore, Boston, MA) from 1.2 mL of conditioned culture medium obtained from subconfluent keratinocytes grown for 24 h in one well of a six-well plate, facilitates observation of a TIMP-1 band after 3 d of reaction. In addition, incubation with shaking (0.06–0.1*g*, 60–80 rpm) is important to detect the inhibitory activity of TIMPs against MMP-9.
21. During the whole procedure of Western blotting analysis, wear gloves to avoid contamination with fingerprints. In addition, avoid drying the PVDF membrane during the procedure after soaking in the methanol.
22. To assemble the blotting sandwich, be careful to remove air bubbles between the layers and move as quickly as possible to avoid band diffusion and resolution loss.
23. Moist papers can be used in the area uncovered by parafilm on the dish with closure of the lid to avoid evaporation during soaking.

Acknowledgment

This work was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science.

References

1 Birkedal-Hansen, H. (1995) Proteolytic remodeling of extracellular matrix. *Curr. Opin. Cell Biol.* **7,** 728–735.
2 McCawley, L. J. and Matrisian, L. M. (2001) Matrix metalloproteinases: they're not just for matrix anymore! *Curr. Opin. Cell Biol.* **13,** 534–540.
3. Stâhle-Bäckdahl, M. and Parks, W. C. (1993) 92-kd gelatinase is actively expressed by eosinophils and stored by neutrophils in squamous cell carcinoma. *Am. J. Pathol.* **142,** 995–1000.
4 Kobayashi, T., Onoda, N., Takagi, T., Hori, H., Hattori, S., Nagai, Y., et al. (1996) Immunolocalizations of human gelatinase (type IV collagenase, MMP-9) and TIMP (tissue inhibitor of metalloproteinases) in normal epidermis and some epidermal tumors. *Arch. Dermatol. Res.* **288,** 239–244.
5 Fisher, G. J., Wang, Z. Q., Datta, S. C., Varani, J., Kang, S., and Voorhees, J. J. (1997) Pathophysiology of premature skin aging induced by ultraviolet light. *N. Engl. J. Med.* **337,** 1419–1428.

6 Liu, Z., Zhou, X., Shapiro, S. D., Shipley, J. M., Twining, S. S., Diaz, L. A., et al. (2000) The serpin α1-proteinase inhibitor is a critical substrate for gelatinase B/MMP-9 in vivo. *Cell* **102,** 647–655.
7 Coussens, L. M., Tinkle, C. L., Hanahan, D., and Werb, Z. (2000) MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. *Cell* **103,** 481–490.
8 Kobayashi, T., Kishimoto, J., Ge, Y., Jin, W., Hudson, D. L., Ouahes, N., et al. (2001) A novel mechanism of matrix metalloproteinase-9 gene expression implies a role for keratinization. *EMBO Rep.* **2,** 604–608.
9 Mohan, R., Chintala, S. K., Jung, J. C., Villar, W. V., McCabe, F., Russo, L. A., et al. (2002) Matrix metalloproteinase gelatinase B (MMP-9) coordinates and effects epithelial regeneration. *J. Biol. Chem.* **277,** 2065–2072.
10. Wilhelm, S. M., Collier, I. E., Marmer, B. L., Eisen, A. Z., Grant, G. A., and Goldberg, G. I. (1989) SV40-transformed human lung fibroblasts secrete a 92-kDa type IV collagenase which is identical to that secreted by normal human macrophages. *J. Biol. Chem.* **264,** 17,213–17,221.
11. Salo, T., Lyons, J. G., Rahemtulla, F., Birkedal-Hansen, H., and Larjava, H. (1991) Transforming growth factor-β1 up-regulates type IV collagenase expression in cultured human keratinocytes. *J. Biol. Chem.* **266,** 11,436–11,441.
12 Kleiner, D. E. and Stetler-Stevenson, W. G. (1994) Quantitative zymography: detection of picogram quantities of gelatinases. *Anal. Biochem.* **218,** 325–329.
13 Kobayashi, T., Hattori, S., Nagai, Y., Sakuraoka, K., and Nishikawa, T. (1997) Secretion of different types of gelatinases from cultured human keratinocytes. *J. Dermatol.* **24,** 213–216.
14 Kobayashi, T., Hattori, S., Nagai, Y., Tajima, S., and Nishikawa, T. (1998) Differential regulation of MMP-2 and MMP-9 gelatinases in cultured human keratinocytes. *Dermatology* **197,** 1–5.
15 Kobayashi, T., Hattori, S., Nagai, Y., and Tajima, S. (2000) Differential regulation of the secretions of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinases-1 from human keratinocytes in culture. *IUBMB Life* **50,** 221–226.
16 Hattori, S., Fujisaki, H., Kiriyama, T., Yokoyama, T., and Irie, S. (2002) Real-time zymography and reverse zymography: a method for detecting activities of matrix metalloproteinases and their inhibitors using FITC-labeled collagen and casein as substrates. *Anal. Biochem.* **301,** 27–34.
17 Oliver, G. W., Leferson, J. D., Stetler-Stevenson, W. G., and Kleiner, D. E. (1997) Quantitative reverse zymography: analysis of picogram amounts of metalloproteinase inhibitors using gelatinase A and B reverse zymograms. *Anal. Biochem.* **244,** 161–166.
18 Kobayashi, T., Hori, H., Kanamori, T., Hattori, S., Takagi, T., Watanabe, H., Nishikawa, T., and Nagai, Y. (1993) Monoclonal antibodies to human polymorphonuclear leukocyte gelatinase (type IV collagenase) are cross-reactive with fibroblast gelatinase. *Biochem. Biophys. Res. Commun.* **193,** 490–496.
19 Vartio, T. and Baumann, M. (1989) Human gelatinase/type IV procollagenase is a regular plasma component. *FEBS Lett.* **25,** 285–289.
20. Kobayashi, T., Nishikawa, T., Hattori, S., Yoshida, N., Takagi, T., Watanabe, H., et al. (2001) Systematic separation and purification of elastase, gelatinase (matrix metalloproteinase 9), and collagenase (matrix metalloproteinase 8) from polymorphonuclear leukocytes in dialyzers previously used by patients with renal failure. *Protein Expr. Purif.* **22,** 45–51.

23

Characterization of Epithelial Cells in the Hair Follicle With S100 Proteins

Kenji Kizawa and Mayumi Ito

Summary

S100 proteins are the largest subgroup of Ca^{2+} binding proteins with the EF-hand structural motif. A unique feature of this protein family is that individual members are localized in specific cellular compartments. For example, various S100 proteins are expressed in very restricted regions of the hair follicle: S100A3 and S100A6 in distinct postmitotic differentiated epithelial cells and S100A4 and S100A6 in the epithelial stem cell compartments. Characterization of epithelial cells by their S100 protein expression profiles is therefore useful for a better understanding of the dynamic cellular events associated with hair follicle development and regeneration. This chapter presents our protocols for probe preparations and histochemical analyses of S100 proteins in hair follicle tissue, including simultaneous detection procedures for pulse-labeled proliferating cells.

Key Words:

S100 protein; hair follicle; *in situ* hybridization; immunohistochemistry; BrdU.

1. Introduction

Hair grows in a cyclic manner (e.g., anagen, the growing phase; catagen, the intermediate phase; and telogen, the resting phase). In the growing follicle, the epithelial matrix cells within the follicle bulb proliferate and ultimately differentiate into six different cell types with unique morphology (e.g., cortex, cuticle and medulla of the hair shaft, and cuticle, Henle's layer, and Huxley's layer of the inner root sheath). However, in the catagen phase, the lower epithelial portion of the hair follicle degenerates to the bilayered epithelial sac of the telogen follicle. Because the hair follicle retains epithelial stem cells throughout hair cycling, new hairs are replenished repeatedly after birth.

The hair follicle is a useful biological system in which to characterize the mechanisms of tissue regeneration, as well as the underlying cellular events, including proliferation, differentiation, and apoptosis. However, because the hair follicle is a complex structure composed of several distinct epithelial layers that dramatically transform during the hair cycle transition, it is often difficult to identify precisely different cell types and layers in the tissue sections. Therefore, a biochemical marker that can identify specifically particular layers of the hair follicle remains an essential tool.

S100 proteins represent the largest subgroup in the EF-hand calcium binding protein family of which some 20 members have been identified ***(1)***. Each member of the

From: *Methods in Molecular Biology, vol. 289:*
Epidermal Cells: Methods and Protocols
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

S100 protein family has been suggested to have various physiological functions that are regulated in a calcium dependant manner. A unique feature of S100 proteins is that individual members are localized in specific cell populations or tissue compartments. A remarkable example of this feature is represented by S100A3 expression in the hair follicle: it is predominantly expressed in the cuticle and cortex ***(2,3)***. However, S100A6, S100A8 and S100A9 have been shown to be expressed specifically in other types of differentiated follicular cells ***(4,5)***. Recently, we found that the stem cell region of the hair follicle (i.e., the bulge) expressed S100A4 and S100A6 proteins in a hair cycle dependant manner ***(6,7)***. Moreover, some studies using S100 expression as a biochemical marker of hair follicle tissues have helped to elucidate novel functions of the genes ***(8)***, as well as the regenerative mechanisms of the hair follicle ***(7)***.

In this chapter, we document protocols for probe preparations and histochemical examination of S100 expression developed for murine hair follicle tissues. We also provide methods for simultaneous detection of the S100 signal with pulse-labeled proliferating cells.

2. Materials

2.1. Probe Preparations

2.1.1. Riboprobe Production

1. RNaid plus kit (Bio 101, Vista, CA; cat. no. 1009-200).
2. Acid phenol mixture: add 70 mL of phenol melted at 50°C to 30 mL phenol buffer supplied in the RNaid plus kit and let it sit overnight before use.
3. Advantage RT for polymerase chain reaction (PCR) kit (Clontech, Palo Alto, CA; cat. no. K1402).
4. Advantage 2 PCR enzyme system (Clontech; cat. no. K1910).
5. DNA purification kit (Toyobo, Osaka, Japan; cat. no. PUR-101).
6. pGEM-T vector system II with JM109 high efficiency competent cells (Promega, Madison, WI; cat. no. A3610).
7. SOC medium (Gibco BRL; Gaithersburg, MD; cat. no. 15544-034).
8. LB medium: autoclave 10 g bacto-tryptone, 5 g bacto-yeast extract, and 5 g of NaCl/1 L (adjust to pH 7.0 with NaOH).
9. LB plates with ampicillin/IPTG/X-gal: autoclave 3 g agar/200 mL LB and cool to 50°C and then supplement with 0.8 mL of filter-sterilized ampicillin (25 mg/mL), 1 mL filter-sterilized 0.1 *M* IPTG, and 0.32 mL of X-gal (50 mg/mL dimethylformamide; Promega; cat. no. V3941). Pour 20 mL medium into 90-mm Petri dishes and let the agar harden.
10. QIAprep spin plasmid kit (Qiagen, Chatsworth, CA; cat. no. 27104).
11. RNA labeling kit (Roche Diagnostics, Mannheim, Germany; cat. no. 1 175 025).

2.1.2. Antibody Production

1. All reagents for peptide synthesis, including *N*-(9-fluorenyl)methoxycarbonyl (Fmoc) multiple antigenic peptide (MAP) resin are available from Applied Biosystems (Foster City, CA; cat. no. 401192 for 4-branching lysine core, 401193 for 8-branching lysine core).
2. Polyflon filter (Advantec, Tokyo, Japan, cat.no. PF060), 3-µm PTFE filter (Advantec; cat. no. T300A).
3. MAP bound ELISA titer plate: dissolve MAP prepared according to **Subheading 3.1.2.1.** in 50 m*M* carbonate buffer, pH 9.6. Place the MAP solution (1 µg/100 µL) in each well of the IMMULON2 ELISA titer plate (Dynatech Laboratory, Chantilly, VA; cat. no. 011 010 3455) at 4°C overnight.

4. Affi-Prep 10 (Bio-Rad, Hercules, CA; cat. no. 153-6099).
5. Protein extraction buffer: 50 m*M* Tris-HCl buffer, pH 7.5, containing 1% Triton X-100, 150 m*M* NaCl, 1 m*M* EGTA, and 1 m*M* phenylmethlsulfonyl fluoride (350 µL of 50 mg/mL isopropanol solution/100 mL buffer).
6. Bicinchoninic acid protein assay kit (Pierce, Rockford, IL; cat. no. 23225).
7. Anode buffer: 24.2 g Tris/1 L adjusted to pH 8.9 with HCl.
8. Cathode buffer: 12.1 g of Tris, 17.9 g tricine, and 1 g SDS/1 L (pH 8.2–8.3).
9. Gel buffer: 72.7 g Tris, 0.6 g SDS/200 mL; adjust to pH 8.45 with HCl.
10. Stacking acrylamide (3%C): 48 g of acrylamide, 1.5 g of bis-acrylamide/100 mL.
11. Separating acrylamide (6%C): 46.6 g acrylamide, 3.0 g bis-acrylamide/100 mL.
12. 10% Ammonium persulphate (APS).
13. *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED; Sigma, St Louis, MO; cat. no. T-7024).
14. Sample buffer: 12 mL glycerol, 10 mL 0.5 *M* Tris-HCl, pH 6.8, 4 g sodium dodecyl sulfate, 2 mL 2-mercaptoethanol/100 mL.
15. Transfer buffer: 12.1 g Tris, 14.4 g glycine, and 200 mL MeOH/1 L.
16. Prestained molecular markers: Kalidoscope polypeptide standard (Bio-Rad; cat. no. 161-0325).
17. Immobilon-P^{SQ} (Millipore, Bedford, MA; cat. no. ISEQ26260): Cut into 8.4 × 6.5 cm and pre-soak in transfer buffer following a dip in MeOH.
18. Blocking buffer A: 1 g BSA/20 mL phosphate-buffered saline (PBS).
19. Blocking buffer B: 0.4 g BSA/40 mL PBS.

2.2. Sample Preparation

1. Hair removing wax: melt 5.5 g of white beeswax and 4.5 g rosin (for treatment of 10 mice) on boiled water bath. Avoid repetitive use.
2. 5-Bromo-2′-deoxyuridine (BrdU) (Sigma; cat. no. B-9285).
3. 4% paraformaldehyde/PBS: dissolve 20 g PFA in 450 mL PBS with a few drops of 10 *N* NaOH solution on hot-plate stirrer for several hours. After cooling and pH adjustment to 7.4, make up the total volume to 500 mL with PBS.
4. Paraffin (solidification point about 56–58°C; Merck, Darmstadt, Germany; cat. no. 1.07164).
5. Superfrost APS-coated micro slide glass (Matsunami, Kishiwada, Japan; cat. no. S8441).

2.3. Histochemistory

2.3.1. In Situ *Hybridization*

1. DAKOPEN (DakoCytomation, Carpintera, CA; cat. no. S2002).
2. Proteinase K (Roche Diagonostics; cat. no. 745 723): store at –20°C in 100-µL aliquots at 20 mg/mL in TE solution.
3. Alkaline phosphatase-conjugated Fab fragments of anti-digoxigenin goat antibody (Roche Diagonostics; cat. no. 1 093 274).
4. TE: 0.1 *M* Tris, 50 m*M* EDTA, pH 8.0.
5. TEA/acetic anhydride: combine 95 mL diethylpyrocarbonate (DEPC)-treated water, 1.5 g triethanolamine, 3 mL 5 *M* NaCl, and 0.4 mL of HCl. Immediately before use, add 250 µL acetic anhydride to the mixture.
6. Hybridization mix: combine 10 mL 0.1 *M* Tris-HCl, pH 8.0, 50 mL formamide, 25 mL 20X standard sodium citrate (SSC), 2 mL 50X Denhardt's solution (Wako, Osaka, Japan; cat. no. 043-21871), 10 g 10% dextran sulfate, 0.2 mL 10% sodium dodecyl sulfate, 25 mg of yeast tRNA, 2 g of blocking reagent, and 100 mg *N*-lauroyl sarcosine, and then make up total volume to 100 mL with DEPC-treated water. Store the 1-mL aliquots at –20°C.

7. Maleate buffer: 0.1 *M* maleic acid, 0.15 *M* NaCl, pH 7.5.
8. NBT/BCIP stock solution (Roche Diagonostics; cat. no. 1 681 451).

2.3.2. Immunostaining

1. Vectastain Elite ABC kit (Vector Laboratory, Burlingame, CA; cat. no. PK-6101 for rabbit IgG, PK-6104 for rat IgG).
2. Vectastain ABC-AP kit (Vector Laboratory; cat. no. AK-5001).
3. DAB substrate kit (Vector Laboratory; cat. no. SK-4100).
4. Vector blue substrate kit (Vector Laboratory; cat. no. SK-5300).
5. Mount-Quick (Daido Sangyo, Toda, Japan).

2.3.3. Detection of BrdU-Labeled Cells

1. Monoclonal mouse antibody against BrdU (Becton Dickinson, San Jose, CA; cat. no. 347580).
2. Monoclonal rat antibody against BrdU (Harlan Sera-Laboratory, Loughborough, UK; cat. no. OBT0030).

3. Methods

3.1. Probe Preparations

Among the 18 bona fide members of the S100 protein family, almost all the human and murine complimentary deoxyribonucleic acid (cDNAs) have been cloned, and their nucleotide and deduced amino acid sequences are available in the public database (e.g., www.ncbi.nlm.nih.gov/Genbank/index.html, **Table 1**). Such information has allowed us to prepare riboprobes and antibodies to S100 proteins required for the analyses of their expression and tissue distribution. This section describes our probe preparation methods adopted for murine S100A3, S100A4, and S100A6 ***(2,3,6)***.

3.1.1. Riboprobe Production

3.1.1.1. Total RNA Preparation With the RNAid Plus Kit

1. Homogenize the excised skin tissue (~200 mg) in 1 mL cell lysis solution.
2. Transfer 750 µL homogenate to a 2-mL Eppendorf tube.
3. Add 75 µL of 3 *M* sodium acetate and 750 µL acid phenol mixture, and vortex.
4. Add 410 µL chlorform-isoamyl alchohol (24 : 1), vortex and incubate on ice for 15 min.
5. Spin at 4°C for 20 min at 10,000*g* and transfer the top phase containing the RNA to a new tube.
6. Add 820 µL of chlorform–isoamyl alchohol and vortex.
7. Spin for 5 min and transfer the top phase to a 1.5-mL Eppendorf tube.
8. Add 20 µL RNAMATRIX and incubate at room temperature with occasional mixing.
9. Spin for 5 min and resuspend the pellet in 500 µL RNA wash solution. Repeat this washing step twice.
10. Spin for 5 min and remove the liquid phase.
11. Add 20 µL of DEPC water and incubate at 55°C for 5 min.
12. Spin for 5 min and store the supernatant containing total RNA at –70°C.

3.1.1.2. cDNA Template Synthesis Using Advantage RT for PCR Kit

1. Combine 1 µL of oligo$(dT)_{18}$ primer (20 µ*M*) to 1 µg total RNA/12.5 µL DEPC water.
2. Heat the tube to 70°C for 2 min and then cool to 4°C.
3. Add 4 µL of 5X reaction buffer, 1 µL of dNTP mix (10 m*M* each), 0.5 µL RNase inhibitor, and 1 µL of moloney-murine leukemia virus reverse transcriptase.

Table 1
Database Accession Numbers of S100 cDNAs

	Human	Mouse	Rat
S100A1	X58079	AF087687	S68809
S100A2	NM_005978		
S100A3	Z18948	AF004941	AF140231
S100A4	M80563	X05835	J03628
S100A5	Z18954	AF087469	
S100A6	M18981	X66449	AF140232
S100A7	M86757	AY582964	
S100A8	Y00278	S57123	L18891
S100A9	M26311	M83219	L18948
S100A10	M38591	M16465	J03627
S100A11	D38583	AK003889	
S100A12	D83664		
S100A13	X99920	X99921	
S100A14	AY007220	AK003669	
S100A15	NM_176823		
S100A16	NM_080388		
S100B	AH002981	NM_009115	X01090
S100P	X65614		

4. Incubate at 42°C for 1 h and then heat to 94°C for 5 min.
5. Dilute with 80 µL of DEPC water and store at –70°C.

3.1.1.3. PCR Primer Preparation

1. Design specific sequence primer sets for each S100 gene with software tools (e.g., primer3; www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi).
2. Synthesize primers using a DNA synthesizer **(Note 1)**.

3.1.1.4. cDNA Amplification Using Taq DNA Polymerase (e.g., Advantage 2 PCR Enzyme System)

1. Combine 40 µL PCR-grade water, 5 µL 10X PCR buffer, 1 µL cDNA template, 2 µL primer set (10 µ*M* each), 1 µL dNTP mix (10 m*M* each), and 1 µL polymerase mix.
2. Commence thermal cycling with the following parameters: 95°C, 1 min; 30 cycles: 95°C, 15 s; and 68°C, 3 min.

3.1.1.5. DNA Purification With a DNA Purification Kit

1. Electrophorese the PCR product on a 1.5% agarose gel.
2. Cut out the DNA band of the appropriate size and transfer the gel to a preweighed 1.5-mL Eppendorf tube.
3. Add 6 *M* NaI (300 µL/100 mg gel) and melt the gel at 55°C for 3 min.
4. Add 5 µL silicamilk, incubate for 10 min on ice, and then spin for 2 min.
5. Resuspend the pellet in 0.5 mL of ice-cold gel wash buffer and spin for 2 min. Repeat this washing step twice.
6. Suspend the pellet in 5 µL of TE buffer, elute DNA at 55°C for 3 min, and then spin for 30 s. Repeat this elution step twice and pool both supernatants.

3.1.1.6. Ligation of the PCR Product into the pGEM-T Vector, Which Contains Promoters for T7 and SP6 RNA Polymerase, With T4 DNA Ligase

1. Combine 10–15 ng of PCR product (200–300 bp), 1 µL T4 DNA ligase 10X buffer, 1 µL pGEM-T vector (50 ng), 1 µL T4 DNA ligase (3 U), and dH_2O to a final volume of 10 µL.
2. Mix the reactions by pipetting, and incubate overnight at 4°C.

3.1.1.7. Transformation and Selection of Transformants With Blue/White Color Screening

1. Add 2 µL of ligation reaction to a sterile 1.5-mL Eppendorf tube on ice.
2. Add 50 µL of just thawed JM109 competent cells and flick the tube to mix.
3. Place the tube on ice for 20 min.
4. Heat for 45–50 s in a water bath at 42°C, and then cool on ice for 2 min.
5. Add 950 µL SOC medium and incubate for 1.5 h at 37°C with shaking (~150 rpm).
6. Plate 100 µL of the transformation culture **(Note 2)** onto LB plates with ampicillin/IPTG/X-gal.
7. Incubate the plates overnight at 37°C.
8. Select single white colonies and grow them overnight at 37°C in 5 mL LB medium.
9. Isolate the plasmid DNA from *Escherichia coli* with a preparation kit (e.g., QIAprep spin plasmid kit).

3.1.1.8. In Vitro Transcription

1. Linearize the plasmid DNA (1 µg) with the appropriate restriction enzyme.
2. Add an equivalent volume of TE saturated phenol to the reaction mixture, spin for 5 min at 10,000*g*, and transfer the top phase to a new tube. Repeat this step twice.
3. Add an equal volume of chloroform/IAA, spin for 5 min, and transfer the top phase to a new tube.
4. Precipitate DNA in the supernatant with EtOH.
5. Dissolve the pellet in 13 µL DEPC-treated distilled water, add the components of an RNA labeling kit (2 µL NTP labeling mixture, 2 µL transcription buffer, 1 µL RNase inhibitor, and 2 µL RNA polymerase SP6 or T7) and incubate at 37°C for 2 h.
6. Add 2 µL 0.2 *M* EDTA, pH 8.0, to stop the reaction.
7. Add 2.5 µL 4 *M* LiCl and 75 µL cold EtOH, then place the tube at –70°C for 2 h.
8. After centrifugation, add 100 µL DEPC-treated water to dissolve the precipitated digoxygenin-labeled RNA probe.

*3.1.2. Antibody Production (**Note 1**)*

3.1.2.1. MAP Syntheses ***(9)***

1. Compute the local hydrophilicity ***(10)*** using the amino acid scale of S100 protein and software tools (e.g., ProtScale, www.expasy.org/cgi-bin/protscale.pl).
2. Identify the amino acid sequence with the highest average scale as the antigenic determinant (**Note 3**).
3. Add sequentially Fmoc-amino acids onto a four- or eight-branching lysine core according to the 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophasphate activation protocol (FastMoc chemistry, Applied Biosystems) using a 431A solid-phase peptide synthesizer.
4. Transfer the MAP resin (100 mg) into a round-bottom flask containing a microstir bar.
5. Add the cleavage mixture (e.g., 4.75 mL of TFA/0.25 mL of ethanedithiol/0.25 mL H_2O; refer to Instruction to cleavage technique, Applied Biosystems) on ice bath.
6. Stir for 3–5 h at room temperature.

7. Transfer the reaction mixture into a glass funnel containing a polyflon filter, and then attach the funnel to a vacuum filtration flask.
8. Wash the resin with 1 mL TFA and 5–10 mL dichloromethan.
9. Condense the volume of the pass-through mixture to 1–2 mL using a rotary evaporator below 40°C.
10. Add 50 mL of cold Et_2O.
11. Remove the solvent by passing through a PTFE filter.
12. Dissolve the MAP precipitated onto the filter with 2 *M* acetic acid.
13. Dialyze at 4°C against 0.2 *M* acetic acid and then lyophilize.

3.1.2.2. Antibody Production

1. Inject the emulsified mixture of the MAP (250 µg/0.5 mL) and 0.5 mL complete Freund's adjuvant for the first injection or incomplete Freund's adjuvant for booster injections into rabbits subcutaneously at 3-wk intervals.
2. Collect the rabbit blood (1–2 mL) from the ear vein 1 wk after each antigen injection and centrifuge at 1000*g* for 15 min.
3. Titrate the resultant serum by enzyme-linked immunosorbent assay (ELISA) using the MAP bound plate.
4. Collect whole rabbit blood (>60 mL) from the carotid artery during the plateau phase of the titre established by ELISA **(Note 4)** and centrifuge to obtain antiserum.
5. Purify the specific antibody from the antiserum using a MAP-bound Affi-Prep 10 column.

3.1.2.3. Examination of Antibody Specificity by Western Blot Analyses **(Note 5)**

1. Lyse the excised small pieces of skin tissue in a two to threefold volume of cold extraction buffer for 10 min.
2. Determine the protein concentration with the BCA method using bovine serum albumin as a standard.
3. Separating gel (16.5% T) preparation: mix 5 mL each of separating acrylamide (6%C), gel buffer and 40% glycerol, and then degas. After the addition of 150 µL of 10% APS and 15 µL of TEMED, the combined mixture was stirred and poured into a glass plate assembly with a spacer (dimensions of $8 \times 8 \times 0.1$ cm). Gently overlay the gel with distilled water, and let stand for 20 min to polymerize the separating gel.
4. Stacking gel preparation: mix 1 mL of stacking acrylamide (3%C), 3.1 mL of gel buffer, and 8.4 mL distilled water containing 150 µL 10% APS and 20 µL TEMED. After decantation of the overlaying water, pour the combined mixture onto the separating gel being careful to completely fill the wells around the comb, and let stand for 10 min to polymerize the stacking gel.
5. Set the gel into the electrophoresis apparatus, fill the cathode and anode buffer chambers, and flush out any unpolymerized acrylamide.
6. Mix the protein extract (10–100 µg), or prestained molecular markers, with sample buffer, heat at 95°C for 2 min, then apply the mixtures to the wells.
7. Set constant current to 12 mA and run the gel for 16 h.
8. In the blotting apparatus (AE-6677; Atto, Tokyo, Japan), stack seven sheets of chromatography paper, a pretreated polyvinyleine difluoride membrane, the gel, and another seven sheets of chromatography paper.
9. Set the current to 110 mA for 1 h to start the electro-blotting.
10. Immerse the transferred polyvinyleine difluoride membrane into following solutions:
    a. Blocking buffer A, 2 h.
    b. 1 µg/mL affinity-purified antibody or 1000- to 10,000-fold diluted antiserum/blocking buffer B, 1 h.

c. PBS-T, 3X, 5 min.
d. Biotinylated goat anti-rabbit IgG antibody/blocking buffer B, 30 min.
e. PBS-T, 3X, 5 min.
f. Avidin–peroxidase/blocking buffer B, 30 min.
g. PBS-T, 3X, 5 min.
h. 4 mg diaminobenzidine/20 mL PBS/10 µL 30% H_2O_2, 2–10 min.
i. Distilled water, 3 min.

3.2. Hair Follicle Tissue Section Preparation

The mouse pelage follicle is the premiere experimental material in hair cycle biology. Using dorsal skin tissue, we can definitively analyze the stage of hair cycling since hairs grow synchronously after birth (e.g., anagen, 6–9 d; catagen, 18–19 d, and telogen, 8 wk) ***(12)***. Moreover, we can experimentally induce the anagen phase by hair plucking during the telogen phase, and the time course of hair cycling induced by plucking is also well established ***(13)***. Administration of BrdU just before sacrifice ensures precise discrimination between cells in the transit amplifying region and the postmitotic differentiated region in the hair follicle as specially described in **Subheading 3.4.**

3.2.1. Experimental Induction of the Anagen Phase

1. Paint the hair-removing wax onto the back of 8-wk-old mice.
2. After the wax hardens, gently remove the embedded dorsal fur.

3.2.2. Pulse-Labeling Procedure With BrdU

1. Administer BrdU intraperitoneally to experimental animals (20 mg/kg body weight).
2. Continue processing through the next steps after at 90 min.

3.2.3. Tissue Preparation

1. Excise dorsal skin tissue after removal of hairs with scissors.
2. Fix the tissues in 4% PFA/PBS for 16 h at 4°C.
3. Dehydrate the tissues 3X in EtOH for 1 h each and 3X in xylene for 15 min each.
4. Infiltrate the tissues 4X with paraffin at 58°C for 1 h each, and then embed.
5. Section the paraffin block into 6-µm-thick sections using a microtome and float the sections in warm DEPC-treated water (37°C) to extend the tissue.
6. Place the sections on APS-coated slides and dry at 37°C overnight.

3.3. Identification of Hair Follicle Tissues From the Distribution of S100 mRNA and Protein

This section describes methods for optimized detection of S100 mRNAs by *in situ* hybridization and S100 protein by immunohistochemistry on hair follicle tissue sections **(Note 6)**. Epithelial matrix cells in the bulb of the anagen follicle differentiate into three layers of the hair shaft (medulla, cortex and cuticle) and three layers of the inner root sheath (cuticle, Huxley's layer and Henle's layer). We have established that S100A3 mRNA and protein is localized predominantly in the cuticle of the hair shaft and to a lesser extent in the cortex (**Fig. 1A**; **ref. *3***). However, S100A6 mRNA is localized in Hexley's layer of the inner root sheath, the innermost cell layer of the outer root sheath and the medulla **(Fig. 1B)**. Staining for neither protein is seen in Henle's layer or the cuticle of inner root sheath. The distribution of S100A3 and S100A6 in the anagen follicle is illustrated in **Fig. 1C**.

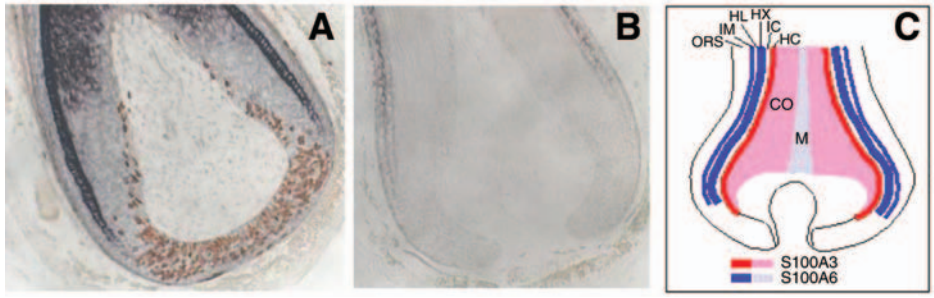


Fig. 1. Localization of S100A3 and S100A6 mRNA in the anagen follicle. **A,** Anagen vibrissa follicles of BrdU-treated rat were subjected to *in situ* hybridization for S100A3 mRNA followed by BrdU immunohistochemistry. Signals are seen in the cuticular layer and cortex of the hair shaft but not in the proliferating matrix cells pulse labeled with BrdU. **B,** A rat anagen vibrissa follicle was subjected to *in situ* hybridization with antisense probe to S100A6 mRNA. Signals are seen intensely in Huxley's layer of the inner root sheath and the innermost layer of outer root sheath, and faintly in the medulla. **C,** Schematic illustration of S100A3 and S100A6 mRNA expression in the anagen follicle. This pattern is conserved between the vibrissa and pelage follicle.

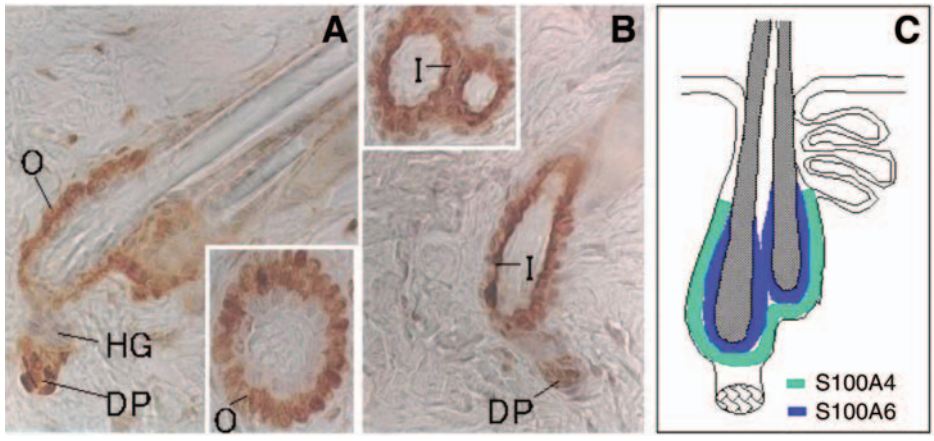


Fig. 2. Localization of S100A4 and S100A6 protein in the telogen follicle. Pelage follicles of 8-wk-old mice were subjected to immunohistochemistry with antibodies to S100A4 **(A)** and S100A6 **(B)**. S100A4 protein is localized in the outer layer of the epithelial sac, whereas S100A6 is preferentially distributed in its inner layer. Staining for both proteins is seen in the dermal papillae, but not in the hair germ. Adapted from ***(6)***. **(C)** Schematic illustration of S100A4 and S100A6 expression in the telogen follicle.

The epithelial portion of the telogen follicle consists of a bilayered epithelial sac surrounding the club hair and the secondary hair germ. The attachment site of the arrector pili muscle is referred to as the bulge area, which is indistinguishable from the outer layer of the epithelial sac. The anti-S100A4 antibody stains specifically this area, but not the secondary hair germ **(Fig. 2A)**. In contrast, the anti-S100A6 antibody stains the inner layer much more intensely than the outer layer **(Fig. 2B)**. Thus, antibodies to S100 proteins biochemically discriminate three epithelial regions of the telogen follicle (i.e., the inner and the outer layers of the epithelial sac, and the hair germ; **Fig. 2C**; **ref. *6,7***).

3.3.1. In Situ *Hybridization*

1. Deparaffinization with xylene **(Note 7)**, 2X, 15 min.
2. Treat sequentially with 100%, 100%, 90%, and 70% EtOH for 5 min each.
3. Wash with PBS, 2X, 15 min.
4. Incubate in 0.2 *M* HCl, 20 min.
5. Treat with proteinase K/TE buffer (20 µg/mL; preincubate for 1 h; **Note 8**), 10 min at 37°C.
6. Wash with PBS, 5 min.
7. Fix with 4% paraformaldehyde/PBS, 20 min.
8. Wash with DEPC-treated dH_2O, 30 s.
9. Incubate with Glycine/PBS (2 mg/mL), 2X, 15 min.
10. Treat with TEA/acetic anhydride, 10 min.
11. Repeat **step 2** but use the alcohol series in reverse order, 5 min each.
12. Air-dry and outline sections with DAKOPEN.
13. Prehybridize in the hybridization mix (use 80 µL per slide; **Note 9**), 2 h at 45°C.
14. Heat digoxigenin-labeled RNA probe for S100 mRNA, 3 min at 85°C.
15. Dilute the probe with hybridization mix (0.2 ng/µL).
16. Hybridize the probe to the sections (use 80 µL per slide), overnight at 45°C.
17. Wash with 5X SSC for 30 s at 45°C.
18. Incubate with 2X SSC/50% formamide for 30 min at 45°C.
19. Incubate with 2X SSC for 20 min at 45°C.
20. Incubate with 0.2X SSC 2X for 20 min at 45°C.
21. Wash with 0.3% Tween-20/maleate buffer for 5 min.
22. Incubate with 1.5% blocking reagent/10% sheep serum/0.005% yeast tRNA/maleate buffer for 60 min.
23. Incubate with alkaline phosphatase-conjugated antidigoxigenin antibody (1000-fold dilution with foregoing blocking buffer) for 30 min.
24. Wash with 0.3% Tween-20/maleate buffer for 15 min.
25. Wash with maleate buffer for 15 min.
26. Wash with 0.1 *M* Tris-HCl buffer (pH 9.5)/0.1 *M* NaCl/0.05 *M* $MgCl_2$ for 3 min.
27. Incubate with NBT/BCIP stock solution (20-fold dilution in foregoing buffer) for 0.5–4 h.
28. Wash in tap water for 10 min.
29. Mount with glycerol.

3.3.2. *Immunohistochemistry*

1. Deparaffinize and rehydrate as for **steps 1–3** of **Subheading 3.3.1.**
2. Treat with proteinase K/TE buffer (20 µg/mL), 10 min at 37°C.
3. Wash with PBS for 5 min.
4. Fix with 4% paraformaldehyde/PBS for 20 min.
5. Treat with 0.3% H_2O_2 in MeOH for 45 min.
6. Wash with PBS for 5 min.
7. Block with 1.5% goat serum/PBS for 20 min.
8. Incubate with affinity purified rabbit anti-S100 antibody/PBS (1 µg/mL) for 45 min.
9. Wash with PBS 2X for 5 min.
10. Incubate with biotinylated goat anti-rabbit IgG antibody/foregoing blocking buffer (2.5 µg/mL) for 30 min.
11. Wash with PBS 2X for 5 min.
12. Incubate with avidin and biotinylated horseradish–peroxidase complex/PBS **(Note 10)** for 45 min.
13. Wash with PBS 2X for 5 min.

14. Treat with 40 µg/mL diaminobenzidine/Tris-HCl buffer (pH 7.5)/0.0004% H_2O_2 for 2–10 min.
15. Wash in tap water.
16. Dehydrate through the series of ethanol and finally clear in xylene.
17. Mount with Mount-Quick.

3.4. Simultaneous Detection of S100 Protein With BrdU Labels

Through the analyses of the spatial distribution of proliferating cells, we can clearly distinguish cells in the transit amplifying region from those in the post mitotic differentiated region within the hair follicle. Detection of proliferating cells together with S100 expression would clarify whether cells in the specific layers of the hair follicle identified by S100 expressions were proliferating at the time of fixation **(Fig. 1A)**. This section describes methods for simultaneous detection of S100 protein and BrdU pulse-labeled proliferating cells on the same tissue section.

3.4.1. Combined BrdU Immunohistochemistry/ In Situ Hybridization

1. Subject tissue sections of BrdU-treated animals (refer to **Subheading 3.2.2.**) to *in situ* hybridization according to the protocol outlined in **Subheading 3.3.1.**
2. After the final visualization step of *in situ* hybridization, place slides in distilled water.
3. Treat with 2 *M* HCl for partial denaturation of nuclear DNA for 1 h at 37°C.
4. Incubate in 0.1 *M* $Na_2B_4O_7$ for 5 min.
5. Wash with PBS 3X for 5 min.
6. Block with 1.5% rabbit serum/PBS for 20 min.
7. Incubate with monoclonal anti-BrdU antibody (1000-fold dilution with PBS; **Note 11**) for 45 min.
8. Wash with PBS 2X for 5 min.
9. Incubate with biotinylated rabbit anti-rat IgG antibody/foregoing blocking buffer (2.5 µg/mL) for 30 min.
10. Wash with PBS 2X for 5 min.
11. Incubate with avidin-biotinylated horseradish–peroxidase complex/PBS **(Note 10)** for 45 min.
12. Wash with PBS 2X for 5 min.
13. Incubate with 40 µg/mL diaminobenzidine/Tris-HCl buffer (pH 7.5)/400 ppm H_2O_2 for 2–10 min.
14. Wash in tap water.
15. Mount with glycerol.

3.4.2. Double Immunostaining for S100 Protein and BrdU

1. Conduct section deparaffinization, antigen retrieval, postfixation, and inactivation of endogenous enzyme according to **steps 1–6** of **Subheading 3.3.2.**
2. Block with 1.5% goat serum/PBS, 20 min.
3. Incubate with rabbit antiserum against S100 antibody/PBS (100-fold dilution) for 45 min.
4. Wash with PBS 2X for 5 min.
5. Incubate with biotinylated goat anti-rabbit IgG antibody/PBS (2.5 µg/mL) for 30 min.
6. Wash with PBS 2X for 5 min.
7. Incubate with avidin-biotinylated alkaline–phosphatase complex/PBS **(Note 12)** for 45 min.
8. Wash with PBS 2X for 5 min.

9. Visualize using Vector blue alkaline phosphatase substrate for 30 min.
10. Place slides in distilled water and then process to **steps 3–15** of **Subheading 3.4.1.** for the detection of BrdU immunoreactivity.

4. Notes

1. Custom service for DNA and peptide syntheses is available (e.g., Qiagen, www.qiagen.com). A specific pathogen-free breeding condition is advantageous to produce polyclonal antiserum with high titer. Custom service is also available for immunization.
2. If a higher number of colonies is required, centrifuge the culture to pellet cells and re-suspend cells in 100 µL of SOC medium.
3. Hexapeptide average is recommended for scaling by Hopp and Woods ***(10)***.
4. Typically, the ELISA titer peaks after three to four injections.
5. Tricine–sodium dodecyl sulfate–polyacrylamide gel electrophoreses ***(11)*** enables the electrophoretic separation of S100 proteins (~10 kDa) with high resolution.
6. The distribution of mRNA versus protein is different in some regions of the hair follicle. For example, S100A4 protein, but not mRNA, is localized in the outer layer of the epithelial sac of the telogen follicle ***(6)***.
7. Deparaffinization, antigen retrieval, postfixation, and washing steps are carried out in a coloration vessel.
8. Proteinase K Stock solution (100 µL) is added to 100 mL TE. The vessel is incubated at 37°C in a water bath.
9. Hybridization steps, antibody application, and staining steps are conducted in a humidified chamber.
10. Add two drops each of Reagents A (avidin) and B (biotinylated horseradish peroxidase) supplied in the Elite ABC kit into 5 mL of PBS and allow forming complex by standing for 30 min before use.
11. Use mouse antibody against BrdU for rat tissues and rat antibody for mouse tissues.
12. Add two drops each of Reagents A (avidin) and B (biotinylated alkaline phosphatase) supplied by the ABC-AP kit into 10 mL of PBS and allow forming complex by standing for 30 min before use.

Acknowledgment

The authors would like to thank other past and present members of the Basic Research Laboratory, Kanebo Ltd, who have contributed to developing the protocols described.

References

1 Heizmann, C. W., Fritz, G., and Schäfer, B. W. (2002) S100 proteins: structure, function and pathology. *Front. Biosci.* **7,** d1356–d1368.
2 Kizawa, K., Uchiwa, H., and Murakami, U. (1996) Highly-expressed S100A3, a calcium-binding protein, in human hair cuticle. *Biochim. Biophys. Acta* **1312,** 94–98.
3 Kizawa, K., Tsuchimoto, S., Hashimoto, K., and Uchiwa, H. (1998) Gene expression of mouse S100A3, a cysteine-rich calcium-binding protein, in developing hair follicle. *J. Invest. Dermatol.* **111,** 879–886.
4 Wood, L., Carter, D., Mills, M., Hatzenbuhler, N., and Vogeli, G. (1991) Expression of calcyclin, a calcium-binding protein, in the keratogeous region of growing hair follicle. *J. Invest. Dermatol.* **96,** 383–387.
5 Schmidt, M., Gillitzer, R., Toksoy, A., Brocker, E.-B., Rapp, U. R., Paus, R., et al. (2001) Selective expression of calcium-binding proteins S100A8 and S100A9 at distinct sites of hair follicles. *J. Invest. Dermatol.* **117,** 748–750.

6 Ito, M. and Kizawa, K. (2001) Expression of calcium-binding S100 proteins A4 and A6 in Regions of the epithelial sac associated with the onset of hair follicle regeneration. *J. Invest. Dermatol.* **116,** 956–963.
7 Ito, M., Kizawa K., Toyoda, M., and Morohashi, M. (2002) Label-retaining cells residing in the bulge region are directed to cell death after plucking followed by healing from the surviving hair germ. *J. Invest. Dermatol.* **119,** 1310–1316.
8 Lin, M.-H., Leimeister, C., Gessler, M., and Kopan, R. (2000) Activation of the Notch pathway in the hair cortex leads to aberrant differentiation of the adjacent hair-shaft layers. *Development* **127,** 2421–2432.
9 Posnett, D. N., McGrath, H., and Tam, J. P. (1988) A novel method for producing anti-peptide antibodies: production of site-specific antibodies to the T cell antigen receptor β-chain. *J. Biol. Chem.* **263,** 1719–1725.
10 Hopp, T. P. and Woods, K. R. (1981) Prediction of protein antigenic determinants from amino acid sequences. *Proc. Natl. Acad. Sci. USA* **78,** 3824–3828.
11. Schägger, H., and von Jagow, G. (1989) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **40,** 91–99.
12. Dry, F. W. (1926) The coat of the mouse (Mus musculus). *J. Genet.* **16,** 287–340.
13. Chase, H. B., Rauch, H., and Smith, V. W. (1951) Critical stages of hair development and pigmentation in the mouse. *Physiol. Zool.* **24,** 1–8.

24

Immunoelectron Microscopic Analysis of Cornified Cell Envelopes and Antigen Retrieval

Akemi Ishida-Yamamoto

Summary

In this chapter, postembedding immunoelectron microscopy methods for studies of cornified cell envelopes are provided. Human epidermal tissue samples are used as the material. The samples are cryo-fixed without chemical fixation, freeze-substituted at a low temperature, and embedded in Lowicryl K11M resin. For immunostaining, colloidal gold-conjugated secondary antibodies are used. Methods for retrieval of masked epitopes are also described.

Key Words:

Immunoelectron microscopy; postembedding method; cornified cell envelopes; keratinocytes; involucrin; loricrin; epitope masking.

1. Introduction

The cornified cell envelope (CE) is a highly insoluble structure that replace the plasma membrane of differentiating keratinocytes ***(1)***. It consists of various cross-linked precursor proteins, including involucrin and loricrin. During the last several years, we have begun to understand the normal assembly process of CE and its alteration in skin disorders. In this chapter, protocols to study the localization of CE proteins using postembedding immunoelectron microscopy will be described in detail ***(2)***. Because some of the epitopes of CE proteins are masked during the assembly of CE or tissue processing, methods for antigen retrieval will also be provided ***(3,4)***. All of the protocols are developed for the use with human skin.

2. Materials

2.1. Antibodies for Immunoelectron Microscopy

1. Rabbit polyclonal antibody against loricrin (AF62; BAbCO, Richmond, CA; cat. no. PRB-145P).
2. Mouse monoclonal antibody against involucrin (SY5; NeoMarkers, Fremount, CA; cat. no. MS-126-P).
3. Rabbit polyclonal antibody against involucrin (BT-601; Biomedical Technologies, Stoughton, MA; cat. no. BT-601).

From: *Methods in Molecular Biology, vol. 289:*
Epidermal Cells: Methods and Protocols
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

2.2. Reagents

2.2.1. Preparation of Resin-Embedded Tissue Samples

1. Lowicryl K11M (TAAB Laboratories, UK; cat. no. L016).
2. Nickel 150-grid mesh (VECO®Plain Nickel Grids, Ernest F. Fullam, Inc. NY; cat. no. 26121).
3. Formvar: 3% Formvar Soluction (LADD Research Industries; cat. no. 12065), dilute at 0.3% in 1,2-dichloroethane. Store at room temperature up to 1 mo.

2.2.2. Immunoelectron Microscopy

1. Fish gelatin (Amersham Biosciences, Buckinghamshire, UK; cat. no. RPN416).
2. Preincubation buffer: 1% bovine serum albumin (BSA), 5% normal goat serum (NGS), 0.1% fish gelatin, 0.02% sodium azid/phosphate-buffered saline (PBS) pH 7.4.
3. First incubation buffer: 1% BSA, 1% NGS, 0.1% fish gelatin, 0.02% sodium azide /PBS.
4. Tris-HCl buffered saline pH 8.2 (TBS): 0.242 g Tris (tris-hydroxmethyl-aminomethane), 0.13 g NaN_3 (sodium azide), 0.9 g NaCl; Adjust pH with 0.1 *N* HCl to 8.2. Add dH_2O to final total volume of 100 mL.
5. Second incubation buffer: 1% BSA, 1% NGS, 0.1% fish gelatin, in TBS.
6. 5-nm Gold-conjugated goat anti-rabbit IgG (Amersham Biosciences, Buckinghamshire, UK; cat. no. RPN420).
7. 10-nm Gold-conjugated goat anti-rabbit IgG (Amersham Biosciences; cat. no. RPN421).
8. 5-nm Gold-conjugated goat anti-mouse IgG (Amersham Biosciences; cat. no. RPN424).
9. 10-nm Gold-conjugated goat anti-mouse IgG (Amersham Biosciences; cat. no. RPN425).

2.2.3. Antigen Retrieval

1. Proteinase K (DAKO, Carpinteria, CA; cat. no. S3020): store at 50 m*M* in Tris–HCl buffer, pH 7.4 at –30°C, up to 3 mo.
2. Trypsin (Difco, Detroit, MI; cat. no. 0152-13-1): stored at 0.25% in PBS at –30°C.

2.3. Equipments and Vials for Cryo-Fixation and Freeze-Substitution

1. Reichert KF80 cryofixation apparatus (Leica, Wien, Austria).
2. Cryogenic vial (Nalge Company, Rochester, NY; cat. no. 5000-0020).
3. Reichert AFS automatic freeze-substitution system (Leica).

3. Methods

3.1. Tissue Preparation for Postembedding Immunoelectron Microscopy

This section describes the method to prepare tissue samples used for postembedding immunoelectron microscopy. By using cryofixation, all components of biological specimens are immobilized without the use of any of chemical fixation procedures and the damaging effects on sensitive and labile antigenic sites can be avoided. The ice in the specimen is then dissolved and replaced by an organic solvent at low temperature by a freeze-substitution method. This enables us to avoid denaturation of proteins that can occur during the curing of resins at higher temperatures.

3.1.1. Cryofixation

1. Immerse skin tissue samples immediately after a biopsy or surgical operation into ice-cold 15% glycerol/PBS for 1 h. Samples should be cut into small pieces less than 1 mm^3.
2. Mount a sample on a bare copper grid (VECO) held with forceps.
3. Plunge it rapidly into liquid propane at –190°C using a cryo-fixation apparatus.
4. Store in a cryogenic vial in liquid nitrogen.

3.1.2. Freeze-Substitution

1. Transfer cryofixed tissue samples to plastic capsules with mesh bottom containing substitution liquid (methanol) placed in the substitution chamber of an automatic freeze-substitution system at –80°C.
2. Continue substitution for 44 h, exchanging substitution liquid twice a day.

3.1.3. Low Temperature Embedding

1. Raise the temperature 5°C/h up to –60°C and start infiltration with embedding medium in the dark.
2. Prepare fresh Lowicry K11M.
3. Exchange the substitution solution against embedding medium by increasing concentration of Lowicryl in methanol as follows: (1) Lowicryl K11M/methanol: 1:2 (vol:vol) 1 h; (2) 1:1 1 h; (3) 2:1 1 h; (4) pure Lowicryl K11M 1 h; (5) pure Lowcryl K11M overnight; and (6) embed in fresh Lowicryl K11M filled in plastic molds.
4. Initiate polymerization under ultraviolet radiation for 48 hrs at –60°C, raise the temperature 5°C/h up to 20°C and continue polymerization for further 48 h.

3.2. Immunostaining

This section describes the section preparation methods and immunostaining methods for postembedding immunoelectron microscopy.

3.2.1. Sectioning Procedure

1. Remove the specimen embedded in Lowicryl K11M from plastic molds.
2. Cut 100-nm thick sections using a ultra-microtome and collect them on Formvar-coated nickel mesh (*see* **Note 1**).
3. Blot on filter paper and air-dry.

3.2.2. Immunostaining Procedure

Immunostaining is performed on drops of solutions placed on a piece of laboratory film. (Parafilm, American Natinal Can, Chicago, IL). (For negative control studies and double labeling, *see* **Notes 2** and **3**, respectively.)

1. Incubate the specimen-mounted grids on a drop of preincubation buffer for 15 min at room temperature.
2. Transfer to a drop of one of the primary antibody solutions (anti-loricrin antibody AF62, 1:500 dilution, anti-involucrin antibody SY5, 1:100 dilution, anti-involucrin antibody BT-601, 1:5 dilution) diluted in first incubation buffer and incubate for 1 h at 37°C.
3. Place on drops of first incubation buffer twice for 5 min at room temperature.
4. Place on drops of second incubation buffer twice for 5 min at room temperature.
5. Incubate on a drop of appropriate colloidal gold-conjugated secondary sera (gold particle size 5 or 10 nm, 1:10 dilution in second incubation buffer) for 1 h at 37°C.
6. Place on drops of second incubation buffer twice for 5 min at room temperature.
7. Place on drops of dH_2O twice for 5 min at room temperature and rinse gently in running dH_2O.
8. Blot on filter paper and air-dry on a piece of laboratory film.
9. The sections were then contrasted with 1.5% uranyl acetate in methanol for 3 min.
10. Rinse in 50% methanol/dH_2O.
11. Rinse in dH_2O.
12. Blot on filter paper and air-dry on a piece of laboratory film.

3.3. Antigen Retrieval

Incubate ultrathin sections of Lowicryl K11M embedded skin tissue with either 0.4 mg/mL proteinase K in Tris-HCl buffer, pH 7.4, for 30 s to 5 min at room temperature or with 0.25% trypsin in PBS, pH 7.4, for 15 min to 60 min at 37°C. Rinse with the buffer (Tris-HCl buffer pH 7.4 and PBS pH 7.4, respectively) and immunostain as described previously.

4. Notes

1. Lowicryl K11M is a hydrophilic resin. Therefore, precautions should be taken to ensure that the block face does not become wet during sectioning. This is best accomplished by sectioning with a low water level in the knife-trough.
2. Negative controls included incubation in the presence of the secondary antibody alone and with primary antibodies against antigens that are not expected to be present in the epidermis.
3. For double labeling, stain with one primary antibody and label with 5-gold-conjugated secondary antibodies followed by staining with another primary antibody raised in a different animal and labeling with 10-nm gold conjugated secondary antibodies.

Acknowledgment

Development of the protocols as described earlier was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labor and Welfare of Japan.

References

1 Ishida-Yamamoto, A. and Iizuka, H. (1998) Structural organization of cornified cell envelopes and alterations in inherited skin disorders. *Exp. Dermatol.* **7,** 1–10.

2 Ishida-Yamamoto, A., Eady, R. A. J., Watt, F. M., Roop, D. R., Hohl, D., and Iizuka, H. (1996) Immunoelectron microscopic analysis of cornified cell envelope formation in normal and psoriatic epidermis. *J. Histochem. Cytochem.* **44,** 167–175.

3 Ishida-Yamamoto, A., Tanaka, H., Nakane, H., Takahashi, H., and Iizuka, H. (1999) Antigen retrieval of loricrin epitopes at desmosomal areas of cornified cell envelopes: an immunoelectron microscopic analysis. *Exp. Dermatol.* **8,** 402–406.

4. Ishida-Yamamoto, A., Kato, H., Kiyama, H., Armstrong, D. K. B., Munro, C. S., Eady, R. A. J., et al. (2000) Mutant loricrin is not crosslinked into the cornified cell envelope but is translocated into the nucleus in loricrin keratoderma. *J. Invest. Dermatol.* **115,** 1088–1094.

IV

Methods and Approaches for the Analysis for Epidermal Function

25

Cell Kinetic Analysis in Artificial Skin Using Immunochemical Methods

Andrea Casasco, Antonia Icaro Cornaglia, Federica Riva, Marco Casasco, and Alberto Calligaro

Summary

Cell kinetic studies provide important information on histogenesis in vivo and in vitro. In this regard, specific antibodies to cell cycle-related antigens have been raised and characterized, thus permitting the study of cell kinetics using immunochemical methods. Recent advances in culture technology permitted the generation of human skin equivalents in vitro. We here provide detailed practical procedures for the study of epidermal cell kinetics in a model of artificial skin using immunohistochemistry and flow cytometry. The combined application of both techniques allows a precise detection of tissue growth sites and a quantitative assessment of cell growth. Moreover, simultaneous analysis of differentiation markers and proliferation antigens may be useful to understand molecular mechanisms that regulate tissue growth and development.

Key Words:

Human artificial skin; biotechnology; cell cycle; immunohistochemistry; flow cytometry; bromodeoxyuridine; proliferating cell nuclear antigen; Ki-67 antigen; cell cycle-related antigens; epidermal stem cells.

1. Introduction

The application of stem cell in culture technology provides the basis for in vitro organ reconstitution. In particular, cultivation of skin keratinocytes permitted the formation of epithelial layers similar to natural epidermis ***(1)***. Subsequently, epidermal layers have been combined with dermal equivalents made of connective tissue to reconstruct the entire skin architecture ***(2–6)***. Epidermal and dermal stem cells can be isolated from different sources ***(7)***. Moreover, it also has been possible to introduce melanocytes, Langerhans cells, blood vessels, and hair in models of engineered skin ***(8–11)***. Information concerning cell kinetics is important to clarify how tissue reconstitution can be obtained in vitro.

In this regard, the production of monoclonal antibodies to cell cycle-related antigens provides the basis for immunochemical analysis of cell kinetics in tissues. Every cellular antigen that is specifically expressed in different phases of the cell cycle may be used to monitor the position of a cell within a cycle using immunochemical methods ***(12,13)***. Immunocytochemistry allows precise tissue localization of replicating cells, whereas flow cytometry defines the exact position of immunoreactive cells in the cell cycle and ensures a quantitative analysis of the growth fraction. Moreover, simultaneous detection

From: *Methods in Molecular Biology, vol. 289: Epidermal Cells: Methods and Protocols*
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

of cell cycle-related antigens and differentiation markers using double immunochemical staining may be used to unravel molecular mechanisms that regulate tissue growth dynamics (e.g., coexpression of receptors or adhesion molecules with proliferation antigens).

Here, we describe detailed practical procedures that we have used to investigate cytokinetics in a model of artificial skin by means of immunohistochemistry and flow cytometry ***(14,15)***.

2. Materials

2.1. Tissue and Antigen Investigation

1. Artificial skin ***(4,5)***: human skin equivalent (HSE) Apligraf (Organogenesis Inc., MA) stored at 4°C.
2. Monoclonal antibody to Ki-67 antigen, clone MIB-1 (BioGenex, CA; cat. no. AM129).
3. Monoclonal antibody to bromo-deoxyuridine (BrdU), clone B44 (Becton Dickinson, CA; cat. no. 7580).
4. Monoclonal antibody to BrdU, clone BU-1. The ready-to-use reagent contains a nuclease to produce single-stranded deoxyribonucleic acid (DNA; Amersham, England; cat. no. RPN202).
5. Monoclonal antibody to proliferating cells nuclear antigen (PCNA), clone PC-10 (Novocastra Laboratories, England; cat. no. NCL-PCNA).
6. Adhesive slides using poly-L-lysine: dissolve 10 mg poly-L-lysine in 10 mL water to make 0.1% solution. Aliquot into 0.5–1-mL portions and store frozen at –20°C; to use, thaw an aliquot completely and stir before use. Replace unused solution in the freezer for storage. Apply a small drop to one end of a clean slide and spread the poly-L-lysine as a thin, even film over the whole surface. The film will dry rapidly and the slide is ready for use. Label the coated side, as the film is invisible. If the solution spreads in droplets instead of an even film, the slide is not clean enough: soak the slides in acid-alcohol overnight, then rinse well in alcohol.

2.2. Immunohistochemistry

1. Paraffin wax, pastillated, congealing point 56°C (BDH, England; cat. no. 361077E).
2. Pepsin A, from porcine stomach mucosa (Sigma-Aldrich, Germany; cat. no. P7125).
3. Normal goat serum (NGS; Sigma-Aldrich, Germany; cat. no. G9023).
4. Bovin serum albumin (BSA; Sigma-Aldrich, Germany; cat. no. A9647).
5. Link antibody (biotinylated anti-Immunoglobulins) (BioGenex; cat. no. HK336-5R).
6. Horseradish peroxidase-labeled streptavidin (super sensitive kit, BioGenex; cat. no. HK320-UK).
7. 3,3′-Diaminobenzidine tetrahydrochloride (DAB; liquid DAB Biogenex; cat. no. HK153-5K).
8. Phosphate-buffered normal saline (PBS), pH 7.4: Sol. A: 5.52 g potassium dihydrogen phosphate ($NaH_2PO_4 \bullet H_2O$). Dissolve in 200 mL water. Sol. B: 28.48 g disodium hydrogen phosphate ($Na_2HPO_4 \bullet 2H_2O$). Dissolve in 800 mL water. To use, mix Sol. A with Sol. B (1 : 4) to obtain 0.2 *M* buffer. Dilute 1 : 1 with water and check pH for a 0.1 *M* buffer.
9. 0.05 *M* Tris-buffered normal saline (TBS), pH 7.4: 6.07 g Tris-(hydroxymethil)methylamine (Tris base), 8.7 g sodium chloride. Dissolve in 900 mL deionized water. Add concentrated hydrochloride acid HCl (12–14 mL) until the pH reaches 7.4 and make up to 1 L with water.
10. 0.05 *M* Tris-HCl, pH 7.4 (substrate buffer): 6.61 g Tris-HCl, 0.97 g Tris base. Dissolve in 900 mL deionized water. Adjust pH with 1 *N* HCl and make up to 1 L with water.
11. 0.01 *M* Citrate buffer, pH 6.0: 1.9 g citric acid. Dissolve in 900 mL water, then add 2 *M* NaOH to adjust pH 6.0. Make up to 1 L with water. The buffer can be kept at room temperature for several days.

12. Diluent of primary antibody and link antibody: 0.1 g BSA, 0.01 g sodium azide (NaN_3). Dissolve in 10 mL TBS, pH 7.4.
13. Diluent of streptavidin-peroxidase (peroxidase label): 0.1 g BSA, 0.001 g thimerosal. Dissolve both powders in 10 mL TBS, pH 7.4 (*see* **Note 1**).
14. DAB solution: dissolve 1 mg DAB in 2 mL substrate buffer to obtain a final DAB concentration of 0.05%. Add 10 µL hydrogen peroxide (H_2O_2) 3%, just before use (final concentration 0.01–0.02%). Make fresh solution each time and use within 10 min (*see* **Note 2**).
15. Paraformaldehyde buffered at pH 7.4 within PBS 0.1 *M*: dissolve 8 g paraformaldehyde in 100 mL water with stirring. Heat to 55°C and add 1 *N* NaOH, one drop a time, until the solution becomes clear. Cool at room temperature and add 100 mL PBS, 0.2 *M*, pH 7.4, to reach a final concentration of 0.1 *M*.

2.3. Cytometric Analysis

1. 1X trypsin type III (Eurobio, France,; cat. no. CEZ DA00-0U).
2. 0.1% nonidet NP-40 (Calbiochem, CA; cat. no. 492015).
3. Propidium iodide (50 µg/mL; Calbiochem, CA; cat. no. 537059).
4. RNase type I 1A, free DNase (50 Kunitz U/mL; Sigma-Aldrich, Germany; cat. no. R7397).
5. Triton X-100 (BDH, Pole, England; cat. no. 30632).
6. BSA (Sigma-Aldrich, Germany; cat. no. A9647).
7. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma-Aldrich, Germany; cat. no. F5387).
8. Mouse Isotype Control antibody IgG2a, Kappa (UPC-10; Sigma-Aldrich, Germany; cat. no. M9144).
9. 0.01 *M* PBS, pH 7.4: 8.7 g sodium chloride, 0.272 g potassium dihydrogen phosphate, 1.136 g disodium hydrogen phosphate. Dissolve salts in water, make up to 1 L, and check pH.
10. Rinse buffers and antibodies diluent (PTA): 10 µL Triton X-100, 0.1 g BSA. Dissolve in 10 mL PBS. Make fresh solution each time.
11. 4% paraformaldehyde in PBS buffer, pH 7.4: dissolve 8 g paraformaldehyde in 100 mL water with stirring. Heat to 55°C with stirring and add 1 *N* NaOH until clear; cool at room temperature and add 100 mL PBS, 0.2 *M*, pH 7.4.

2.4. Instruments

1. FACStar Cell Sorter (Becton Dickinson, CA).
2. Partec PAS V (Basel, Switzerland).
3. Light microscope (Axiophot, Zeiss, Germany).

3. Methods

3.1. BrdU Incorporation

1. Incubate living HSE for 2 h with 5 mL medium containing 200 µ*M* BrdU to label S phase cells.
2. Wash with 0.1 *M* PBS and proceed with the fixation.

3.2. Immunohistochemistry for Cell Cycle-Related Antigens

This section describes the immunohistochemical method used to detect expression of proteins that are typical of proliferating cells ***(16–21)***.

3.2.1. Sample Preparation

1. Fix skin specimens (5 × 5 mm) with the fixative solution (4% buffered paraformaldheyde) for 6 h.

2. Wash in TBS three times for 15 min and start to dehydrate through graded alcohols: 70% ethanol for 2 h; 85% ethanol for 2 h; 100% ethanol for 2 h; xylene 10 min.
3. Embed samples in liquid paraffin.
4. Cut paraffin sections (5–10 µm). Float on warm (not hot) water and pick up on poly-L-lisine coated slides. Dry for several hours at 37°C.
5. Remove paraffin in two changes of xylene and take through graded alcohol to water.
6. Block endogenous peroxidase: treat with 0.3% H_2O_2 in water for 30 min (a shorter time may be adequate).
7. Place slides in TBS and proceed with immunostaining according to indirect immunoperoxidase protocol (**refs.** ***22*** and ***23***; *see* **Note 3**).

3.2.2. Staining Procedure for Paraffin Sections With Monoclonal Antibodies to BrdU, PCNA, and Ki-67

3.2.2.1. Antibody Anti-BrdU, Clone BU-1

1. Dip the section within a solution of 0.5% pepsin in 0.01 *M* HCl for 30 min at 37°C.
2. Wash well with tap water for 5 min, then wash in TBS for 5 min.
3. In the humidified box treat with NGS 1:20 in TBS for 30 min to reduce nonspecific background staining; then remove excess serum from sections.
4. Incubate the section with primary antibody (at dilution 1:2 in specific buffer) at 4°C overnight in a humidified chamber then rinse well with TBS for 10 min three times.
5. Incubate with link antibody (biotinylated anti-immunoglobulins), diluted 1:50 in buffer, at room temperature for 20 min, and then rinse well with TBS for 10 min three times.
6. Incubate with peroxidase-labeled streptavidin (at dilution 1:50 in specific diluent) at room temperature for 20 min, then rinse well with TBS for 10 min three times.
7. Add enough DAB solution to cover completely the sections and incubate for 3–5 min.
8. Wash with TBS for 10 mins and then counterstain the section with hematoxylin.
9. Rinse slides with tap water for 10 min and dehydrate the sections through graded alcohol and xylene.
10. Mount the sections in permanent medium.

3.2.2.2. Ki-67

1. Place the slide in a plastic slide carrier and immerse them completely in 0.01 *M* citrate buffer.
2. Microwave at 750 W for 5 min (the buffer must boil) for antigen retrieval ***(24)***.
3. Let the buffer cool down until it is possible to remove slides without danger of buffer salts precipitating upon the section as they dry. Then, wash in TBS buffer for 5 min twice.
4. In the humidified box, treat with NGS 1:20 in TBS for 30 min to reduce nonspecific background staining; then, remove excess serum from sections.
5. Incubate with mouse monoclonal antibody to Ki-67 antigen (diluted 1:50 in specific buffer) at 4°C overnight. Then, rinse well with TBS for 10 min three times.
6. Incubate with link antibody link antibody (biotinylated anti-immunoglobulins), diluted 1:50 in buffer at room temperature for 20 min.
7. Wash in TBS buffer three times for 10 min.
8. Incubate with horseradish streptavidin-peroxidase (diluted 1:50) at room temperature for 20 min, and then wash in TBS buffer for 10 min three times.
9. Add enough DAB solution to cover completely the sections and incubate for 3–5 min.
10. Wash with TBS for 10 min and counterstain the section with hematoxylin.
11. Rinse slides with tap water for 10 min.
12. Dehydrate the sections through graded alcohol and xylene.
13. Mount the section with permanent medium.

3.2.2.3. PCNA

1. In the humidified box treat with NGS 1 : 20 in TBS for 30 min to reduce nonspecific background staining.
2. Remove excess serum from sections and incubate with mouse monoclonal antibody anti-PCNA (diluted 1 : 200 in specific buffer) at 4°C overnight.
3. Rinse well with TBS for 10 min three times.
4. Incubate with link antibody (biotinylated anti-immunoglobulins) diluted 1 : 50 in buffer at room temperature for 20 min; then, wash in TBS buffer for 10 min three times.
5. Incubate with horseradish streptavidin-peroxidase (diluted 1 : 50) at room temperature for 20 min and then wash in TBS buffer for 10 min three times.
6. Add enough DAB solution to cover completely the sections and incubate for 3–5 min.
7. Wash with TBS for 10 min and counterstain the section with hematoxylin.
8. Rinse slides with tap water for 10 min and then dehydrate the sections through graded alcohol and xylene.
9. Mount the section with permanent medium.

3.2.2.4. Controls

To check that a tissue sample is being immunostained specifically, a negative control must be performed, consisting of substitution of the primary antiserum with nonimmune serum or antibody diluent ***(25)***. All other conditions must be identical with those for the test. It is essential that a positive control sample that is known to contain the antigen in question is included.

3.3. Staining Procedure for Cytometric Analysis (26)

1. Trim samples of HSE in small pieces.
2. Immerse in 80% ethanol at 4°C for 10 min.
3. Wash with Triton X-100 (0.1% final concentration) in PBS for 15 min.

3.3.1. Propidium Iodide DNA Staining

1. Centrifuge cell suspensions at 400*g* and stain pellet with a solution of 50 µg/mL propidium iodide, 0.1% nonidet P-40, and type 1A RNase (50 Kunitz U/mL) in PBS for 30 min at room temperature.
2. Conserve samples at 4°C for about 24 h.
3. Before flow analysis, filter cells to remove aggregates.

*3.3.2. Biparametric Analysis by Flow Cytometry (Indirect Immunofluorescence Procedures; **Fig. 1**)*

3.3.2.1. BrdU Cytometric Analysis

1. To detect BrdU, centrifuge the cell suspension (400*g*) and treat the pellet ($1–2 \times 10^6$ cells) with 1 mL HCl 2 *N* for 30 min at room temperature to denature the DNA.
2. Neutralize with 1 mL sodium tetraborate 0.1 *M* for 15 min then centrifuge for 3 min at 1200 rpm at room temperature.
3. Wash with PBS for 10 min, centrifuge, and incubate pellet with PTA for 30 min to reduce nonspecific background staining.
4. Centrifuge for 3 min at 400*g* at room temperature; then, incubate the pellet with BrdU-antibody B44, diluted 1 : 10 in PBS containing 0.5% NGS for 30 min at room temperature. Shake each tube containing samples every 10 min to avoid sedimentation.
5. Add 1 mL PTA in each tube and centrifuge for 3 min at 400*g* at room temperature; then, remove supernatant and rinse pellet well with PTA for 10 min twice.

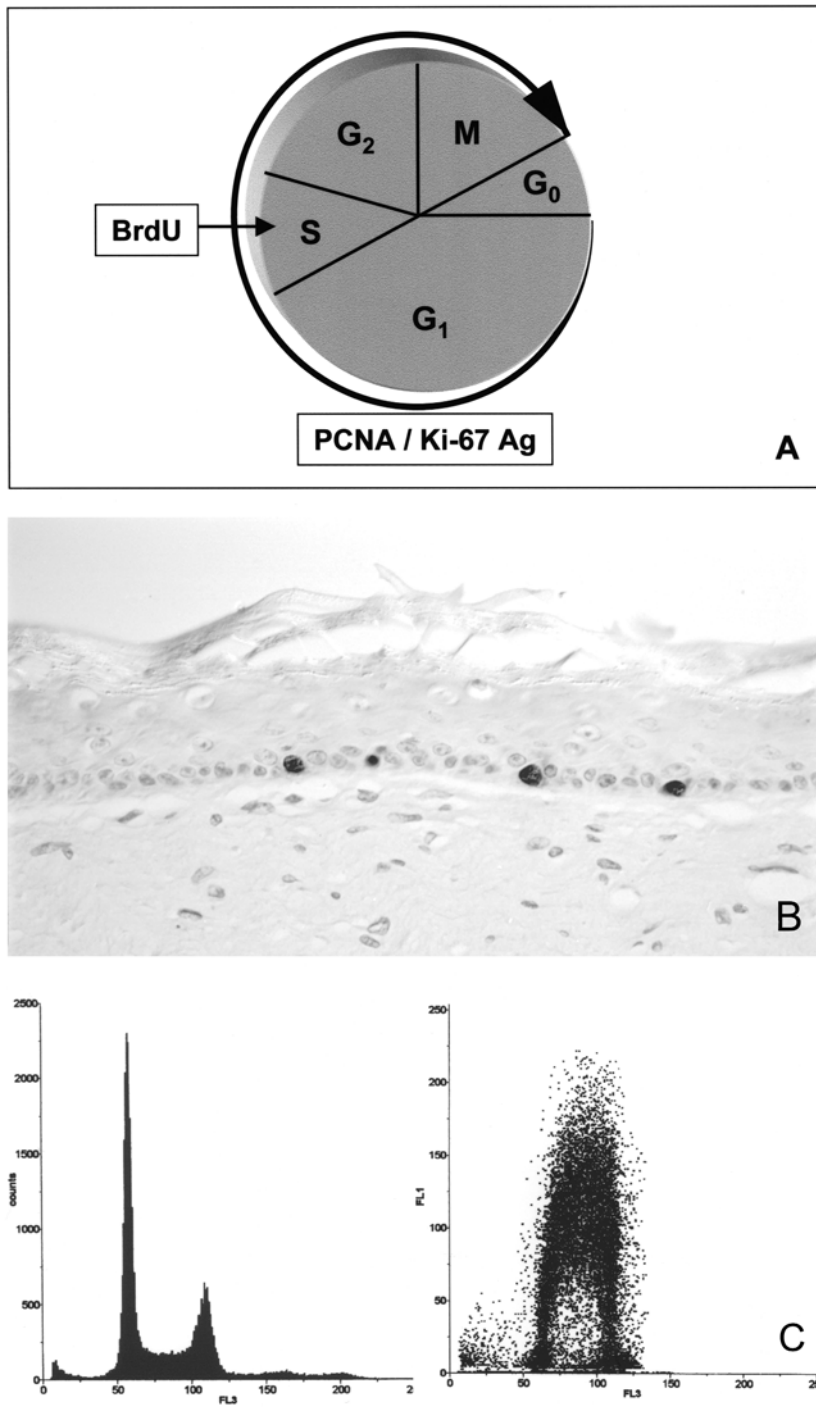


Fig. 1. **A,** Temporal expression patterns of some cell cycle-related antigens. PCNA and Ki-67 antigen immunoreactivities define the G1, S, and G2-M cell populations of the cell cycle, whereas BrdU immunostaining is restricted to the S phase. **B,** Immunohistochemical identification of replicating cells in human skin equivalent. Ki-67 antigen expression reveals G1, S, and G2-M cells. Within the epithelium, the nuclei of some basal cells are immunostained (arrows). Magnification, ×400. **C,** Flow cytometric analysis of S phase cells. The diagrams show the measurements of DNA content (left) and BrdU immunofluorescence vs DNA content (right).

6. Incubate cells with secondary antibody FITC-conjugated goat anti-mouse, diluted 1 : 100 in PBS, for 30 min and shake each tube with the samples every 10 min to avoid sedimentation.
7. Add 1 mL PTA in each tube and centrifuge for 3 min at 400*g* at room temperature; then, remove supernatant and rinse well pellet with PTA for 10 min twice.
8. Centrifuge for 3 min at 400*g* at room temperature and incubate pellet with a solution of 5 µg/mL propidium iodide, 0.1% nonidet P-40, and type 1A RNase (50 Kunitz U/mL) in PBS for 30 min at room temperature to stain DNA.
9. Store samples at 4°C for about 24 h.
10. Before flow analysis, filter cells to remove aggregates using filter.

3.3.2.2. Ki-67 and PCNA Cytometric Analysis

1. Wash with PBS for 10 min and centrifuge at 400*g*.
2. Incubate pellet with PTA for 30 min to reduce nonspecific background staining and then centrifuge for 3 min at 400*g* at room temperature.
3. Incubate cells with primary antibody (Ki-67 or PC-10) diluted 1 : 100 in 100 µL PTA for 1 h at room temperature and keep shaking each tube with the cells.
4. Add 1 mL PTA in each tube and centrifuge for 3 min at 400*g* at room temperature.
5. Remove surnatant and rinse well pellet with PTA for 10 min twice.
6. Incubate cells with secondary antibody FITC-conjugated goat anti-mouse, diluted 1 : 100 in 100 µL PBS, for 30 min and keep shaking each tube with the cells.
7. Add 1 mL PTA in each tube and centrifuge for 3 min at 400*g* at room temperature; then, remove surnatant and rinse well pellet with 1 mL PTA for 10 min.
8. Centrifuge for 3 min at 400*g* at room temperature and incubate pellet with a solution of 6 µg/mL propidium iodide, 0.1% nonidet P-40, and type 1A RNase (50 Kunitz U/mL) in 1 mL PBS for 30 min at room temperature to stain DNA.
9. To measure samples, wait at least 30 min after staining at room temperature or keep them overnight at 4°C for about 24 h and, before flow analysis, filter cells to remove aggregates.

3.3.2.3. Controls

To perform the negative controls to evaluate the unspecific binding of monoclonal antibodies, incubate the cells with an isotypic-irrelevant antibody as the primary antibody, and with FITC-conjugated secondary antibody.

3.4. Quantitative Evaluation of Immunohistochemical Data

Immunolabeled cells for cell cycle-related antigens were observed by conventional light microscopy. Tissue sections were scored for the number of immunoreactive cells by at least two independent observers (AC, AIC). Bromodeoxyuridine-, Ki-67-antigen- and PCNA-labeling indices (i.e., percent of labeled cells) were evaluated by counting immunoreactive cells in three different sections of different samples ($n = 5$). Labeling indices (± standard error) were evaluated in different tissue compartments, that is, basal layer of the epidermal equivalent, epidermis, and dermis ***(14,15)***.

3.5. Quantitative Evaluation of Flow Cytometric Data (see Note 4)

3.5.1. Single-Parameter DNA Measurements

1. For single-parameter DNA measurements, analyze at least 10,000 cells for each sample using a flow cytometer.
2. Display the data as frequency histograms, showing the measurements of cell DNA content versus total number cells. In this way, it's possible to know the number of cells in G1, S, and G2+M phases of cell cycle and measure the growth fraction of S phase cell.
3. Compare the results with the negative control.

3.5.2. Biparametric Analysis

1. Analyze with a flow cytometry and measure for each sample a total of 10^4 cells.
2. Perform with FACStar Cell Sorter two parameter flow cytometry analysis (FITC-green vs propidium iodide-red) of BrdU (or PCNA or Ki-67) incorporation and of nuclear DNA content.
3. Evaluate fluorescence intensity signals of BrdU (or PCNA or Ki-67) immunostaining in each phase of the cell cycle by gating the cells according to their DNA content value, as determined by PI fluorescence.
4. Use electronic gating provided by instruments software program (Consort 30 software) for exclusion of cell doublets.
5. Collect data with the program software running on a computer system displaying results as dual parameter contour density plots. Plots show the cell cycle distribution of immunofluorescence relative to specific protein in the different phases of cell cycle (G1, S, G2+M phases).

4. Notes

1. Sodium azide is not recommended as a preservative because it will inhibit peroxidase activity.
2. *Caution:* DAB is classified as potential carcinogens and can cause skin irritation upon contact.
3. TBS or methanol may be used instead of water to dilute H_2O_2. Methanol is itself a blocker of peroxidase and may be used with up to 3% H_2O_2 for persistent enzyme activity.
4. It is possible to analyze the cells with a FACStar Cell Sorter (Becton Dickinson, CA) or Partec PAS V (Basel, Switzerland). However, other flow cytometers (such as Epics XL, Coulter, or FACScan, Becton Dickinson) may be used. All instruments are equipped with an argon laser tuned at 488 nm for fluorescence excitation. Electronic correction for doublets was used with each apparatus. Statistical analysis of the percentage of cells in each phase of the cell cycle was performed by the software provided with each instrument.

Acknowledgments

We thank Dr. Giuliano Mazzini (C.N.R., Pavia) and Mrs. Aurora Farina (University of Pavia) for helpful discussion and valuable technical help. This research was supported by grants from the University of Pavia (F.A.R.).

References

1 Rheinwald, J. G. and Green, H. (1975) Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell* **6,** 331–344.

2 Bell, E., Ehrlich, H. P., Buttle, D. J., and Nakatsuji, T. (1981) Living tissue formed in vitro and accepted as skin-equivalent tissue of full thickness. *Science* **211,** 1052–1054.

3. Asselineau, D., Bernard, B. A., Bailly, C., and Darmon, M. (1986) Three-dimensional culture of human keratinocytes on a dermal equivalent as a model system to study environmental modulation of epidermal physiology in vitro: effect of air-exposure. *Br. J. Dermatol.* **31,** 126–127.

4 Parenteau, N. L., Nolte, C. M., Bilbo, P., Rosenberg, M. , Wilkins, L. M., Johnson, E. W., et al. (1991) Epidermis generated in vitro: pratical considerations and applications. *J. Cell Biochem.* **45,** 245–251.

5 Parenteau, N. L., Bilbo, P., Nolte, C. J., Mason, V. S., and Rosenberg, M. (1992) The organotypic culture of human skin keratinocytes and fibroblasts to achieve form and function. *Cytotechnology* **9,** 163–171.

6 Zacchi, V., Soranzo, C., Cortivo, R., Radice, M., Brun, P., and Abatangelo, G. (1998) In vitro engineering of human skin-like tissue. *J. Biomed. Mater. Res.* **40,** 187–194.

7 Eaglstein, W. H. and Falanga, V. (1997) Tissue engineering and the development of Apligraf, a human skin equivalent. *Clin. Therapeutics* **19,** 894–905.

8 De Luca, M., Franzi, A. T., D'Anna, F., Zicca, A., Albanese, E., Bondanza, S., et al. (1988) Coculture of human keratinocytes and melanocytes: differentiated melanocytes are physiologically organized in the basal layer of the cultured epithelium. *Eur. J. Cell Biol.* **46,** 176–180.

9 Regnier, M., Staquet, M. J., Schmitt, D., and Schmidt, R. (1997) Integration of Langerhans cells into a pigmented reconstructed human epidermis. *J. Invest. Dermatol.* **109,** 510–512.

10 Black, A. F., Berthod, F., L'Heureux, N., Germain, L., and Auger, F. A. (1998) In vitro reconstruction of a human capillary-like network in a tissue-engineered skin equivalent. *FASEB J.* **12,** 1331–1340.

11 Michel, M., L'Heureux, N., Pouliot, R., Xu, W., Auger, F. A., and Germain, L. (1999) Characterization of a new tissue-engineered human skin equivalent with hair. *In Vitro Cell Dev. Biol. Anim.* **35,** 318–326.

12 Prosperi, E., Stivala, L. A., Scovassi, A. I., and Bianchi, L. (1997) Cyclins: relevance of subcellular localization in cell cycle control. *Eur. J. Histochem.* **41,** 161–168.

13 Darzynkiewicz, Z., Gong, J., Juan, G., Ardelt, B., and Traganos, F. (1996) Cytometry of cyclin proteins. *Cytometry* **25,** 1–13.

14 Casasco, A., Casasco, M., Icaro Cornaglia, A., Zerbinati, N., Mazzini, G., and Calligaro, A. (2001) Cell kinetics in a model of artificial skin. An immunohistochemical and flow cytometric analysis. *Eur. J. Histochem.* **45,** 125–130.

15 Casasco, A., Casasco, M., Zerbinati, N., Icaro Cornaglia, A., and Calligaro, A. (2001) Cell proliferation and differentiation in a model of human skin equivalent. *Anat. Rec.* **264,** 261–272.

16 Gratzner, H. G. (1982) Monoclonal antibody to 5-bromo and 5-iododeoxyuridine: a new reagent for detection of DNA replication. *Science* **218,** 474–475.

17 Gerdes, J., Lemke, H., Baisch, H., Wacker, H. H., Schwab, U., and Stein, H. (1984) Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *J. Immunol.* **133,** 1710–1715.

18. De Fazio, A., Leary, J. A., Hedley, D. W., and Tattersall, M. H. N. (1987) Immunohistochemical detection of proliferating cells in vivo. *J. Histochem. Cytochem.* **35,** 571–577.

19 McCormick, D., Chong, H., Datta, C., and Hall, P. A. (1993) Detection of the Ki-67 antigen in fixed and wax-embedded sections with the monoclonal antibody MIB1. *Histopathology* **22,** 355–360.

20 Casasco, A., Giordano, M., Danova, M., Casasco, M., Icaro Cornaglia, A., and Calligaro, A. (1993) PC10 monoclonal antibody to proliferating cell nuclear antigen as probe for cyclng cell detection in developing tissues. *Histochemistry* **99,** 191–199.

21. Casasco, A., Casasco, M., and Calligaro, A. (1997) Microscopical immunodetection of cell proliferation antigens. *Microsc. Anal.* **48,** 25–27.

22 Hsu, S. M., Raine, L., and Fanger, H. (1981) Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J. Histochem. Cytochem.* **29,** 577–580.

23. Polak, J. M. and Van Noorden, S. (1986) *Immunocytochemistry, Modern Methods and Applications*, 2nd ed., Butterworth Heinemann, Oxford (originally John Wright and Sons, Bristol), UK.

24 Shi, S. R., Key, M. E., and Kalra, K. L. (1991) Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue section. *J. Histochem. Cytochem.* **39,** 741–748.

25. Polak, J. M. and Van Noorden, S. (1987) Specificity problems and essential controls, in *An Introduction to Immunocytochemistry: Current Techniques and Problems* (Hammond, C., ed.), Oxford University Press, Microscopical Society, Oxford, UK, pp. 33–36.
26. Mazzini, G. and Danova, M. (1994) Citometria a flusso. Applicazioni cliniche dell'analisi del DNA in oncologia. Ed. SSOSB-Scuola Internazionale di Oncologia e Medicina sperimentale, Genova, I.

26

Proliferation, Differentiation, and Inflammation in Normal and Hyperproliferative Skin Using Multiparameter Flow Cytometry

Piet E. J. van Erp

Summary

Our knowledge of the etiology and pathogenesis of skin diseases characterized by abnormal growth is relatively limited. Even more important, still very little is known of how epidermopoeisis is controlled in normal epidermis. There is no cure for skin diseases caused by abnormal growth control, such as psoriasis. Mechanisms are complex, additional models for epidermal growth and differentiation, and specific techniques to analyze these processes, are needed. Therefore, we have developed flow cytometric techniques to study epidermal growth over the past two decades. A prerequisite for accurate and reliable flow cytometric analysis is a high quality of epidermal single-cell suspensions. In this chapter, protocols are described for preparation of single-cell suspensions and protocols for the multiparameter flow cytometric analysis of growth behavior in normal and hyperproliferative epidermis.

Key Words:

Epidermis; skin disease; hyperproliferation; multiparameter flow cytometry.

1. Introduction

Skin is an organ; "it possesses an unusual shape, a variety of appendages and it encloses or supports all other organs, but it is nevertheless a single integrated organ" ***(1)***. Its main function is acting as a barrier between the internal milieu of the organism and the external environment. However, the organ acts only partly as a physical and static barrier by producing a dead layer of protein and lipid material; it is also a controlling and communicating device with a high degree of organization.

The outer part of the skin is the epidermis and is composed mainly (90–95%) of keratinocytes. It is this part of the skin that produces the outermost protective barrier of cross-linked protein and neutral lipids. Furthermore, it contains specialized cells with specific functions, such as immunoprotection by antigen-presenting Langerhans cells and protection against UV irradiation by melanin-producing melanocytes. Epidermis is a continuously renewing epithelium; cells in the lower layers divide and replace the differentiated and dead corneocytes that scale off from the skin surface, leaving the organic physical barrier for protection. This process of controlled cell production is called epidermopoeisis ***(2)***.

From: *Methods in Molecular Biology, vol. 289: Epidermal Cells: Methods and Protocols*
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

Our knowledge of the etiology and pathogenesis of skin diseases characterized by abnormal growth is relatively limited. Even more important, still very little is known of how epidermopoeisis is controlled in normal epidermis. There is no cure for skin diseases caused by abnormal growth control, such as psoriasis. Mechanisms are complex, additional models for epidermal growth and differentiation, and specific techniques to analyze these processes are needed. Therefore, we have developed novel immunohistochemical and flow cytometric techniques to study epidermal growth over the past two decades ***(3–10)***. A prerequisite for accurate and reliable flow cytometric analysis is a high quality of epidermal single-cell suspensions. In this chapter, protocols are described for preparation of single-cell suspensions and protocols for the multiparameter flow cytometric analysis of growth behavior in normal and hyperproliferative epidermis.

2. Materials

2.1. Clinical Material

Skin samples are obtained from healthy volunteers without signs of skin diseases and from the lesional skin of patients with the hyperproliferative skin disease psoriasis vulgaris. All subjects have to give informed consent prior to biopsy.

Microbiopsy specimens (3-mm diameter and about 2-mm thick) are taken freehand using a razor blade in conjunction with a metal guard. When larger areas are needed, dermatotome biopsies are taken from normal or lesional psoriatic skin. After induction of local anesthesia with ethyl chloride spray (Maxxim Medical Europe BV, Oss, The Netherlands; cat. no. PD2175/1), a small dermatotome (Coriotome, Aesculap, Tuttlingen, Germany; cat. no. 6B333) with a metal guard is used to obtain skin samples with an area of 1 cm^2 and a thickness of 0.2 mm (normal skin) or 0.4 mm (psoriatic skin). Punch biopsies (3 or 4 mm) are taken from normal and psoriatic skin. Local anesthesia was induced with xylocain/adrenalin 1 : 100,000.

2.2. Antibodies for Immunocytochemistry

1. Mouse anti-keratin 10, clone RKSE60, isotype IgG1 (Monosan, Uden, Netherlands; cat. no. MON3010). Dilution to be used is 1 : 20 in final incubation volume. This monoclonal antibody reacts in immunoblots with the 56.6-kDa keratin protein (no. 10 in the Moll catalog). It reacts specifically with keratinizing squamous epithelia, and it can be used for the recognition of keratinizing cells in squamous cell carcinomas from epidermis, lung, bladder, cervix, and esophagus. Furthermore, this antibody can be applied in research of skin diseases because it reacts only with the keratinizing (suprabasal) cells of the epidermis and not with the basal cells. Cross-reactivity is observed with keratin from rat, mouse and bovine tissues.
2. Mouse anti-keratin 16, clone K8.12, isotype IgG1 (Sigma-Aldrich, St. Louis, MO; cat. no. C7034). Dilution to be used is 1 : 20 in final incubation volume. This monoclonal antibody reacts with keratins 13, 15, and 16. It labels nonkeratinizing squamous epithelium, the basal layer of pseudo-stratified epithelium, and transitional epithelium. K8.12 does not react with normal epidermis or most simple epithelial cells. It reacts with all squamous cell carcinomas tested, but not with adenocarcinomas. Furthermore, the suprabasal epidermis of hyperproliferative skin is positively stained as well.
3. Mouse anti-human vimentin, clone Vim3B4, isotype IgG2a (Novocastra Laboratories Ltd, Newcastle Upon Tyne, United Kingdom; cat. no. NCL-VIM). Dilution to be used is 1 : 50 in final incubation volume. This monoclonal antibody is directed against the human

vimentin intermediate filament subunit. It recognizes all nonkeratinocytes in human epidermis, including the inflammatory infiltrate in diseased skin.

4. Mouse anti-human vimentin, clone V9, isotype IgG1 (Monosan, Uden, Netherlands; cat. no. MON3005). Dilution to be used is 1:20 in final incubation volume. Vimentin is the major subunit protein of the intermediate filaments of mesenchymal cells, and has a molecular weight of 57 kDa. Immunohistochemical staining for Vimentin is thus characteristic of sarcomas (of neural, muscle, and fibroblast origin) compared with carcinomas, which are generally negative. Melanomas, lymphomas, and vascular tumors may all stain for Vimentin. Thus, V9 recognizes all nonkeratinocytes in human epidermis, including the inflammatory infiltrate in diseased skin. The antibody stains the 57-kDa vimentin band in immunoblots performed on a lysate of normal human lymphocytes. It does not stain mouse or rat vimentin and there is no detectable crossreactivity with keratin, GFAP, neurofilamin and desmin.
5. Mouse anti-keratin 6, clone LHK6B, isotype IgG2a (Novocastra Laboratories Ltd, Newcastle Upon Tyne, United Kingdom; cat. no. NCL-CK6). Dilution to be used is 1 : 20 in final incubation volume. The antibody reacts with a human keratin intermediate filament protein of 56 kDa identified as cytokeratin 6. Some cross-reactivity with smooth muscle may be observed. The protein is not detectable in normal epidermis, whereas strong suprabasal expression is observed under hyperproliferative skin conditions.

2.3. Reagents

2.3.1. Preparation of Cell Suspensions, Microbiopsy Method

1. Phosphate-buffered saline (PBS; 1 L): 8.2 g NaCl (Merck, Darmstadt, Germany; cat. no. 106404); 1.9 g Na_2HPO_4 $2H_2O$ (Merck; cat. no. 106580); 0.3 g $NaH_2PO_4 \cdot 2H_2O$ (Merck; cat. no. 106345); and pH between 7.2–7.4.
2. Trypsin-DTE solution, freshly prepared (10 mL): 100 mg 1% trypsin 250 (Difco, Detroit, MI; cat. no. 0152-15) and 30 mg 0.3% dithioerythritol (Sigma-Aldrich; cat. no. D-8255). Dissolve in 10 mL PBS.

2.3.4. Preparation of Cell Suspensions, Dermatome Method

1. PBS (*see* **Subheading 2.3.1.**).
2. Trypsin–DTE solution, freshly prepared (10 mL): 2.5 mg trypsin (Sigma-Aldrich; cat. no. T-4665) and 30 mg dithioerythritol (Sigma-Aldrich; cat. no. D-8255). Dissolve in 10 mL PBS.
3. PBS containing 20% calf serum.
4. RNAse solution (A), store aliquots of 1 mL at –20°C (10 mL): 25 mg Ribonuclease A (Sigma-Aldrich; cat. no. R-4875). Dissolve in 10 mL PBS.

2.3.3. Preparation of Epidermal Cell Suspensions Using an Optimized Thermolysine-Trypsin Protocol

1. PBS-Dulbecco with Ca and Mg (Seromed, Berlin, Germany; cat. no. L-1815).
2. Thermolysin solution, freshly prepared (10 mL): 5 mg thermolysin (Protease type X, Sigma-Aldrich; cat. no. P-1512). Dissolve in 10 mL PBS–Dulbecco.
3. PBS without Ca and Mg (NPBI, Amsterdam, Netherlands; cat. no. D 1011, pH 7.4).
4. Trypsin-DTE solution, freshly prepared (10 mL): 2.5 mg trypsin (Sigma-Aldrich; cat. no. T-8253) and 30 mg dithioerythritol (Sigma-Aldrich; cat. no. D-8161). Dissolve in 10 mL PBS.
5. Newborn heat-inactivated calf serum (NHCS, Life Technologies, Paisley, United Kingdom; cat. no. 26010-033).

2.3.4. Staining Procedure

1. PBS (*see* **Subheading 2.3.1.**)
2. RAM–FITC: Fluorescein isothiocyanate (FITC)-conjugated secondary antibody (5 mL): 200 µL mouse immunoglobulins/FITC rabbit F(ab′)2 (Dako, Copenhagen, Denmark; cat. no. F0313); 200 µL normal rabbit serum; and 4.6 mL PBS.
3. Propidium iodide (PI) staining solution, store at 4°C in the dark (500 mL): 20 mg PI (Calbiochem, San Diego, CA; cat. no. 537059). Dissolve in 500 mL PBS.
4. RNAse solution (B), store aliquots of 1 mL at –20°C (10 mL): 100 mg Ribonuclease A (Sigma-Aldrich; cat. no. R-4875). Dissolve in 10 mL PBS.
5. PBS containing 1% NHCS.
6. Secondary antibody cocktail. For 3450 mL (10 tubes): 21 µL phycoerythrin (PE)-conjugated goat F(ab′)2 anti-mouse IgG1 (Southern Biotechnology Associates Inc., Birmingham, AL; cat. no. 1072-09); 106 µL FITC-conjugated goat F(ab′)2 anti-mouse IgG2a (Southern Biotechnology Associates Inc.; cat. no. 1082-02); 106 µL normal goat serum; and 3217 µL PBS.
7. TO-PRO-3 iodide (TP3) staining solution; store at 4°C in the dark (50 mL): 100 µL TO-PRO-3 iodide, 1 m*M* in DMSO (Molecular Probes, Eugene, OR; cat. no. T-3605) and 50 mL PBS.
8. DAPI/Triton X-100 staining solution, freshly made (10 mL): 10 µL 1 mg/mL DAPI in water stock solution (4′,6-diamidino-2-phenylindole, dihydrochloride, Molecular Probes; cat. no. D-1306; can be stored several months in dark or foil-wrapped bottles at –20°C) and 10 mL 0.1% Triton X-100 in PBS.

2.4. Instrument, Setup, and Software

For preparation of cell suspensions using method in **Subheading 3.1.**, a standard sonicator is used (Sonifier 250, Branson Sonic Power Company). Samples are analyzed on a Coulter flow cytometer (Beckman-Coulter, Luton, United Kingdom; model Epics Elite). The flow cytometer is equipped with three lasers for excitation, an air-cooled 488-nm argon laser set at 15 mW (for FITC, PE, and PI staining, forward scatter and right angle scatter signals), a 10-mW 633-nm HeNe laser for TP3 staining, and a tunable water-cooled argon laser set at 80 mW 356 nm excitation for DAPI staining. When 356 nm and 488 nm excitation are used simultaneously, the two laser beams are spatially separated, resulting in fluorescent signals 40 µs apart. In this way spectral crosstalk of the DAPI and FITC fluorescence is avoided.

Immunophenotyping is performed using the Coulter Elite standard optical filters with a 525-nm bandpass filter (Beckman-Coulter; cat. no. 3814134) for the detection of FITC emission and a 575-nm bandpass filter (Beckman-Coulter; cat. no. 3814135) for the detection of PE emission. For PI, TP3, and DAPI emission, a 630-nm long-pass filter (Ortho; cat. no. 4800A-354), a 675-nm bandpass filter (Beckman-Coulter; cat. no. 3814139), and a 450-nm bandpass filter (Zeiss; cat. no. 467984) are used, respectively. For measuring relative cell size blue side scatter (right angle scatter) is collected using a 488-nm bandpass (narrow-band) filter (Beckmann-Coulter; cat. no. 3814137) and a photodiode is used for collecting forward scatter.

For (deoxyribonucleic acid [DNA]) histogram standardization we use both Flow-Check fluorespheres (Beckmann-Coulter; cat. no. 6605359) and ethanol-fixed human lymphocytes. The equipment is focused in a way that if Flow-Check is used, the half peak CV is less than 1.5, and if human lymphocytes are used the half peak CV is less than 3. Moreover, before the assessment of all stained cell suspensions, a sample with normal human keratinocytes is assessed for adjusting the amplifications of the signals.

Using WinList flow cytometry analysis software version 5.0 (Verity Software House, Inc., Topsham, ME), gates are set to calculate the proportion of cells expressing a specific intermediate filament protein or a combination of proteins. In general, a count stop is set at 20,000 keratinocytes, or when possible 10,000 basal keratinocytes (if the proper monoclonal antibodies are used). Cell kinetic characteristics are calculated from the DNA histograms using ModFit DNA analysis software version 3.0 (Verity Software House, Inc., Topsham, ME).

3. Methods (*see* Note 1)

3.1. Preparation of Epidermal Cell Suspension From Razor Blade Microbiopsy Specimens

1. Skin specimens (usually of the back or upper arm) are obtained by shave biopsy using a razorblade in conjunction with a 15 × 2 cm metal guard. A 5-mm diameter hole is punched in the metal guard to get reproducible sized biopsies (approx 0.2-mm thick and 3-mm diameter).
2. Place the biopsies, dermis downwards, in a small Petri dish containing a few drops of trypsin/DTE solution and incubate for 20 min at 37°C.
3. After incubation, transfer the pieces of skin to a small test tube containing 500 µL PI solution and sonicate for 2 s to dissociate the epidermis.
4. Add 100 µL fetal calf serum (to inhibit traces of trypsin).
5. Wash the cell suspension once with PBS and filter through gauze (mesh size approx 120 µm). The stratum corneum and the dermis remain intact during this procedure, yielding a single cell suspension with little admixture of dermal cells.
6. Pellet the cells by centrifugation at 500*g*, resuspend, and fix in 1 mL ice-cold 70% ethanol under continuous vortexing. Fixed cell suspensions are stored at –20°C until further use.

3.2. Preparation of Epidermal Cell Suspensions From Keratotome Biopsies

1. Cool the skin with an ethyl chloride spray and cut skin specimens using a small dermatome in conjunction with a steel guard limiting the area of biopsy to about 1 cm^2. Set the dermatome to give an average thickness of 0.2 mm for normal skin and 0.4 mm for a psoriatic lesion.
2. Float the biopsy, dermal face downwards, in a plastic Petri dish (60-mm diameter) containing a Trypsin/DTE solution (**Subheading 2.3.2.**) for 30 min at 37°C.
3. Transfer the intact specimen to a second Petri dish containing 2 mL PBS/20% calf serum. Peel the dermis from the epidermis and discard. Transfer the remaining contents of the dish (epidermis and some detached cells in PBS/serum) to a tube and agitate gently on a Vortex mixer for 1 min.
4. Remove the transparent horny layer, add 50 µL RNAse solution (A) and incubate the tube for a further 30 min at 37°C.
5. Filter the contents of the tube through gauze (mesh approx 120 µm) and wash the residue with 2 mL PBS/serum.
6. Pellet the cells by centrifugation (100*g* for 10 min), resuspend, and fix in an appropriate volume of 70% ice-cold ethanol (usually 1–2 mL). Fixed cell suspensions are stored at –20°C until further use.

3.3. Preparation of Epidermal Cell Suspensions Using an Optimized Thermolysine-Trypsin Protocol (see Note 2)

1. Collect routine clinical punch biopsies of 3 or 4 mm taken under local anesthesia with xylocain/1% adrenalin.

2. Wash in PBS without Ca and Mg. Shorten the dermis when necessary with a scalpel (length of biopsy should be approx 2 mm). Transfer the biopsy to a small test tube containing 1 mL thermolysin solution and incubate overnight (16–20 h) at 4°C.
3. After thermolysin incubation, separate the dermis and epidermis using fine forceps. Transfer the epidermis to a new test tube containing 2–3 mL trypsin/DTE solution and incubated for a further 30 min at 37°C. The remaining dermis is discarded.
4. Stop the trypsin/DTE incubation by putting the test tube in ice and adding 100 µL NHCS per milliliter of solution and agitate the test tube containing the epidermis on a Vortex mixer for 1 min. Remove the transparent horny layer and pellet the cells by centrifugation (100*g* for 10 min), resuspend, and fix in an appropriate volume of 70% ice-cold ethanol (usually 1–2 mL). Fixed cell suspensions are stored at –20°C until further use.

3.4. Dual-Parameter Flow Cytometric Analysis Combining Monoclonal Antibodies Against Skin-Specific Intermediate Filament Proteins and the DNA-Specific Dye PI

1. Use aliquots of ethanol-fixed cells (100,000–200,000 cells) in suspension. Centrifuge 5 min at 1500*g* at room temperature and remove the supernatant.
2. Resuspend the pellet in 2 mL PBS and centrifuge as before.
3. Resuspend the cell pellet in the rest volume (rest volume is approx 175 µL) and add 325 µL primary monoclonal antibody at the appropriate dilution (**Subheading 2.2.**), rinse, and incubate for 30 min at room temperature in the dark. Control cells are treated identically but incubated with either the isotypic antibody at the same titer or with 325 µL PBS.
4. After 30 min, wash the incubation mixture with 2.5 mL PBS and remove the supernatant by centrifugation as before.
5. Resuspend the pellet and add 325 µL RAM-FITC solution. Rinse the suspension and incubate for 15 min on ice in the dark.
6. After 15 min, wash the suspension using 2.5 mL PBS and remove the supernatant by centrifugation as before.
7. Suspend the cell pellet in 400 µL PI staining buffer and add 50 µL RNAse solution. Incubate 10 min at room temperature in the dark before FCM measurement.
8. Set up the flow cytometer and adjust for excitation with blue light (488 nm) from a 15-mW air-cooled argon laser. Use a 525-nm bandpass filter for detection of FITC emission and a 630-nm long-pass filter for PI emission.
9. Measure the intermediate filament protein-associated green fluorescence of FITC (log-scale) and DNA-associated red fluorescence of PI and collect both area and peak value of the red fluorescence signal. The ratio area/peak is an excellent discriminator between artifacts because of doublets of diploid cells and real single tetraploid cells (*see* **Note 3**). Store the data in list mode and analyze using Verity software (*see* **Subheading 2.4.**). An example of the staining is given in **Fig. 1**.

3.5. Triple Parameter Flow Cytometric Characterization Using the Optimized Thermolysine-Trypsin Protocol

1. Prepare the starting material as 200,000 fixed cells in less than 500 µL of 70% ethanol.
2. Before staining, wash the cell suspensions in 2.5 mL PBS and centrifuge for 5 min at 1500*g*. Remove the supernatant and resuspend the cells on a vortex in the rest volume (~175 µL).
3. To every sample, add 150 µL RKSE60, an IgG1-isotype monoclonal antibody directed against keratin 10. Add another 150 µL VIM 3B4, an IgG2a-isotype monoclonal antibody directed against vimentin.
4. Incubate for 30 min in the dark at room temperature. After 30 min, add 2.5 mL PBS/1% HNCS and wash the cells again.

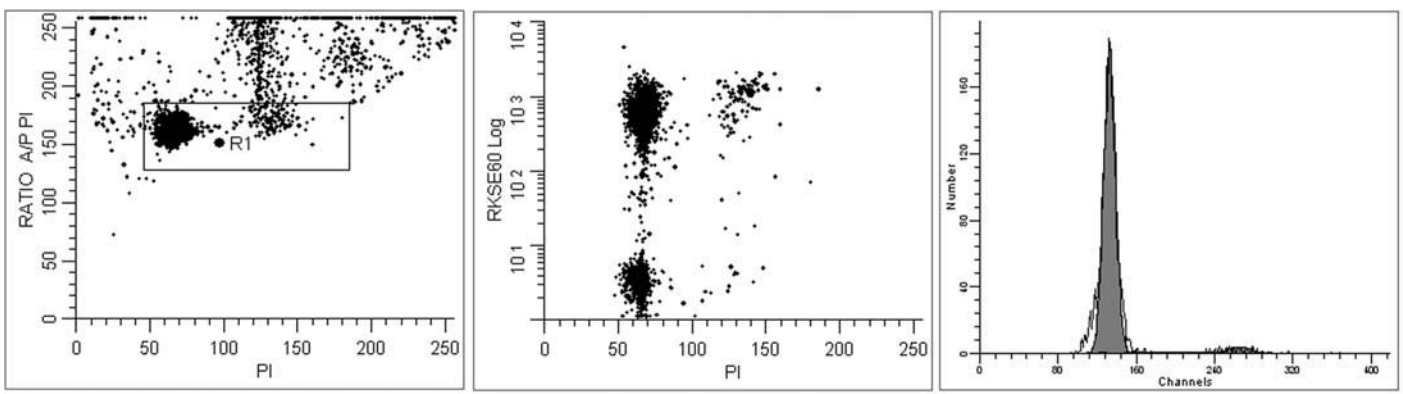


Fig. 1. Example of the method in **Subheading 3.4.**: epidermal cells derived from normal skin labeled with the monoclonal antibody RKSE60 (keratin 10). **A** shows that the ratio area/peak of the PI fluorescent signal (*y* axis) is an excellent discriminator between artifacts owing to doublets of diploid cells and real single tetraploid cells. At the *x* axis, the integrated area of the PI fluorescent signal is plotted, which is used for the relative DNA content per cell. **B** shows the clear discrimination of RKSE60-positive and RKSE60-negative cells (*y* axis). At the *x* axis, again the integrated area of the PI fluorescent signal is plotted. Finally, **C** shows the DNA histogram from which the percentage cells in G0G1, S, and G2M phase are calculated.

5. Subsequently, add 325 µL of secondary antibody cocktail, containing isotype-specific FITC and PE-conjugated secondary antibodies, and incubate the suspension for 15 min in the dark on ice. Final dilutions of the antibodies are 1 : 50 and 1 : 250, respectively.
6. Wash the cell suspensions with 2.5 mL PBS containing 1% NHCS, centrifuge as before, and resuspend the pellets. At this point, the cell suspensions can be stored overnight at 4°C in the dark.
7. Add 300 µL TP3 staining buffer (*see* **Note 4**) and 50 µL RNAse solution and incubate 10 min at room temperature in the dark before FCM measurement.
8. Set up the flow cytometer and adjust for simultaneous excitation with blue light (488 nm) from a 15-mW air-cooled argon laser and red light (633 nm) from a 10-mW air-cooled Helium-Neon laser. A 525-nm bandpass filter is used for detection of FITC emission, a 575-nm bandpass filter for detection of PE emission, and a 675-nm bandpass filter for TP3 emission.
9. Measure the intermediate filament protein-associated green fluorescence of FITC (log-scale) and orange fluorescence of PE (log-scale) together with the DNA-associated far red fluorescence of TP3. Collect both area and peak value of the far red fluorescence signal. Additionally, measure forward and side scatter signals and store the data from the six-parameter assay in list mode and analyze using Verity software (**Subheading 2.4.**).

3.6. A Functional Multiparameter Flow Cytometric Assay to Characterize Proliferation in Skin (see Notes 5 and 8)

1. Using a sample containing approx 200,000 cells of each cell suspension (as in **Subheading 3.5.**); wash, centrifuge, and resuspend the pellet.
2. Incubate with 100 µL PBS containing RKSE60, LHK6B, and V9 (*see* **Note 6**). The final dilution of the monoclonal antibodies is 1 : 20. Incubate the cell suspension with the monoclonal antibody cocktail in the dark for 30 min at room temperature.
3. Wash with PBS containing 1% NHCS and label with the secondary antibody cocktail, containing isotype-specific FITC- and PE-conjugated secondary antibodies for 15 min in the dark on ice. Final dilutions of the antibodies are 1 : 50 and 1 : 250, respectively.
4. Wash the cell suspensions as before and resuspend. At this point the cell suspensions can be stored overnight at 4°C in the dark.
5. For DNA staining add 400 µL DAPI/Triton X-100 staining solution to each specimen (*see* **Note 7**) and wait 10 min before flow cytometric measurement.
6. Set up the flow cytometer and adjust for excitation with 80-mW UV light (356 nm) from a Spectra Physics water-cooled argon laser in combination with a 15-mW air-cooled argon laser for simultaneous excitation with blue light (488 nm). The two laser beams are spatially separated, resulting in fluorescent signals 40 µs apart. A 525-nm bandpass filter is used for detection of FITC emission, a 575-nm bandpass filter for detection of PE emission, and a 450-nm bandpass filter for DAPI emission.
7. Measure the intermediate filament protein-associated green fluorescence of FITC (log-scale) and orange fluorescence of PE (log-scale) together with the DNA-associated blue fluorescence of DAPI. Collect both area and peak value of the blue fluorescence signal. Additionally, measure forward and side scatter signals. The forward scatter or side scatter signal is essential for separating keratin 10 and vimentin staining. Store the data from the seven-parameter assay in list mode and analyze using Verity software (**Subheading 2.4.**). An example of the staining is given in **Fig. 2**.

4. Notes

1. The method in **Subheading 3.1.** is fast and simple. However, the methods in **Subheadings 3.2.** and **3.3.** are much more reproducible. The thermolysine-trypsin protocol (**Subheading 3.3.**) is preferable because it makes use of a clinically routine biopsy procedure with punch

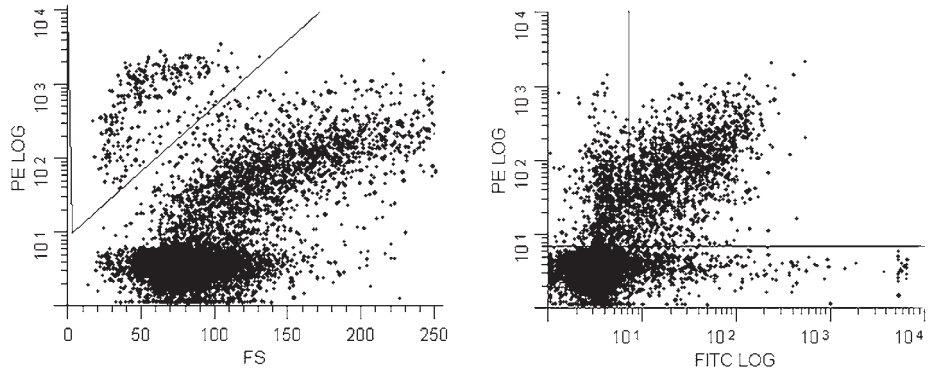


Fig. 2. Example of the method in **Subheading 3.6.**: epidermal cells derived from psoriatic lesional skin labeled with the monoclonal antibodies RKSE60 (keratin 10), LHK6B (keratin 6), and V9 (vimentin), simultaneously. Cells gated by the triangle in the upper left part of **A** are small (low FS, *x* axis) and highly PE fluorescent, representing the vimentin-positive nonkeratinocytes (mostly infiltrating leukocytes). The biggest cells (high FS) are positive for PE too, being the differentiated keratin 10-positive keratinocytes. In **B**, the nonkeratinocytes are gated out, leaving a bivariate histogram of LHK6B (keratin 6, FITC-LOG, *x* axis) vs RKSE60 (keratin 10, PE-LOG, *y* axis.). Unlabeled keratinocytes can be found in the lower left quadrant, RKSE60 single-labeled cells in the upper left quadrant, LHK6B single labeled cells in the lower right quadrant and double labeled cells in the upper right quadrant.

biopsies of 3–4-mm diameter. Furthermore, it selectively obtains the whole epidermis, not only in normal skin, but also in hyperproliferative skin disorders. Because only the epidermis is required, a relatively superficial biopsy that comprises the epidermis and at least a small specimen of dermis is sufficient. The amount of cells obtained per biopsy is around 150,000–300,000 for normal skin and 600,000–1,000,000 for skin derived from hyperproliferative skin disorders. When more cells are preferred 1 cm^2 dermatotome biopsies can be used.

2. Using the thermolysine-trypsin protocol, the vimentin-positive cells are epidermis-derived. In the case of hyperproliferative skin disorders this means it will consist mainly of epidermal infiltrate cells.
3. Flow cytometric data are collected in list-mode with debris elimination gates set: to discriminate between doublets of cells in the G1 phase of the cell cycle and cells, which are in G2+M phase of the cell cycle, the ratio area/peak of the DNA signal (PI, TP3, DAPI) is used, combined with scatter parameters. Although the issue of appropriate hardware gating versus software compensation for aggregate corrections remains controversial ***(11)***, we have used this hardware methodology at our department for years, and it is in accordance with a method described previously ***(12)***. Thus, debris is not calculated mathematically from the DNA histogram, but it is rather excluded physically by determination of aggregates and debris from the list-mode file. As aggregates and debris have already been excluded, the percentage histogram background aggregates and debris (%BAD), defined as the ratio of model estimated aggregates and debris to total cellular events in the histogram, can not be calculated. For physical debris elimination, criteria for accepting a satisfactory S-phase analysis are applied similar to the guidelines for implementation of clinical DNA cytometry, being less than 20% of aggregates and debris ***(11)***.
4. The DNA-specific stain TP3 (**Subheading 3.5.**) gives good results with no spectral overlap with PE. More over, compared with PI, often considered the standard for DNA stain-

ing, TP3 shows similar results. It is suggested that TP3 intercalates with double-stranded regions of nucleic acids similar to PI, but it also affects RNA. For this reason, RNAse was added to each specimen before flow cytometric measurement. Others have suggested to use the 488 nm excitable dye 7-amino actinomycin D for staining DNA. However, the binding of 7-amino actinomycin is markedly affected by differences in chromatin structure. This is especially the case when staining heterogeneous epidermal cell suspensions containing a mixture of basal and differentiated cells.

5. To establish a simple staining procedure and a clinically relevant flow cytometric assay (**Subheading 3.6.**), we tested several combinations of isotype specific monoclonal antibodies against cytokeratin 10 and 6 and vimentin. As we are interested in expanding our protocols by adding more parameters, we searched for a combination of antibodies, which allowed assessment of all three markers while labeled with only two fluorchromes. We found that isotype specific selection of monoclonal antibodies against cytokeratin 10 (RKSE60) and vimentin, allowed quantification of both cell populations although both populations were labeled with PE.
6. For the simultaneous measurement of the IgG1 specific anti-keratin 10 marker RKSE60 and a hyperproliferation marker other than vimentin (**Subheading 3.5.**), we focused on a hyperproliferation marker that was not IgG1specific. Although the anti-keratin 16 marker LL025 is a well-documented hyperproliferation marker, we were not able to use this marker because it is IgG1 specific. LHK6B (NCL-CK6 from Novocastra Laboratories) is an IgG2a-specific monoclonal antibody that is directed against the hyperproliferation-associated keratin 6. As LHK6B is of the IgG2a isotype and as it is a well-described hyperproliferation marker, we focused on this label.
7. Among the variety of DNA-specific dyes, the binding of DAPI to DNA *in situ* is the least influenced by nuclear proteins. DAPI, therefore, appears to be the most suitable fluorochrome for stoichiometric staining. The disadvantage, however, is the need for an additional UV laser. Changing TP3 (**Subheading 3.5.**) for DAPI leaves the red end of the emission spectrum for a third fluorochrome coupled to an antibody, such as the resonance energy transfer dye Cy5-phycoerythrin. This is an extremely bright dye, primarily because of the very low autofluorescence in the region where it fluoresces. It is easily excited at 488 nm, and emits at 680 nm and can therefore be used in combination with FITC and PE.
8. A frequently used method for clinical assessment of psoriasis disease severity is the Psoriasis Area and Severity Index. In this scoring system, the percentage involvement and the degree of erythema (inflammation), induration (proliferation) and desquamation (differentiation) is estimated. These are the hallmarks of this hyperproliferative skin disorder. Using a formula, an ordinal value between 0 and 72 can be calculated. However, the index cannot be regarded as an exact numerical value because the severity rating is subjective. Therefore, there is a need for objective measurements of psoriasis severity. The protocols presented in this chapter, especially in **Subheading 3.5.**, are the result of the development of multiparameter flow cytometric assays with simultaneous staining of markers for epidermal proliferation (DNA content), differentiation (anti-keratin 6 and 10), and inflammation (anti-vimentin). In contrast to clinical scoring flow cytometry permits quantitative analysis of different parameters (*see* **Table 1**). We have performed correlation analysis on 166 paired values obtained from 83 patients using the methods in **Subheading 3.5.** ***(8)***. A highly significant correlation was observed between erythema and the percentage of vimentin-positive cells, between desquamation and the percentage of keratin 10-positive cells, and between induration and the number of basal keratinocytes in S- and G2M phase of the cell cycle. In conclusion, multiparameter flow cytometry has been shown to be a sensitive tool to evaluate the growth inhibiting, anti-inflammatory, and keratinization-enhancing effects of antipsoriatic therapies.

Table 1
Measured Percentages of Positive Cells for the Different Monoclonal Antibodies in Normal and Psoriatic Epidermal Cells

			Percentage positive cells					
			Subheading 3.4. ***(5)***		**Subheading 3.5.** ***(7,13)***		**Subheading 3.6.**[a]	
Clone	Specificity	Isotype	N	PL	N	PL	N	PL
RKSE60	Keratin 10	IgG1	57.2 ± 3.5	46.6 ± 8.3	70.0 ± 3.6	27.4 ± 3.2	70.7 ± 2.1	42.5 ± 3.7
K8.12	Keratin 16 ***(13,15)***	IgG1	1.8 ± 1.0	40.4 ± 19.5	N.D.	N.D.	N.D.	N.D.
LHK6B	Keratin 6	IgG2a	N.D.	N.D.	0.6 ± 0.1	18.0 ± 3.9	6.9 ± 0.9	29.0 ± 4.0
Vim3B4	Vimentin	IgG2a	N.D.	N.D.	7.2 ± 0.6	15.1 ± 2.4	N.D.	N.D.
V9	Vimentin	IgG1	N.D.	N.D.	N.D.	N.D.	5.1 ± 0.6	3.4 ± 0.4

[a]Unpublished data.

Acknowledgment

The author would like to thank other present and past members of the Dermatology research laboratory at the UMCN who have contributed to developing the foregoing protocols.

References

1. Mier, P. D. and Cotton, D. W. K. *The Molecular Biology of Skin*, Blackwell Scientific Publications, Oxford, 1976.
2 Bauer, F. W. (1986) Cell kinetics, in *Textbook of Psoriasis* (van de Kerkhof PCM, ed.), Churchill Livingstone, Edinburgh, pp. 100–112.
3. Bauer, F. W., Crombag, N. H., de Grood, R. M., and de Jongh, G. J. (1980) Flow cytometry as a tool for the study of cell kinetics in epidermis. *Br. J. Dermatol.* **102,** 629–639.
4 van Erp, P. E., Brons, P. P., Boezeman, J. B., de Jongh, G. J., and Bauer, F. W. (1988) A rapid flow cytometric method for bivariate bromodeoxyuridine/DNA analysis using simultaneous proteolytic enzyme digestion and acid denaturation. *Cytometry* **9,** 627–630.
5 van Erp, P. E., Rijzewijk, J. J., Boezeman, J. B., Leenders, J., de Mare, S., Schalkwijk, J., et al. (1989) Flow cytometric analysis of epidermal subpopulations from normal and psoriatic skin using monoclonal antibodies against intermediate filaments. *Am. J. Pathol.* **135,** 865–870.
6 de Mare, S., van Erp, P. E., Ramaekers, F. C., and van de Kerkof, P. C. (1990) Flow cytometric quantification of human epidermal cells expressing keratin 16 in vivo after standardized trauma. *Arch. Dermatol. Res.* **282,** 126–130.
7 Glade, C. P., Seegers, B. A., Meulen, E. F., van Hooijdonk, C. A., van Erp, P. E., and van de Kerkof, P. C. (1996) Multiparameter flow cytometric characterization of epidermal cell suspensions prepared from normal and hyperproliferative human skin using an optimized thermolysin-trypsin protocol. *Arch. Dermatol. Res.* **288,** 203–210.
8 Glade, C. P., van Erp, P. E., Boezeman, J. B., and van de Kerkof, P. C. (1997) Multiparameter flow cytometry as a tool to evaluate antipsoriatic therapy. *Br. J. Dermatol.* **137,** 367–375.
9 Mommers, J. M., Goossen, J. W., van De Kerkhof, P. C., and van Erp, P. E. (2000) Novel functional multiparameter flow cytometric assay to characterize proliferation in skin. *Cytometry* **42,** 43–49.
10 Seegers, B. A., Glade, C. P., van Hooijdonk, C. A., van Erp, P. E., de Jongh, G. J., and van de Kerkof, P. C. (2000) Flow-cytometric characterization of normal versus psoriatic epidermis using improved cell separation methodology. *Exp. Dermatol.* **9,** 266–270.
11 Shankey, T. V., Rabinovitch, P. S., Bagwell, B., Bauer, K. D., Duque, R. E., Hedley, D. W., Mayall, B. H., Wheeless, L., et al. (1993) Guidelines for implementation of clinical DNA cytometry. International Society for Analytical Cytology. *Cytometry* **14,** 472–477.
12. Bauer, F. W. and Boezeman, J. B. (1983) Flow cytometric methods in human skin with respect to cell cycle kinetics, in *Psoriasis: Cell Proliferation* (Camplejohn, R. S., ed.), Churchill Livingstone, Edinburgh, pp. 104–116.

27

Fluorimetric DNA Assay of Cell Number

William R. Otto

Summary

This fluorimetric assay has utility for the accurate assessment of cultured epidermal cell numbers by virtue of their deoxyribonucleic acid content, which is the most significant correlate available. The improvement in fluorochromes in the recent past makes PicoGreen the dye of choice for this, with its greatly increased sensitivity (± 50 cells) over the Hoechst and DAPI stains and which remains linear over several orders of magnitude with a single dye concentration. The assay involves minimal liquid handling to achieve cell disruption by sodium dodecyl sulfate in saline sodium citrate buffer, and PicoGreen staining is rapidly assayed by a multiwell plate reading fluorimeter, which can be automated for robotic high throughput use. Highly fibrous cells like epidermal keratinocytes can be disrupted using 8 *M* urea and assayed after dilution. The assay is also compatible with tritiated thymidine incorporation.

Key Words:

DNA assay; cell growth; fluorimetry; fluorochrome; fluorescence; PicoGreen; Hoechst 33258; Hoechst 33342; cell culture; epidermis; keratinocyte; tritiated thymidine; scintillation spectrometry; high throughput.

1. Introduction

There are a great many assays for cell number that have been developed over the years. The simplest is a cell count with hemacytometer, but this is time-consuming and prone to inaccuracies of mixing and cannot provide a high throughput. For cell populations that can be separated into single cells, there are flow cytometers in which a stream of cells is excited by a laser and passes a photomultiplier detector, which records a variety of cellular parameters that correlate to cell size (i.e., through light scatter), but that can be combined with fluorochromes to measure individual cellular contents of an increasing number of molecular, organelle or cell type-specific moieties (i.e., DNA, mitochondria, transport vesicles). These also provide a way to separate cells based on their assayed contents by imparting a charge to each cell in the stream and passing the flow of droplets between electrically charged plates to deflect certain ones into separate tubes, the basis of fluorescent-activated cell sorting (FACS). These powerful methods need both a dedicated laboratory to run them and a large budget. There are a number of other methods using similar fluorescent techniques that can be performed on microplates that are therefore amenable to high throughput at much less cost and with much meaningful information. It is generally recognized that fluorescent assays are

From: *Methods in Molecular Biology, vol. 289:*
Epidermal Cells: Methods and Protocols
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

many-fold more sensitive than spectrophotometric assays, and this chapter describes an assay for the fluorimetric estimation of DNA.

Many assays purporting to quantify cell number rely on estimations of intracellular molecules, either in general, such as total protein content, or by enzyme activities, e.g., dehydrogenases or phosphatases. Such assays can be quick and accurate if the content of such molecules *per cell* does not change with any purturbation in the experiment. However, it may also be the case that only a subset of cells in a population retains the ability to respond to an external signal (i.e., growth factor, proapoptotic agent), and so could lead to false cell number outputs. For epidermal keratinocytes this is a critical problem because as the cells stratify, the protein content per cell rises, which would lead a protein assay of "cell growth" to overestimate cell number (*see* **Note 1**). There is a further problem in that keratinocytes are programmed to cornify and die once reaching the granular layer and above. This involves the activation of nucleases, which must, by definition, reduce the nucleic acid content of the cornifying cells, which can lead to further errors. This is less of a problem than the protein content per cell because cornified cells can be counted ***(1,2)***. The problems associated with non-DNA estimations of cell number have been discussed previously ***(3,4)***.

The cellular component that most accuately reflects cell number is DNA. This holds true within a species generally and usually within all cells in any one individual, with a few well known exceptions, such as epidermal keratinocytes, which have reached the granular layer and have begun degrading their intracellular contents, red blood cells, and their immediate precursors, as well as megakaryocytes (polyploid) and their products the blood platelets ***(5)*** and also the fusion of macrophages into giant nucleated cells, or osteoclasts (*see* **Note 2**). One important cell that can have regular polyploid variations is the hepatocyte, which can reach up to 128 *N* in mice ***(6)***, and assays for cell number by DNA assay alone here could be reasonably inaccurate. Polyploidy in cancer has been reviewed in detail recently ***(7)***. Even tumor cells can retain a relatively stable karyotype and consequently DNA content, although heterokaryons may be quite unstable, with variable chromosome numbers. Researchers should be aware of such confounding issues and make suitable decisions on their assay.

Fluorimetric DNA assays are more sensitive and specific than spectrophotometric methods (*see* **Table 1)** and may be automated and performed in multiwell formats. The most sensitive assays use adenine–thymine (AT)-binding fluorochromes, such as Hoechst 33258 (bisbenzamide: 2-[2-(4-hydroxyphenyl)-6-benzimidazolyl]-6-(1-methyl-4-piperazyl)-benzimidazole.3HCl) ***(8–14)*** or Hoechst 33342 ***(9,15–17)***, DAPI ***(18)***, and more recently the highly sensitive PicoGreen ***(8,10,13,14,16,19,20)***. These dyes are DNA specific, with a substantial preference for double-stranded DNA and with which proteins and RNA do not interfere. In this they are far superior to phenanthridinium fluorochromes, such as ethidium bromide ***(13,21,22)***, propidium iodide ***(16)***, which bind to RNA as well as DNA and require RNase or DNase as appropriate to estimate the nucleic acid of interest. Acridine orange fluoresces with RNA and DNA at different wavelengths and so can be used as a dual-assay reagent, but its sensitivity is less than the Hoechst dyes or Picogreen for DNA (**ref. *23***; *see* **Note 3**). The TOTO dimeric series of dyes and their less sensitive TO-PRO monomers are cyanine compounds and are recent additions to the nucleic acid assay repetoire, but the TOTO series may take several hours to reach DNA binding equilibrium and so may be less convenient than Picogreen or the Hoechst dyes, which show rapid binding, within 5 min ***(24)***. The SYTO

Table 1
Detection Limits for Several DNA Assays

Method[a]	Detection limit[b]	Reference
Diphenylamine	10 µg/mL	***47***
Diaminobenzoic acid	1 µg/mL	***39***
Absorbance at 260 nm	250 ng/mL[c]	***46***
Acridine orange	40 ng/mL	***23***
Hoechst 33258	5 ng/mL	***12***
Ethidium homodimer	1 ng/mL[d]	***21***
Picogreen	25 pg/mL	***14***

[a]Diphenylamine and routine absorbance assays are performed on a spectrophotometer. The other assays use a fluorimeter at specified wavelengths (*see* **Table 2** for details).

[b]For dsDNA in free solution.

[c]Highly dependent on the purity of DNA, with significant interference from RNA and proteins. The ratio of absorbance at 260/280 nm should be 1.8 for pure DNA, anything less indicating impurities.

[d]Ethidium also fluoresces significantly with RNA.

series of dyes bind both DNA and RNA and are not the fluorochromes of choice for single-species assays, but benefit from their cell permeability and may be useful for live imaging. Of the series, SYTO-16 has some twofold greater fluorescent signal for DNA over ribonucleic acid (RNA), whereas the reverse is true for SYTO 12 or 14. The main spectral characteristics of several nucleic acid-binding dyes are given in **Table 2**. From this, it is clear that the major fluorochromes useful for DNA-specific work are the Hoechst dyes, SYBR-1, PicoGreen, and although DAPI continues to be used for DNA assays, there is some interference from AU-rich RNA. The most sensitive of these dyes is PicoGreen, by up to 400-fold over Hoechst derivatives ***(14)***, whereas SYBR-1 comes close ***(13)***. No truly specific dye exists yet for RNA, but RiboGreen shows some specificity in this regard ***(25,26)***. Picogreen has become useful in whole cell cytometric assays ***(27)***, 96-well assays ***(10,28)*** or in polymerase chain reaction-based amplifications ***(19,29)***.

Assays other than direct cell number or DNA assessment have also been developed using these dyes, and many overlap with histological and microscopical techniques. For example, Picogreen can be used to assay telomerase activity ***(30)***, DNA damage and unwinding ***(31)***, DNase I activity ***(32)***, microbial pathogen DNA fingerprinting by flow cytometry ***(27)***, serum DNA in cancer patients ***(33)***, plasma nucleosome concentrations ***(34)***, chitosan nanoparticle DNA transfections ***(35)***. The assays depend on the ability of the intercalated dyes to fluoresce at specific wavelengths in a DNA concentration-dependent manner. Sensitivities of 25 pg/mL DNA can readily be achieved using PicoGreen ***(14)***, which is about 400-fold greater than that for the Hoechst dyes (*see* **Notes 1** and **2**). Spectrophotometric assays cannot compete with these levels and are further hindered by competing signals from RNA and proteins where these are not selectively removed (*see* **ref. *4***). There are fluorochromes that can differentiate simultaneously between nucleic acid types (i.e., acridine orange) which enable both to be assayed without separation, but these are not as sensitive as Picogreen (*see* **Note 3**). The fluorimetric methods described here are suited to microanalysis and

Table 2
Spectral Characteristics of Selected Nucleic Acid-Binding Fluorochromes (After Haugland 2002; ref. *24*)

Fluorochrome	Excitation (nm)	Emission (nm)	DNA binding	RNA binding	Cell permeability
Acridine (DNA)	500	526	Yes	Yes	Yes
Orange (RNA)	460	650	Yes	Yes	
DAPI	358	461	Yes (AT)	Little (AU)	Semi
Ethidium bromide	518	605	Yes	Yes	No
Hoechst 33258	352	461	Yes (AT)	No	Yes
Hoechst 33342	350	461	Yes (AT)	No	Yes
PicoGreen	502 (485)[a]	523 (530)[a]	Yes	No	NT
Propidium iodide	535	617	Yes	Yes	No
RiboGreen	500	520	Yes	Yes	NT
SYBR Green I	494	521	Yes	No	NT
SYBR Green II	492	513	Yes (ss)	Yes	NT
SYTO-16	488	588	Yes	Yes[b]	Yes
SYTO-12	500	522	Yes[b]	Yes	Yes
TOTO-I	514	513	Yes (ds/ss) (esp 5′CTAG3′ sites)	Yes	No

Cautionary note: Beware that most DNA-binding dyes are mutagenic and should be handled with care in an appropriate environment by skilled personnel.

DNA binding not sequence-specific unless stated; DAPI, 4,6-diamidino-2-phenylindole, which is more photostable but less bright than the Hoechst dyes.

[a]Suitable wavelengths for Cytofluor or other plate-reading fluorimeter, using the fluorescein channels.

AT, adenine–thymine selective intercalator; AU, adenine–uracil; NT, not tested; ds, double-stranded, ss, single-stranded; SYTO dyes are cell-permeant for live cell assays, but background cytoplasmic fluorescence may be high.

[b]Twofold lower binding than the other nucleic acid; TOTO-I binding to DNA may take up to 2 h to reach equilibrium. Avoid glass tubes for TOTO-I and related dyes, as they adsorb readily.

do not require mechanical homogenization of samples unless tissue fragments are used, but do depend on cell disruption by detergent. Earlier fluorimetric assays, which have fallen into disuse by virtue of their lower sensitivity, such as that using diaminobenzoic acid, have been reported ***(4)***, and interested readers are referred there. Although now not considered a method of choice, the use of tritiated thymidine (^{3}H-TdR) to assay a population of cells synthesizing DNA via the salvage pathway could be considered, with autoradiography and individual nuclear scoring by microscopy as an endpoint (**ref. *3*; *see* Note 4).** Clearly, such an assay cannot measure the whole population DNA content nor the DNA of non-S-phase cells (except those undertaking DNA repair), but it may indicate whether there are actively proliferating cells in a population, and it has the advantage of preserving the cells and their relative positions, which assays undertaking any form of homogenization cannot provide. A similar assay may be performed by immunocytochemical means by exposing cells to the thymidine analog bromodeoxyuridine (BrdU) (*see* **Note 3),** and assaying for its incorporation. This can be achieved by anti-BrdU antibodies, either by imaging positive nuclei on microscope slide fixed preparations (or tissue culture

plastic) using precipitating chromogens, such as diamino benzidine for peroxidase or an alkaline phosphatase chromogen such as 4-nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate, depending on the final antibody conjugate of choice. Alternatively, a soluble product can be obtained using suitable compounds. These include, for peroxidase, the chromogens tetramethyl benzidine, 2,2′-azino-di-(3-ethyl-benzthiazoline-6-sulphonic acid) or fluorogens, such as fluorescein diacetate, and resorufin, or light emission by enhanced chemiluminescence by 5-amino-2,3-dihydro-1,4-phthalazinedione [luminol] in a 96 (or other) -well plate format ***(3)*** using the appropriate plate reader (spectrophotometer, fluorimeter or luminometer respectively). Equivalent dyes with soluble products exist for alkaline phosphatase conjugated antibodies: the chromogen paranitrophenol phosphate, the fluorogens fluorescein diphosphate or difluorinated methylumbelliferone phosphate, or the luminescent substrate 1, 2-dioxetane [4-chloro,]3-(4-methoxyspiro{1,2-dioxetane-3, 2′-(5′-chloro)tricyclo-[3.3.1.1^{3,7}] decan}-4-yl) phenyl phosphate).

The Hoechst-, DAPI-, or PicoGreen-based assays are essentially two-step procedures once samples are ready for analysis. For in vitro cell assays, culture medium is washed off, cells are dissolved in sodium dodecyl sulfate (SDS), the dye is added and the fluorescence measured in a suitable fluorimeter. The sensitivities of Hoechst 33258 ***(12)*** and PicoGreen ***(14,36)*** to SDS are different, and these should be factored into any assay for DNA. Hoechst dye assays can tolerate up to 0.02% final concentration of SDS, whereas PicoGreen is sensitive to concentrations above 0.01% (*see* **Note 5**). This means that any cell (or virus) lysis step at concentrations above these limits needs to be diluted to reach these levels at the time of assay. This is conveniently achieved by the lysis being performed first, followed by addition of an SDS-free solution of buffer containing the fluorochrome which simultaneously reduces the SDS level appropriately. 96-Well plates are available with white sides and a clear plastic or glass bottom to allow visualisation of cells during the growth period, but that eliminate fluorescent crosstalk between wells during the quantification steps (e.g., Nunc 96 Microwell, Packard Viewplate). Liquid handling is thus minimal and can be automated, resulting in high precision and accuracy.

2. Materials

2.1. Buffer Formulae

1. Phosphate-buffered saline (PBS-A, calcium- and magnesium-free). For 1 L: 8 g NaCl, 0.2 g KCl, 2.9 g Na_2HPO_4;$12H_2O$, 0.24 g KH_2PO_4, and Milli-Q. A "Biocet" or "synthesis" system water (nuclease-free, 18.2 Mohm. cm. Millipore Corporation) or double-distilled water to 900 mL. pH solution to 7.4 with concentrated HCl and make to 1000 mL with MilliQ or double-distilled water. Sterilize by autoclaving at 15 lbs per square inch (psi) for 20 min and store at room temperature (RT).
2. Saline sodium citrate (SSC). For 1 L of 20X stock: 175.3 g NaCl, 88.2 g Na_3 citrate, and MilliQ or 800 mL double-distilled water. Adjust pH solution to 7.0 with a few drops of 10 *M* NaOH and make to 1000 mL with MilliQ or double-distilled (dd) water. Sterilize by autoclaving at 15 psi for 20 min. Dilute 5 mL to 100 mL (1 in 20) in sterile MilliQ or ddH_2O to use.
3. Tris-EDTA (TE). 10 m*M* Tris-HCl, with added 1 m*M* EDTA. For 1 L: 1.21 g Tris base, 0.372 g EDTA Na_2, and MilliQ water or 800 mL double-distilled. Adjust pH to 7.5, make up to 1000 mL, sterilize by autoclaving at 15 psi for 20 min, and store at 4°C.

4. Phosphate-buffered EDTA (Versene). For 1 L: 8 g NaCl, 0.2 g KCl, 2.9 g $Na_2HPO_4 \cdot 12H_2O$, 0.24 g KH_2PO_4, 0.2 g EDTA-Na_2, 1.5 mL 5% phenol red, and MilliQ or 900 mL double-distilled water. Adjust pH to 7.4 and make up to 1000 mL with MilliQ or ddH_2O. Sterilize aliquots by autoclaving at 15 psi for 20 min. The solution should be orange–red in color.

2.2. Growth of Cultured Cells

1. 8- or 12-Channel or repeating pipet with sterile tips.
2. Sterile reagent troughs, or square Petri dishes.
3. Cells in culture flasks (*see* **Note 1**).
4. Multi-well plates (typically 96-well, though many formats can be used in several plate readers).
5. PBS, calcium and magnesium-free.
6. Trypsin solution in versene. Make trypsin solution fresh, at 0.5 to 2.5 mg/mL, as required. Sterilize by 0.2-µ filtration. Presterilized 10X trypsin concentrates can be stored frozen at –20°C.
7. Culture medium, stored at 4°C.
8. Fetal calf serum or other serum, stored frozen in convenient aliquots, commonly 20–50 mL.
9. Hemacytometer, cover slips, and counting tally.
10. Inverted phase-contrast microscope.
11. Growth promoting or inhibiting agents at 1000X final concentration, stored frozen in 0.2-µ sterilized aliquots, usually with carrier protein such as 0.1% BSA (*see* **Note 6**). If volumes are too small to presterilize, then full medium can be sterilized just before use. Prewet the filter with medium containing fetal calf serum or bovine serum albumin at the same concentration to minimize adsorption of diluted peptides to the filter. Carefully remove any liquid from the sterile luer underside of the filter to minimise dilution of the factor.
12. Incubator at 37°C, 5–10% CO_2 as appropriate for your cells.

2.3. Fluorimetric DNA Assay Using PicoGreen

1. 10% SDS in distilled water (dw). Store at RT (*see* **Note 5**).
2. Saline sodium citrate (SSC).
3. Stock calf thymus DNA solution in distilled water or 0.9% saline (1 mg/mL). Dissolve overnight at 4°C with gentle shaking and store aliquotted at –20°C.
4. Freshly diluted range of working DNA concentrations (0–20 µg/mL) diluted in 0.02% sodium dodecyl sulfate in SSC.
5. Stock PicoGreen (or DAPI, Hoechst 33258 or 33342) in SSC (1 mg/mL), stored aliquoted at –20°C, protected from light. Working dilutions of PicoGreen of 0.8 µ*M*, or of the DAPI or Hoechst dyes of 0.1 µg/mL, in SSC should be made. Beware of possible mutagenic effects of DNA-intercalating dyes, wear gloves and avoid aerosol formation at all times.
6. Fluorimeter (i.e., Cytofluor 4000; Perseptive Biosystems, Foster City, CA) with appropriate filter sets (*see* **Table 2**). Beware that some optical sensors are adjustable for assays above or below a multiwell plate. Black and white plates can only be assayed from above, clear plates from both sides.

3. Methods

3.1. Growth of Cultured Cells

This assay presumes the experiment is to measure cell growth and so begins with a low cell number per well. For growth inhibition or apoptotic studies a larger starting population would be appropriate.

1. Trypsinize cells as for routine subculture (commonly 2–5 min), quench enzyme with serum-containing medium, spin-wash cells at 100–150*g* for 5 min at RT, resuspend in medium, and count a small aliquot in a hemacytometer.
2. Resuspend appropriate cell number for the whole experiment (allow a modest excess for pipetting errors) at 10^4/mL and dispense 100 µL (1000 cells) to each well if using 96-well plates. Thorough cell mixing and identical volumes dispensed at this stage are critical to obtain uniform cell numbers per well. Adjust cell numbers for your specific requirements or known growth rate (*see* **Note 1**). For cell toxicity assays, a high starting cell number will be required as cells will be lost into the supernatant during the assay. These may be collected by gentle swirling and aspiration by pipet for further assay, though a DNA estimation may not be an appropriate method for apoptotic events, and the TUNEL ***(37)*** or annexin V-biotin ***(38)*** assays should be considered. Such initial cultures may contain commonly up to 10^5 cells for 96-well plates. The serum is often 10% when plating cells, to allow for efficient attachment and spreading, though this can be altered by design, and cultures switched to lower serum levels for subsequent treatments, often at 24 h. Use a separate plate per time point to allow for several time points.
3. After cells have spread, experimental media are substituted as appropriate until just pre-confluent for the positive control group, or to a maximum of about 10^5 per well for a 96-well plate (*see* **Note 6**).
4. Cell harvest: at each time point, carefully aspirate medium from each well under low suction. Eight-channel manifolds are available for convenience (i.e., Nunc Immuno Washers), with plates held at a high angle (up to 60°C) to ensure complete aspiration. Avoid contact by the mainfold or pipet with the cell monolayer. A slow suction force is less damaging to the cultures. For nonadherent cells, a V-bottomed plate may be used and the plates centrifuged at 200*g* before aspirating medium. For assay of detaching or apoptotic cells, these should be removed quantitatively into a V-bottomed plate by aspirating with an 8- or 12-channel pipet and rinsing each well again with at least one change of PBS-A (or other suitable buffer). These are then combined and spun as for non-adherent cells to create a pellet for DNA assay and/or cornified envelope assay ***(1)***. When sterility is not important at the end of adherent cell assays, one may use a "flick" method (rapid plate inversion) to remove medium, and then blot the plate onto tissue paper. Take care to wear gloves and not to make aerosols that may contain toxic materials and cells or debris if using this latter method.
5. Rinse out each well twice with PBS-A, aspirate and store at –20°C until DNA assay. A freezing step has the advantage of bursting most cells on thawing, which improves the subsequent lysis procedure (*see* **Note 7**).

3.2. Fluorimetric DNA Assay Using PicoGreen (12,14,36)

1. Make working dilutions of stock DNA (0 to 20 µg/mL) in 0.02% SDS in SSC. Greater SDS concentrations used with Picogreen produce a high-background fluorescence. Pipet 100 µL in quadruplicates into a fresh plate or into spare lanes of experimental plates. Avoid making bubbles as these act as lenses/diffusers during fluorimetry and lead to aberrant results. "Reverse" pipetting is a useful way to achieve this when using Gilson or equivalent pipets. Electronic multichannel pipets usually avoid bubble formation and generally deliver more reproducible volumes.
2. To lyse cells in each experimental well, add 100 µL 0.02% SDS in SSC. Higher concentrations of SDS may be used to lyse more cells. However, the final concentration, after dye addition, should be 0.01% or less when reading in the fluorimeter. The maximum working volume for a 96-well plate is about 280 µL per well. For fibrous cells, such as strati-

fying epidermal or gingival keratinocytes, it is necessary to include 8 *M* urea in the lysis buffer. The final urea concentration when PicoGreen is added should be 2 *M* ***(14)***, and account made of the dilutions during cell number calculations (*see* **Note 7**).

3. Incubate at 37°C for 30 min on a rotary shaker to ensure cell dissolution.
4. Add 100 µL PicoGreen stock solution diluted 1 : 200 in SSC to each well. Wear gloves and avoid aerosol production when handling possibly mutagenic DNA-binding dyes.
5. Protecting from light, swirl the plate gently for 5 min to mix and stabilize the dye–DNA complexes. Fluorescence can be read in the clear 96-well plate at this point, but for improved sensitivity, remove 180 µL (or other known fraction of the cell or standards solution) from each well into a white plate.
6. Read the fluorescence of each sample in a microplate-reading fluorimeter at λ excitation (ex) 502 nm, λ emission (em) 523 nm, or use the fluorescein filter set (λ ex 494, λ em 518 nm), which is sufficient for most requirements. Subtract blank readings to arrive at DNA-specific fluorescent signals, plot standards to check for linearity, and calculate the unknowns from the slope of the standard line. The R^2 of the slope should approach unity.

4. Notes

1. This assay should facilitate the assay of the growth rate of many cell types. The regression of fluorescence units to DNA content reliably approaches unity. The assay may be combined with parallel assay plates using fluorimetric enzyme-linked immunosorbent assay (ELISA) for cellular antigens to establish correlations between several parameters simultaneously.
2. A further factor to consider is the ploidy level of the cells, which by definition affects DNA values, but which could confuse the calculation of true cell numbers. A titration of PicoGreen fluorescence of a known cell number against a DNA standard curve will allow the mean DNA content per cell to be calculated. Normal human diploid cells contain about 7 pg total DNA ***(39)***, but up to 2 pg of this can be mitochondrial. Phase-contrast microscopy of cells will reveal how many are multinicleate, while fluorescence microscopy with PicoGreen, DAPI or a Hoechst dye to stain the nuclei will reveal polyploid cells by their brighter images. Flow cytometry of cells would provide a statistical readout of the whole population, which may be useful, particluarly for liver cells, which can often have more than one nucleus and several copies of the genome in each one, particularly in the mouse. Recent reviews of how ploidy can vary in different tissues ***(5,6)*** or cancer ***(7)*** have appeared. Ploidy is not usally an issue for epidermal keratinocytes, though researchers should recall the need to monitor desquamating cornified envelopes with greatly reduced contents of DNA.
3. The assay described here may be adapted to use the metachromatic acridine orange for simultaneous estimation of DNA and RNA, choosing suitable wavelengths (λ ex 485 nm, λ em 530 nm for DNA, and λ ex 485 nm, λ em 620 nm for RNA). However this dye has a lower nucleic acid sensitivity. Ethidium bromide also binds both DNA and RNA, but is not metachromatic, and a specific DNA assay requires an extra step, the removal of RNA with RNase ***(40,41)***. The two Hoechst dyes (33258, 33342) and DAPI bind AT base pairs, unlike PicoGreen which binds CG-rich DNA equally as well as AT-rich DNA. These four dyes are nevertheless broadly specific for DNA, with far lower fluorescence signals from RNA and single-stranded DNA ***(14)***. However, there are occasions when cells may be exposed for short periods to bromodeoxyuridine (BrdU) to assay for cells synthesizing DNA. However, this thymidine analogue inhibits Hoechst and DAPI binding and would result in an underestimate of the DNA content ***(42)***. Whether this is also true for PicoGreen is as yet unreported.
4. Cellular tritiated thymidine (^{3}H-TdR) incorporation may be assessed without affecting the fluorimetric DNA assay. Care should be taken of its potential hazards and experimental problems ***(43)***. Cells receive a 1–4 h pulse of 37kBq (1 µCi)/mL ^{3}H-methyl-TdR (specific

activity 5 GBq mmol-1) just before harvest, unincorporated label is removed by three washes in PBS-A, and the above DNA assay is followed. After reading the fluorescence values an aliquot of the cell digest is assayed by scintillation spectroscopy, and the counts related to the micrograms of DNA assayed in the same wells. However, such an assay will not take into account acid-extractable intracellular pools of nonincorporated or stored label, and so will overestimate the ^{3}H-thymidine incorporation. It would be better to precipitate the DNA in an aliquot with 5% TCA and measure the thymidine in that, but that would mean many more steps per well, or require a parallel assay plate (*see* **ref. *4*** for details). Another important shortcoming when using ^{3}H-TdR is that only cells synthesizing DNA take up label, and this may not reflect the behavior of all cells in the culture. Investigators are encouraged to read older but still relevant critiques of ^{3}H-thymidine assays ***(43–45)***.

5. For maximum sensitivity the final %SDS in the lysis buffer should not exceed 0.01% after addition of PicoGreen, or 0.02% with Hoechst dyes or DAPI. Other lysis agents, such as NaOH, EDTA, Triton X-100, NP-40, or higher SDS levels are unreliable or give very high blank values and lower sensitivity ***(12,14,36)***. DNA standards should be treated identically with the unknown samples. Once added, all the fluorochromes suggested for this assay bind almost immediately and change little with time if protected from light. Nevertheless, it is prudent to read fluorescence values at a standard time after adding the dye to improve interexperimental comparisons.
6. Growth of cells in vitro is usually serum-dependent, and studies on growth promoters require a dose-response to serum to be established, unless serum-free conditions are known for your cells. Serum concentrations from 0.1 to 10% are sufficient for most cell types. A level of 1% serum should permit slow growth of cells over a 7-d period, and to such a baseline are added growth factors or inhibitors. Most cells show a "lag" period of 1–2 d after plating before log-phase growth. It is usual to include a known growth promoter (epidermal growth factor, or a cocktail of several factors) to the basal growth medium to act as a positive control if testing unknown factors. This will show that the cells remain responsive to a known agent. Similarly, it is useful to include cells at 10–20% serum to show maximal growth. For growth inhibitors, one needs to consider conditions where cells would proliferate strongly and then assess lower growth of cells over a time course.
7. After performing a growth curve with several time points it is often convenient to store washed and aspirated samples at –20°C pending assay. This results in no loss of DNA, and after thawing, nucleases from lysed cells are considerably inhibited by the citrate in the SSC buffer or, less so, by the EDTA in TE buffer ***(46)***. It may be convenient to retain a blank row on experimental plates to use with DNA standards at the time of assay. The assay is well-suited to small samples, has a sensitivity of around ±50 cells, and the 96-well plate format allows cell growth and analysis to be undertaken in the same place. If culture flasks were used it would be critical to ensure complete mixing of the lysed cell mix when pipetting samples for DNA assay into a 96-well plate, although larger volumes would be well suited to a full size cuvet fluorimeter (2 mL). Suitable minimum volumes of the lysis buffer are 50 µL per well for 96-well plates, or 250 µL per well for 24-well plates or 2 mL for 6-well plates. The assay is compatible with colorectal or breast carcinoma cell lines and fibroblasts. When stratifying keratinocyte cultures, or overconfluent other cell types, may be assayed, the initial lysis volume and/or %SDS concentration should be doubled to ensure complete dissolution of cells, though for keratinocytes, the SSC is best augmented with 8 *M* urea and then diluted to 2 *M* when using PicoGreen. Bubble-free mixing of samples is also important to prevent corruption of the fluorescent signals away from the detecting optics. "Reverse" pipetting is useful in this instance. If using Hoechst dyes or DAPI,

then the dye concentration should be raised to 4 µg/mL to accommodate DNA levels over 10 µg/mL. The assay using Hoechst dyes or DAPI is less affected by the concentration of urea than the Picogreen assay.

References

1. Rice, R. H. (1994) Assays for involucrin, transglutaminase and ionophore-inducible envelopes, in *The Keratinocyte Handbook* (Leigh, I. M. and Watt, F. M. E., eds.), Cambridge University Press, Cambridge, UK, pp. 157–165.
2. Takahashi, H., Ibe, M., Kinouchi, M., Ishida-Yamamoto, A., Hashimoto, Y., and Iizuka, H. (2003) Similarly potent action of 1,25-dihydroxyvitamin D3 and its analogues, tacalcitol, calcipotriol, and maxacalcitol on normal human keratinocyte proliferation and differentiation. *J. Dermatol. Sci.* **31,** 21–28.
3. Otto, W. R. (1993) Assays in cell proliferation, in *Cell and Tissue Culture: Laboratory Procedures* (Doyle, A., Griffiths, J. B., and Newell, D. G. E., eds.), John Wiley, Chichester, UK, pp. 10E11.11–10E11.15.
4. Otto, W. R. (1993) Fluorimetric and spectrophotometric measures of cell number, in *Cell and Tissue Culture: Laboratory Procedures* (Doyle, A., Griffiths, J. B., and Newell, D. G. E., eds.), John Wiley, Chichester, UK, pp. 4B2.1–4B2.10.
5. Ravid, K., Lu, J., Zimmet, J. M., and Jones, M. R. (2002) Roads to polyploidy: the megakaryocyte example. *J. Cell Physiol.* **190,** 7–20.
6. Gupta, S. (2000) Hepatic polyploidy and liver growth control. *Semin. Cancer Biol.* **10,** 161–171.
7. Duesberg, P. and Rasnick, D. (2000) Aneuploidy, the somatic mutation that makes cancer a species of its own. *Cell Motil Cytoskeleton* **47,** 81–107.
8. Bachoon, D. S., Otero, E., and Hodson, R. E. (2001) Effects of humic substances on fluorometric DNA quantification and DNA hybridization. *J. Microbiol. Methods* **47,** 73–82.
9. Cosa, G., Focsaneanu, K. S., McLean, J. R., McNamee, J. P., and Scaiano, J. C. (2001) Photophysical properties of fluorescent DNA-dyes bound to single- and double-stranded DNA in aqueous buffered solution. *Photochem. Photobiol.* **73,** 585–599.
10. McGowan, K. B., Kurtis, M. S., Lottman, L. M., Watson, D., and Sah, R. L. (2002) Biochemical quantification of DNA in human articular and septal cartilage using PicoGreen and Hoechst 33258. *Osteoarthritis Cartilage* **10,** 580–587.
11. Papadimitriou, E. and Lelkes, P. I. (1993) Measurement of cell numbers in microtiter culture plates using the fluorescent dye Hoechst 33258. *J. Immunol. Methods* **162,** 41–45.
12. Rao, J. and Otto, W. R. (1992) Fluorimetric DNA assay for cell growth estimation. *Anal. Biochem.* **207,** 186–192.
13. Rengarajan, K., Cristol, S. M., Mehta, M., and Nickerson, J. M. (2002) Quantifying DNA concentrations using fluorometry: a comparison of fluorophores. *Mol. Vis.* **8,** 416–421.
14. Singer, V. L., Jones, L. J., Yue, S. T., and Haugland, R. P. (1997) Characterization of PicoGreen reagent and development of a fluorescence-based solution assay for double-stranded DNA quantitation. *Anal. Biochem.* **249,** 228–238.
15. Belkacemi, Y., Piel, G., Rat, P., Julia, F., Touboul, E., Housset, M., and Warnet, J. M. (2000) Ionizing radiation-induced death in bovine lens epithelial cells: mechanisms and influence of irradiation dose rate. *Int. J. Cancer* **90,** 138–144.
16. Blaheta, R. A., Kronenberger, B., Woitaschek, D., Weber, S., Scholz, M., Schuldes, H., et al. (1998) Development of an ultrasensitive in vitro assay to monitor growth of primary cell cultures with reduced mitotic activity. *J. Immunol. Methods* **211,** 159–169.
17. Richards, W. L., Song, M. K., Krutzsch, H., Evarts, R. P., Marsden, E., and Thorgeirsson, S. S. (1985) Measurement of cell proliferation in microculture using Hoechst 33342 for the

rapid semiautomated microfluorimetric determination of chromatin DNA. *Exp. Cell Res.* **159,** 235–246.

18 Hukkelhoven, M. W., Vromans, E., Markslag, A. M., and Vermorken, A. J. (1981) A simple fluorimetric microassay for DNA in hair follicles or fractions of hair follicles. *Anticancer Res.* **1,** 341–344.

19 Serth, J., Kuczyk, M. A., Paeslack, U., Lichtinghagen, R., and Jonas, U. (2000) Quantitation of DNA extracted after micropreparation of cells from frozen and formalin-fixed tissue sections. *Am. J. Pathol.* **156,** 1189–1196.

20 Lindstrom, E. S., Weisse, T., and Stadler, P. (2002) Enumeration of small ciliates in culture by flow cytometry and nucleic acid staining. *J. Microbiol. Methods* **49,** 173–182.

21 Markovits, J., Roques, B. P., and Le Pecq, J. B. (1979) Ethidium dimer: a new reagent for the fluorimetric determination of nucleic acids. *Anal. Biochem.* **94,** 259–264.

22 Royce, P. M. and Lowther, D. A. (1979) Fluorimetric determination of DNA in papain digests of cartilage, using ethidium bromide. *Connect. Tissue Res.* **6,** 215–221.

23 Li, D. H., Chen, X. L., Fang, Y., and Xu, J. G. (2001) Determination of nucleic acids based on shifting the association equilibrium between tetrasulfonated aluminium phthalocyanine and acridine orange. *Analyst* **126,** 518–522.

24. Haugland, R. P. (2002) *Handbook of Fluorescent Probes and Research Products*, 9th ed. Molecular Probes, Eugene, OR.

25 Jones, L. J., Yue, S. T., Cheung, C. Y., and Singer, V. L. (1998) RNA quantitation by fluorescence-based solution assay: riboGreen reagent characterization. *Anal. Biochem.* **265,** 368–374.

26 Monpoeho, S., Dehee, A., Mignotte, B., Schwartzbrod, L., Marechal, V., Nicolas, J. C., et al. (2000) Quantification of enterovirus RNA in sludge samples using single tube real-time RT-PCR. *Biotechniques* **29,** 88–93.

27 Larson, E. J., Hakovirta, J. R., Cai, H., Jett, J. H., Burde, S., Keller, R. A., et al. (2000) Rapid DNA fingerprinting of pathogens by flow cytometry. *Cytometry* **41,** 203–208.

28 Gilbert, R. L., Rider, J. R., Turton, J. R., and Pamphilon, D. H. (2003) Detection of residual donor leucocytes in leucoreduced red blood cell components using a fluorescence microplate assay. *J. Immunol. Methods* **274,** 17–25.

29 Romppanen, E. L., Savolainen, K., and Mononen, I. (2000) Optimal use of the fluorescent PicoGreen dye for quantitative analysis of amplified polymerase chain reaction products on microplate. *Anal. Biochem.* **279,** 111–114.

30 Gelmini, S., Caldini, A., Becherini, L., Capaccioli, S., Pazzagli, M., and Orlando, C. (1998) Rapid, quantitative nonisotopic assay for telomerase activity in human tumors. *Clin. Chem.* **44,** 2133–2138.

31. Elmendorff-Dreikorn, K., Chauvin, C., Slor, H., Kutzner, J., Batel, R., Muller, W. E., et al. (1999) Assessment of DNA damage and repair in human peripheral blood mononuclear cells using a novel DNA unwinding technique. *Cell Mol. Biol. (Noisy-le-grand)* **45,** 211–218.

32 Choi, S. J. and Szoka, F. C. (2000) Fluorometric determination of deoxyribonuclease I activity with PicoGreen. *Anal. Biochem.* **281,** 95–97.

33 Chang, C. P., Chia, R. H., Wu, T. L., Tsao, K. C., Sun, C. F., and Wu, J. T. (2003) Elevated cell-free serum DNA detected in patients with myocardial infarction. *Clin. Chim. Acta* **327,** 95–101.

34 Jiang, N., Reich, C. F., Monestier, M., and Pisetsky, D. S. (2003) The expression of plasma nucleosomes in mice undergoing in vivo apoptosis. *Clin. Immunol.* **106,** 139–147.

35 Corsi, K., Chellat, F., Yahia, L., and Fernandes, J. C. (2003) Mesenchymal stem cells, MG63 and HEK293 transfection using chitosan-DNA nanoparticles. *Biomaterials* **24,** 1255–1264.

36 Murakami, P. and McCaman, M. T. (1999) Quantitation of adenovirus DNA and virus particles with the PicoGreen fluorescent dye. *Anal. Biochem.* **274,** 283–288.
37 Li, C., Issa, R., Kumar, P., Hampson, I. N., Lopez-Novoa, J. M., Bernabeu, C., et al. (2003) CD105 prevents apoptosis in hypoxic endothelial cells. *J. Cell Sci.* **116,** 2677–2685.
38 Koopman, G., Reutelingsperger, C. P., Kuijten, G. A., Keehnen, R. M., Pals, S. T., and van Oers, M. H. (1994) Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood* **84,** 1415–1420.
39 Johnson-Wint, B. and Hollis, S. (1982) A rapid in situ deoxyribonucleic acid assay for determining cell number in culture and tissue. *Anal. Biochem.* **122,** 338–344.
40 Karsten, U. and Wollenberger, A. (1977) Improvements in the ethidium bromide method for direct fluorometric estimation of DNA and RNA in cell and tissue homogenates. *Anal. Biochem.* **77,** 464–470.
41 Otto, W. R., Barr, R. M., Dowd, P. M., Wright, N. A., and Greaves, M. W. (1989) 12-Hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) does not stimulate proliferation of human neonatal keratinocytes. *J. Invest. Dermatol.* **92,** 683–688.
42 Latt, S. A. and Stetten, G. (1976) Spectral studies on 33258 Hoechst and related bisbenzimidazole dyes useful for fluorescent detection of deoxyribonucleic acid synthesis. *J. Histochem. Cytochem.* **24,** 24–33.
43. Dover, R. (1992) Basic methods for assessing cellular proliferation, in *Cell Proliferation in Clinical Practice* (Hall, P. A., Levison, D. A., and Wright, N. A., eds.), Springer-Verlag, London.
44 Maurer, H. R. (1981). Potential pitfalls of [3H]thymidine techniques to measure cell proliferation. *Cell Tissue Kinet.* **14,** 111–120.
45. Wright, N. A. and Alison, M. (1984) *The Biology of Epithelial Cell Populations*, Vol. 1, Oxford University Press, Oxford, pp. 97–196 and 223–246.
46. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Vol. 3, 3 vols, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, p. Appendix E5.
47. Burton, K. (1956) A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* **62,** 315–323.

28

Keratinocyte Transient Transfections

Anthony M. Flores and Brian J. Aneskievich

Summary

The goal of this chapter is to give the reader a concise and easy-to-follow guide to proven transient transfection techniques for primary strains and continuous lines of keratinocytes. The emphasis is on readily available and inexpensive resources that also allow for repeatability and adaptability to scale, experimental conditions, and sample replicates. In addition, basic cell culture techniques necessary to achieve optimal transfection results are provided. Although specified for keratinocytes, these techniques also work well for simple epithelial lines and common laboratory lines, such as COS-7 and CV-1. The standard precipitation protocol is suitable for many experimental designs, and the poly-L-ornithine protocol is intended for situations where calcium is contraindicated. The extended notes section provides necessary technical details to guide the novice user and reminders to the experienced scientist that success with the protocol will depend on several quantitative and qualitative factors including promoter strength, cell culture practices and plasmid isolation.

Key Words:

Calcium phosphate; poly L-ornithine, HaCaT; SCC; receptor plasmid.

1. Introduction

Gene transfer to keratinocytes presents a significant opportunity to advance our understanding of transcriptional control of expression and by extension epidermal cell growth, physiology, and differentiation. By their nature, transient transfections have no selective pressure to remove cells not receiving the DNA constructs of interest, which therefore dilute any reporter signal. As a result, efforts over the last several years have emphasized increased efficiency in delivering the plasmid constructs as well as increased sensitivity in detection of reporter gene expression. Biological gene construct carriers (i.e., retrovirus, adenovirus, and adenovirus-associated virus vectors) have been used for keratinocyte cell strains and continuous cell lines with success ***(1)***. However, the biological safety issues and the need to isolate and titer recombinant virus have limited their widespread use for routine transient transfection application. Electroporation, although efficient, requires a significant investment in equipment.

Commercially prepared lipid-based DNA carriers have gained popularity recently for both keratinocyte and nonkeratinocyte cell types ***(2)*** for both cell culture and *in situ* delivery to tissue. Others have also summarized in-house preparation techniques for "lipidfection" ***(3)***. However, this protocol has received limited use potentially because

From: *Methods in Molecular Biology, vol. 289:*
Epidermal Cells: Methods and Protocols
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

of the variability of in-house made lipids and the extra labor needed to quality control for this. Commercial preparations are more consistent but represent a significant cost in doing high-volume work. Although these preparations are cited frequently in the literature, there is little agreement on which, if any, of several lipid or polycation based methods are optimal for transient transfection of primary and/or continuous lines of keratinocytes. Indeed, this parallels our own experience. We found little predictability for similar efficiency of lipid-based carriers among continuous lines of keratinocytes.

Interestingly, a recent review of the literature suggested that for transient transfection purposes (as opposed to selection of stable transfectants) calcium phosphate coprecipitation remained a common method especially when large-scale throughput, robust keratinocyte-specific promoters, or more sensitive detection systems were used. The protocols we provide here reflect this finding and also include an efficient poly-L-ornithine alternative ***(4)*** should noncalcium-mediated approaches be required. Although luciferase-based constructs have gained popularity, many laboratories have a repertoire of CAT-based reporter plasmids and these protocols are geared toward that system ***(5)***. However, many of the cell culture husbandry and transfection methods stressed here will easily carry over to that system. As there are several commercially available detection systems available for both CAT and luciferase expression we have not detailed those protocols here. We do include a main and alternative protocol for detection in cell lysates of β-galactosidase expression as it is commonly used as an internal transfection reference. Finally, we note that no one standard protocol can suffice for all experimental needs but these protocols should provide generally applicable methods, which could be fine-tuned (cell density, exposure time to plasmid DNA, posttransfection time) for individual circumstances. To facilitate this, we include a convenient *in situ* staining protocol for β-galactosidase activity to determine transfection efficiency.

2. Materials

2.1. Culture Media and Phosphate-Buffered Saline (PBS)

1. Keratinocyte growth media (KGM): obtained from Cambrex (Clonetics) and used as per supplier's directions.
2. Keratinocyte media, serum-supplemented: Dulbecco's modified Eagle's medium, Invitrogen, cat. no. 12100-038 (three 1-L packets; high glucose, with L-glutamine, with pyroxidine HCl, without sodium pyruvate, without sodium bicarbonate), F12 (Ham's) Invitrogen, cat. no. 21700-026 (one 1-L packet; with L-glutamine, without sodium bicarbonate), 12.28 g sodium bicarbonate, Sigma, cat. no. S5761. Slowly add powdered media to 3.6 L high-quality water (*see* **Note 1**), dissolve completely, then add the sodium bicarbonate, cover with foil, and stir for 40 min to 1 h. Titrate pH to 7.1 and immediately filter sterilize. Tighten bottle caps well and store at 4°C protected from light. For use, supplement with penicillin–streptomycin (Invitrogen, cat. no. 15140-122; final concentration 100 U/mL, 100 µg/mL, respectively) and fetal bovine serum (which has been screened to support optimal keratinocyte growth) to a final concentration of 10% (*see* **Note 2**).
3. 1X PBS: 4.31 m*M* Na_2HPO_4, 1.47 m*M* KH_2PO_4, 2.68 m*M* KCl, 137 m*M* NaCl. Slowly add the chemicals in the order listed. Allow each to dissolve completely before adding the next. Complete to final volume and mix thoroughly. Aliquot to 500-mL bottles and autoclave on liquid cycle for 25 min.
4. Trypsin: 0.1% trypsin, 0.1% glucose, 0.02% EDTA with 100 U/mL penicillin, 100 µg/mL streptomycin in PBS.

2.2. Standard Calcium Phosphate Coprecipitate (see Note 1)

1. 2X HEPES-buffered saline (HBS; 250 mL): 50 m*M* *N*-(2-hydroxylethyl) piperazine-*N*′-(2-ethanesulfonic acid), 280 m*M* NaCl, 1.5 m*M* Na_2HPO_4. Dissolve the chemicals (*see* **Note 1**) in the order given in pyrogen-free water (*see* **Note 1**). Adjust the pH of the final solution to 7.05 (*see* **Note 3**). Filter-sterilize (Nalgene 0.2 µm or similar) and aliquot to volume as needed for typical experiments, usually 5–10 mL. Store at –20°C. Mix thoroughly after thawing.
2. 5X $CaCl_2$ (100 mL): 1.25 *M* calcium chloride dihydrate. Slowly add the calcium chloride to stirring water. Dissolve completely and complete to final volume. Filter sterilize (Nalgene 0.2 µm or similar) and aliquot to volume as needed for typical experiments, usually 1–2 mL. Store at –20°C. Mix thoroughly after thawing.
3. Minicentrifuge, Fisher, cat. no. 05-090-124 or similar.

2.3. Low CO_2 Transfection

1. 2X BBS–BES-buffered saline (250 mL): 50 m*M* *N*, *N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid BES, (Sigma, cat. no. B4554), 280 m*M* NaCl, 1.5 m*M* Na_2HPO_4 anhydrous. Dissolve the chemicals (*see* **Note 1**) in the order given in pyrogen-free water (*see* **Note 1**). Adjust the pH of the final solution to 6.95 (*see* **Note 3**). Filter sterilize (Nalgene 0.2 µm or similar) and aliquot to volume as needed for typical experiments, usually 5–10 mL. Store at –20°C. Mix thoroughly after thawing.
2. 10X $CaCl_2$ (50 mL): 2.5 *M* calcium chloride dihydrate. Slowly add the calcium chloride to stirring water. Dissolve completely and complete to final volume. Filter sterilize (Nalgene 0.2 µm or similar) and aliquot to volume as needed for typical experiments, usually 1–2 mL. Store at –20°C. Mix thoroughly after thawing.

2.4. Poly-L-Ornithine-Mediated

1. Poly-L-ornithine stock solution, 10 mL (PLO): the 10 mg/mL solution should be made under aseptic conditions with sterile water. Transfer 100 mg (Sigma, cat. no. P3655) from a previously unopened bottle to a 50-mL sterile tube using an autoclaved spatula if necessary. Add 10 mL sterile water and allow to mix on an end-over-end tube rotator. Aliquot to 1-mL units and store the stock at 4°C.
2. Tissue culture-tested DMSO, Sigma, cat. no. D2650 or similar.

2.5. Buffer and Substrate for β-Galactosidase (β-gal) Assay With ONPG

1. 250 mL Z-buffer: 60 m*M* Na_2HPO_4, 40 m*M* NaH_2PO_4, 10 m*M* KCl, 1 m*M* $MgSO_4$. Adjust the solution to pH 7.0. Add β-mercaptoethanol to a final concentration of 50 n*M* fresh the day of the assay (2.7 µL per milliliter of buffer).
2. 4 mg/mL 2-nitrophenyl β-D-galactopyranoside (ONPG), Sigma, cat. no. N1127. Dissolve in Z-buffer, aliquot, and store at –20°C.
3. Stop solution 1 *M* Na_2CO_3 (10.6 g in 100 mL water, final volume).

2.6. Buffer and Substrate for β-gal Assay With CRPG

1. 2X CRPG assay buffer (100 mL final volume) consists of 160 m*M* sodium phosphate. Combine water and the stock phosphate solutions (7.07 mL 1 *M* Na_2HPO_4 plus 2.63 mL 1 *M* NaH_2PO_4) to approx 90 mL, adjust to pH 7.3, and complete volume to 96.8 mL. Supplement with $MgCl_2$ and β-mercaptoethanol the day of use (*see* **Subheading 3.6.**).
2. 20 m*M* chlorophenol red-β-D-galactopyranoside monosodium salt (CRPG), Roche, cat. no. 84 308. Dissolve 250 mg in 20.58 mL water and store in aliquots at –20°C.

2.7. β-*Gal* In Situ *Staining*

1. β-gal *in situ* staining solution (100 mL): supplement 100 mL PBS with the following: 0.1646 g potassium ferricyanide, (Sigma, cat. no. P8131), 0.2112 g potassium ferrocyanide, (Sigma, cat. no. P9387), 200 µL 1 *M* $MgCl_2$, 20 µL NP-40, (Sigma, cat. no. I8896), 0.01 g sodium deoxycholate.
2. X-gal: dissolve X-gal (Sigma, cat. no. B4252 or similar) stock concentration at 20 mg/mL in DMF (*N*,*N*-dimethyl formamide). Store at –20°C in foil-wrapped glass container (no plastic). Stable for approx 3 mo.

3. Methods

3.1. Maintenance of Stock and Preparation of Transfection Cultures

1. Prepare 100-mm diameter stock plates (*see* **Note 4**) for subculture (*see* **Note 6**) to transfection plates/wells by rinsing with PBS–0.02% EDTA.
2. Add 8–10 mL PBS–0.02% EDTA. Incubate at 37°C for approx 5–7 min (*see* **Note 4**).
3. Aspirate the rinse and release cells from plates using 2 mL of freshly thawed trypsin for approx 5 min.
4. Resuspend cells with the addition of 8 mL serum-supplemented media and repeated pipetting. Count from this cell suspension or a dilution of it as necessary and assume full recovery at the next step.
5. Pellet the cells for 5 min at 200*g*.
6. Aspirate off the trypsin/media supernatant and resuspend the cells in 10 mL serum-supplemented media by repeated pipetting. Further dilute this in more serum-supplemented media (*see* **Note 5**) to obtain suspension concentrations of cells to seed culture vessels as indicated in **Table 1**.
7. Transfection 18–24 h after plating is typical (*see* **Note 6**), but some continuous lines require additional time to spread and establish an actively growing culture. SCC13 cultures are typically made at lower initial densities but 48 h in advance.

3.2. Standard Calcium Phosphate Coprecipitate

Each transfection mixture **(Table 2)** will require a labeled Falcon 2059 tube and a correspondingly labeled sterile 1.5-mL microfuge tube (*see* **Note 7**).

1. Combine H_2O and DNAs (*see* **Note 8**) in a sterile 1.5-mL microfuge tube (*see* **Note 9**) for general handling. Mix by tapping and add 1.25 *M* $CaCl_2$. Vortex on medium 2–3 s and spin in the microfuge approx 10 s to bring all liquid to bottom of tube.
2. Measure 2X HBS into each of the Falcon 2059 tubes. In turn, place each 2X HBS tube into styrofoam holder affixed to a vortex mixer (*see* **Note 10**). Set the mixer to constant-on at the lowest speed. Slowly drip the DNA/$CaCl_2$/H_2O mixture into the 2X HBS. The rate of DNA delivery is historically considered important. Let individual drops form at the end of the pipet tip and wait 1–2 s before delivering the next drop.
3. Cap the 2059 tube and allow the precipitate to form for 20 min. Because generating a large number of individual precipitation mixes can take several minutes, we typically mark the time on the individual 2059 tubes to assure all mixture have had a consistent 20-min period before adding to the cells. A faint, fine white precipitate should be visible compared to starting buffers.
4. Before adding the precipitates to the plates/wells, vortex to resuspend and immediately drip across the surface of the media. Swirl plates/wells two to three times in a figure-eight motion and immediately return them to the incubator for 14–16 h (*see* **Note 11**).

Table 1
Transfection Culture Seeding Densities

Cell type	Plate type/volume	Cell number
primary keratinocytes	6-well/3 mL	5.0×10^5
″	60-mm/5 mL	9.0×10^5
HaCaT	12-well/2 mL	1.1×10^5
″	6-well/3 mL	2.5×10^5
″	60-mm/5 mL	6.6×10^5
SCC13	12-well/2 mL	0.6×10^5
″	6-well/3 mL	1.1×10^5
″	60-mm/5 mL	2.1×10^5

Table 2
Reagent Amounts for Coprecipitation

Component	Per well of 6-well tray	Per well of 12-well tray
Sterile water	120 µL minus X µL	80 µL minus Y µL
Plasmid DNA	3.0–3.5 µg total in X µL	1.2–1.4 µg total in Y µL
1.25 *M* $CaCl_2$	30 µL	20 µL
2X HBS	150 µL	100 µL

5. Examine and record the appearance of the cultures and precipitate after the overnight incubation (*see* **Note 12**). With the extended overnight exposure to the precipitate, we have not found a significant increase in transfection efficiency from a subsequent shock step (*see* **Note 13**). Swirl the plates/wells and aspirate off the media/precipitate mixture. Rinse twice with room temperature PBS and refeed plates/wells with pre-warmed serum-supplemented media or media supplemented as per experimental needs. For our experiments dealing with nuclear receptor activation we refeed the plates 24 h later more out of concern of ligand breakdown than media depletion by cellular metabolism (*see* **Note 6**). Total time post-transfection is typically 60–65 h.
6. Harvest cells as required for your choice of reporter constructs.

3.3. Low CO_2 Calcium Phosphate Coprecipitate

1. Combine H_2O and $CaCl_2$ in a Falcon 2059 tube or similar as listed in **Table 3**. Mix by tapping and add plasmid DNA (*see* **Note 8**). Vortex on medium 2–3 s. Add the 2X BBS directly to this tube. Unlike the standard calcium-phosphate coprecipitation method, speed of addition does not seem to affect the transfection.
2. Cap the 2059 tube and allow the precipitate to form for 20 min. Timing should be consistent for all tubes. Unlike the standard calcium-phosphate coprecipitation method, any precipitate formed during this time is not macroscopically visible ***(6)***.
3. Vortex the transfection mixtures to resuspend and immediately drip across the surface of the media. Swirl plates/wells two to three times in a figure-eight motion and immediately return them to the 3% CO_2 incubator for 14–16 h (*see* **Note 11**).
4. Examine and record the appearance of the cultures and precipitate after the overnight incubation (*see* **Note 12**). Fine, phase contrast dark precipitate should be visible over the surface of the cells and open areas of the plates/wells. Swirl the plates/wells and aspirate off

Table 3
Reagent Amounts for Low CO_2 Coprecipitation

Component	Per well of 6-well tray	Per well of 12-well tray
Sterile water	135 µL minus X µL	90 µL minus Y µL
2.5 *M* $CaCl_2$	15 µL	10 µL
Plasmid DNA	3.5 µg total in X µL	1.4 µg total in Y µL
2X BBS	150 µL	100 µL

Table 4
Reagent Amounts for PLO-Mediated Transfection

Component	Per 60-mm plate	Per well of 6-well tray
KGM basal media	1.8 mL minus X µL	810 µL minus Y µL
Plasmid DNA	10 µg total in X µL	4.5 µg total in Y µL
10 mg/mL PLO	2.16 µL	0.98 µL

the media/precipitate mixture. Rinse twice with room temperature PBS and refeed plates/wells with prewarmed serum-supplemented media or media supplemented as per experimental needs. Total time posttransfection is typically 48–65 h.

5. Harvest cells as required for your choice of reporter constructs.

3.4. Poly-L-Ornithine-Mediated (see Note 14)

1. Examine plates and record approximate per cent confluence. Warm serum-free media and PBS to 37°C. Prepare the plasmid/PLO/media mixes as indicated in **Note 8**. Combine the components listed in **Table 4** to perform at least duplicate transfections per experimental condition. Mix the media/DNA/PLO gently but thoroughly by inversion. Make sufficient transfection mixture for at least one extra plate/well to allow for volume loss during handling.
2. Aspirate off growth media and rinse plates/wells once with prewarmed PBS. Remove the maximum of PBS but do not let plates dry. Add the media/DNA/PLO mixes to the corresponding plate/well and immediately rock to distribute. Return plates/wells to the incubator and time exposure for 4–5 h. Approximately once an hour, rock the plates/wells to redistribute the transfection mixture.
3. Towards the end of the fifth hour, bring KGM (without growth additives) to room temperature and supplemented KGM to 37°C. Prepare DMSO/basal KGM "shock" mix for each transfection plate/well at volumes of 2 mL per 60-mm plate and 1 mL per well of a 6-well tray. The DMSO final concentration is 24%. Use tissue culture-tested DMSO. Because timing the shock period is critical, work in subsets rather than trying to shock and rinse all plates at the same time.
4. Aspirate off the transfection mix and add the DMSO/media mix. Time for 4 min, then immediately aspirate off the shock mix and gently rinse twice with basal KGM. Feed with supplemented KGM as needed and incubate for 24–48 h.
5. Harvest cells as required for your choice of reporter constructs.

3.5. β-Gal Assay With ONPG

1. Combine 140 µL Z-buffer, cell extract (20–40 µg), and water to a total of 160 µL in a microtiter plate (Falcon 353915 or similar). Cell extract volume may be up to 80 µL.

Prepare a blank with extract buffer and Z-buffer. Seal plate with adhesive cover or plastic wrap.

2. Prewarm plate to 37°C for 5 min. To each well add 40 µL ONPG and shake on vortex mixer at lowest speed for 5 min. Reseal plate, cover with aluminum foil, return to 37°C incubator and record start time.
3. Check color development in approx 30 min; reaction time varies depending on transfection efficiency and amount of protein. Allow a moderate yellow to develop, typical times are approx 90–120 min. The color will intensify upon addition of the stop solution.
4. To stop reaction add to each well 50 µL of 1 *M* Na_2CO_3 and record time. Read as soon as possible as the reaction may continue to progress slowly.
5. If the microtiter plate reader has an automix function, perform two runs of 20 s each to mix the well contents; alternatively shake on vortex mixer at lowest speed for approx 1 min. Plates are read at 420 nm.
6. Calculations:

$$\text{specific activity} = (\text{nmol ONPG cleaved/min})/\text{mg lysate protein}$$
$$\text{nmol ONPG cleaved} = A_{420}\ /.0045$$

3.6. β-*Gal Assay With CRPG (see Note 15)*

1. Add the $MgCl_2$ and β-ME fresh to the phosphate buffer the day of the assay as follows: 12.1 mL 2X phosphate buffer, 225 µL 1 *M* $MgCl_2$, 177 µL neat β-mercaptoethanol, for final concentrations of 160 m*M*, 18 m*M*, and 204 m*M*, respectively, to complete the 2X CRPG reaction buffer.
2. Combine 100 µL 2X CRPG reaction buffer with lysate and water to 160 µL per well of a microtiter plate. Set up separate wells with buffer and extract from mock-transfected cells to serve as reaction blanks and background controls, respectively. All samples, blanks, and controls should be made in duplicate.
3. Add 40 µL of 20 m*M* CRPG for a total volume of 200 µL and seal with an adhesive cover. Mix thoroughly for approx 5 min at the lowest speed on vortex mixer. Cover with foil and incubate at 37°C for 10 min and examine degree of color change after this time. Incubation may need to proceed for a total of 30–60 min. Check color change periodically; this assay proceeds faster than the ONPG-based one.
4. Read plates at 570 nm. If the microtiter plate reader has an automix function, perform two runs of 20 s each to mix the well contents; alternatively shake on vortex mixer at lowest speed for approx 1 min. Be sure color in wells is thoroughly mixed before saving the plate reading as a data set. Normalize data as A_{570} /µg protein.

3.7. β-*Gal* In Situ *Staining*

This protocol provides a convenient complement to the lysate assays to determine transfection efficiency during optimization of transfection procedures.

1. Aspirate media from transfected cells and wash twice with PBS. Fix for 15 min at 4°C in 2% paraformaldehyde-0.2% glutaraldehyde in PBS. Wash twice with PBS.
2. Per 60-mm dish, add 1.8 mL β-gal reaction solution for 5 min at room temperature, then add 0.20 mL X-gal in DMF for final concentration of 2 mg/mL X-gal. Incubate at 37°C and time the color development. CMV-driven constructs develop in approx 30 min. Plates/wells may be stored in 50% glycerol for a few days at 4°C.

4. Notes

1. References to any particular supplier is done for convenience only and is not an endorsement of any particular source. All plasticware and glassware, including spatulas and stir

bars for tissue culture solution preparation, are kept separate from general use labware and are autoclaved before use. All water for solutions should be pyrogen-free from commercial suppliers or tested in-house supply of double-distilled, NanoPure, or similar quality.

2. This is a minimalist recipe that is suitable for many continuous human keratinocyte cell lines ***(5)***. For details on serum-free, other serum-supplemented, or specialized media for mouse or human keratinocytes, the reader is directed to the recipes compiled by Leigh and Watt ***(3)*** or commercially prepared media.
3. Perform a two-point calibration of the pH meter used for solution preparation, preferably one that reports back probe efficiency as percent slope. Because pH meters may vary, batches of the 2X HBS and 2X BBS varied at 0.05 pH unit may be tested for transfection efficiency.
4. If dealing with primary keratinocytes grown on 3T3 feeder layers, repeatedly pipet the PBS–0.02% EDTA solution over the surface of the plate to remove the fibroblasts before continuing. Better reattachment and more consistent transfection results are obtained from carefully maintained stock cultures for both primary strains and continuous lines of keratinocytes. Primary cultures should not be more than 70–80% confluent and exhibit minimal stratification as judged by phase contrast microscopy. Keratinocyte lines (SCC13, HaCaT or similar) give more consistent transfection efficiencies if harvested when just prior to confluence ***(5)***. Dense, postconfluent plates may yield more cells but in our experience give erratic transfection results both for efficiency as judged by generic reporter constructs, for example, CMV-β-gal, and strength of keratinocyte-specific promoters. For primary keratinocytes, transfection plates are made from second to fourth passage cultures. For keratinocyte cell lines, repeat experiments are done from consecutive passages. Frozen stocks of continuous lines should be generated to enable transfections to occur with cells from the lowest passages possible.
5. Calculate the total number of cells needed for all plates/wells with the serum-supplemented media needed for all plates/wells and increase both by the cells and media needed for two extra wells to allow for pipetting error. For example, if you are planning to seed four six-well trays (24 wells) of HaCaT cells, base cell number and media calculations on 26 wells total. Record the passage number. Sufficient plates/wells should be set to assay DNA combinations at least in duplicate, preferably triplicate, along with mock (receiving carrier DNA only) or untransfected (receiving no precipitate) wells/plates.
6. For any timing, for example, postplating to transfection or posttransfection to washing, pick one interval and be consistent with it for repeat experiments. The total posttransfection time must be individually determined for specific combinations of different cell and reporter types. For our studies of nuclear receptor reporter activation, this is usually 62 h, the last 48 h of which have been in the presence of control or ligand-supplemented media. Other robust reporter constructs, such as those derived from extensive lengths of keratinocyte-specific gene promoters, may be harvested sooner.
7. Components for our most frequently used transfection sizes are provided with individual methods. Calcium phosphate precipitates may be scaled up or down; the final volume is calculated as 10% of the media volume on the plate/well used for the transfection culture (5% water, plasmid DNA volume and $CaCl_2$; 5% 2X HBS). For reference, each well of a six-well tray receives 0.5–1.5 μg CAT reporter constructs, effector plasmids (various expression constructs containing cDNAs for promoter-regulating proteins) with 0.1 μg CMV-β-gal as an internal transfection standardization control along with carrier plasmid DNA (e.g., KS^+ Bluescript) to the totals indicated. Although several methods of CAT expression detection are available, we have found a commercially available enzyme-linked immunosorbent assay suitable for sensitivity, ease, and independence from radioactive materials with their associated disposal problems. The assay is performed as per the supplier's instructions.

8. Plasmid DNA quality is critical to efficient transfection. Perform the following as quality controls:
   a. Preparation. We have done side-by-side comparisons of twice-banded CsCl gradient-prepared plasmid with the same construct isolated from commercially available adsorption columns (Qiagen). The column-prepared plasmids worked as well or better than the cesium gradient preparations offering the advantage of speed and eliminating the need for ethidium bromide from the gradient as hazardous waste. Irrespective of the isolation method used, for any constructs being used in the same transfection experiment, determine the plasmids' absorption at 260 and 280 nm at the same time with similar dilutions. Those with significantly varying 260:280 ratios should be repurified. Plasmids prepared under aseptic conditions in 10 m*M* Tris-HCl, pH 8, do not need to be sterilized for transient transfections. Limit the amount of Tris added to the precipitate mix by preparing plasmids at ≥1 μg/μL and dilute if necessary immediately before transfection with sterile water.
   b. Physical comparison. For functional comparison of similar constructs, i.e., different promoter regions of the same promoter driving CAT or expression of wild-type vs mutant transcription factors, we perform an additional quality control to assure that data read-outs from the expression assay are not because of variations in quality of plasmid DNA isolates. Dilute each plasmid to 25 ng/μL and run 50, 150 ng, and 250 ng of undigested plasmid per lane on an appropriate percentage gel, typically 0.6–0.8%. Compare relative intensities among the different constructs and within any one preparation compare the different species of the plasmids, i.e., supercoiled, relaxed circle and concatamer. The supercoiled band should predominate in each isolate and be of similar intensity between isolates allowing some variance for plasmid size. A fast migrating, thin leading species is probably due irreversibly denatured DNA from extended alkali treatment of the original bacterial lysate and should not be used.
9. All mixing steps are performed in a laminar flow hood. To reduce traffic in and out of the hood set up a vortex mixer and minicentrifuge along with all required labeled tubes, solutions, etc., at the beginning of operations. A minifuge takes a minimum amount of space and provides sufficient *g* force for these steps. For all transfection methods, plates/wells to be transfected should spend a minimum time out of the incubator to limit pH drift of the media.
10. A small number of precipitates may be mixed by hand. However, for comparison of effector construct dosage, wild-type vs mutant constructs etc we often have 8–10 different plasmid combinations leading to worker fatigue and inconsistent mixing. The standard test-tube cup provided with vortex mixers can be easily adapted to hold a Falcon 2059 tube. Slice a width from a 50-mL tube styrofoam rack so as to have three complete tube holes. Invert and secure onto the vortex mixer by pressing the test-tube cup into the center hole. Push the 2059 tube through the styrofoam into one of the side holes.
11. Once cultures with transfection mixtures have been returned to the incubator for the overnight exposure, restrict the traffic in and out of that incubator. Media pH for both the standard and low CO_2 methods is critical for efficient precipitate formation. Depending on incubator air handling and CO_2 sensor design there may be a significant lag time before the expected CO_2 level is restored throughout the incubator chamber allowing some drift to the media pH. For either procedure CO_2 readings on incubator gauges should be confirmed by routine Fyrite testing (Bacharach).
12. Macroscopically, a floating, flaky iridescence to the media surface may mean a pH problem with either the 2X HBS or media and usually turns into poor transfection results. Microscopically, most keratinocyte lines we have dealt with are not obviously affected by the precipitate but cell rounding/detachment in other lines may indicate less exposure time

to the precipitate is appropriate. Some precipitate may remain coating the cell surface or the open areas of the plates/wells after PBS rinsing but this does not seem detrimental and usually dissolves over the next 24 h.

13. Since no one protocol can anticipate the optimal conditions for various keratinocyte lines or the physiological state of the cells best suited for the expression of the constructs under study ***(5)***, an optional post-transfection osmotic "shock" step is provided here. Aspirate the media/precipitate mix from the plates/wells and add 10% glycerol (or 10% DMSO depending on the sensitivity of the particular line, 1 mL per 60-mm plate or 0.5 mL per well of a six-well tray). Evenly distribute the solution and allow it to remain in contact with the cells for 3 min. Timing is crucial so work with a limited number of plates/wells. Add prewarmed PBS (2 mL per 60-mm plate or 1 mL per well of a six-well tray) to the glycerol solution, swirl gently to dilute and aspirate off immediately. Wash twice with additional prewarmed PBS. Feed with keratinocyte serum-supplemented media.
14. The transfection runs over the course of the day but only requires hands-on time at the beginning and end of the procedure with adding the plasmid DNA early in the morning, incubation and occasional rocking of the plates/wells during the day and then rinsing and refeeding the plates at the end of the day. The recipe may be scaled up or down but a DNA : PLO mass ratio of 0.42–0.46 should be maintained ***(4)***.
15. Although the CRPG substrate is more expensive than the ONPG, this assay is approx 10 times more sensitive for the detection of β-gal activity. Extracts may be made via 3× freeze/thaw method or with Roche CAT assay extraction buffer. For SV40- or CMV-driven β-gal reporters, approx 20–40 μg of soluble cell extract protein is sufficient for a 30-min assay.

Acknowledgments

Many of these protocols were optimized during work supported by USPHS/NIH grants to the authors from NIAMS and NIEHS.

References

1 Ghazizadeh, S. and Taichman, L. B. (2000) Virus-mediated gene transfer for cutaneous gene therapy. *Hum. Gene Ther.* **11,** 2247–2251.
2 Dass, C. R. (2002) Cytotoxicity issues pertinent to lipoplex-mediated gene therapy in-vivo. *J. Pharm. Pharmacol.* **54,** 593–601.
3. Leigh, I. M. and Watt, F. M. (1994) *Keratinocyte Methods*, Cambridge University Press, Cambridge.
4 Nead, M. A. and McCance, D. J. (1995) Poly-L-ornithine-mediated transfection of human keratinocytes. *J. Invest. Dermatol.* **105,** 668–671.
5 Aneskievich, B. J. (2001) Deletion of RAR carboxyl terminus reveals promoter- and receptor-specific AF-1 effects. *Biochem. Biophys. Res. Commun.* **289,** 950–956.
6. Chen, C. A. and Okayama, H. (1988) Calcium phosphate-mediated gene transfer: a highly efficient transfection system for stably transforming cells with plasmid DNA. *Biotechniques* **6,** 632–638.

29

Tetracycline-Regulated Gene Expression in Epidermal Keratinocytes

Richard B. Presland and Philip Fleckman

Summary

The tetracycline-regulated expression system developed by Gossen and Bujard is a powerful genetic tool that permits the expression of any gene construct introduced into either cultured cells or transgenic animals to be precisely controlled. It involves two components, a regulatory component based on the prokaryotic tetracycline repressor (TetR) and a response plasmid that expresses the gene of interest under control of the tetracycline-response element. In this paper, we review the Tet system methodology, discuss the available vector systems, and describe how to prepare and characterize keratinocyte cell lines that express a gene under tetracycline control. The methodology involves the development of stable cell lines expressing the TetR protein (either tTA or rtTA, expressed as a fusion with the VP16 activation domain), and a second set of double-stable cell lines that contain both TetR and the response plasmid (tetracycline-response element-gene X) expressed under tetracycline control. As an example of this methodology, we discuss our recently developed keratinocyte cell lines that express human filaggrin in a tetracycline-regulated manner. This technique, now also available in retrovirus and adenovirus-based vectors, is applicable both to the study of genes that are toxic to cells and more generally to understand how genes regulate cell structure/function, growth, and differentiation.

Key Words:

Keratinocyte; tetracycline-inducible system; stable transfection; doxycycline; regulated gene expression.

1. Introduction

The stable expression of transfected genes in cultured keratinocytes is a powerful method for studying gene function and how a particular protein influences growth and differentiation. However, a major drawback of this technique is that constitutively expressed genes often have toxic or deleterious effects on cells; in addition, it often is difficult to distinguish between the direct and indirect effects of a particular gene product (*see* **refs.** ***1*** and ***2***, for example). To overcome these limitations, a number of laboratories have devised inducible, regulated systems in which the gene of interest is expressed only upon addition of a drug or compound. The most widely used of these systems is the tetracycline-regulated system developed by Gossen and Bujard ***(3–5)***. In addition to conventional in vitro plasmid transfection, the tetracycline-regulated expression system has been adopted for introduction by both retroviruses and adenoviruses and for use in transgenic mice and plants, demonstrating its general applicability to the

From: *Methods in Molecular Biology, vol. 289:*
Epidermal Cells: Methods and Protocols
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

study of gene expression both in vitro and in vivo ***(6–11)***. Other advantages of this system include the absence of cytotoxic effects of tetracycline on eukaryotic cells at the low concentrations used (typically 1–2 μg/mL) and the ability to manipulate transgene expression in a dose-dependent manner by varying tetracycline concentration ***(4,10)***.

The tetracycline-regulated system involves two key components, a tetracycline repressor or regulator (TetR) that binds the antibiotic tetracycline and its derivatives and a tetracycline-regulated promoter that contains tetracycline operator (tetO) sequences (also known as a TRE, or tetracycline-responsive element), which binds TetR and regulates expression of the gene downstream **(Fig. 1)**. In most versions of the TetR used in mammalian cells, it is expressed as a fusion protein with the C-terminal activation domain of VP16 from herpes simplex virus. The VP16 activation domain allows the TetR regulator protein to activate eukaryotic genes ***(3,4)***. Two main versions of the tetracycline-regulated system are available, now termed the Tet-On™ and Tet-Off™ systems. These systems differ primarily in the nature of the tetracycline regulator that is expressed in mammalian cells, which in turn determines whether expression of the regulated transgene occurs in the *presence* or *absence* of tetracycline. In the earlier-described Tet-Off system, the TetR-VP16 protein (referred to as tTA) is able to bind to the TRE and activate transgene expression in the absence of tetracycline ***(3)***. In the presence of tetracycline the tTA-VP16 protein switches *off* transcription of gene X **(Fig. 1)**. In the Tet-On system, the TetR-VP16 protein (referred to as rtTA, or reverse TetR) binds to the TRE in the presence of tetracycline, with induction of transgene expression ***(4)*** **(Fig. 1)**. The rtTA version was generated by in vitro mutagenesis of the native tTA gene. Both versions of the system are available commercially.

In this chapter, we will describe protocols for developing keratinocyte lines that can be used to express an introduced transgene under tetracycline control. This system has been used to study the expression of a number of keratinocyte genes both in vitro ***(1,12)*** and in transgenic mouse epidermis ***(13–15)***, demonstrating its usefulness in addressing problems in keratinocyte biology. To simplify discussion of the methods, we will focus primarily on the Tet-On system, which we have used to inducibly express human filaggrin in epidermal keratinocytes ***(1)***. Much of the same methodology is used for the Tet-Off system, with the major difference being that expression of tetracycline-responsive gene(s) is switched on in the absence of tetracycline, that is, after removal of tetracycline from the medium.

Several key steps are involved in setting up this system in your laboratory, as follows: 1) transfection of keratinocytes with the TetR regulator plasmid and preparation of stable (neomycin-resistant) cell lines that express TetR; 2) analysis of neomycin-resistant cell lines for TetR expression, and their inducibility with tetracycline after transient transfection with a TRE reporter gene, for example, TRE-β-galactosidase; 3) transfection of the appropriate, that is, highly inducible, cell lines with the TRE-transgene construct (response or target plasmid), and preparation of double-stable cell lines carrying the TRE transgene; 4) verification that the response gene (gene X) is inducibly expressed in the presence of tetracycline; and 5) characterization of cell lines. Some investigators have circumvented the two-step transfection approach, transfecting both the TetR regulator and response plasmid (TRE gene X) in the same experiment. This saves time and money, but the results are often unsatisfactory and is generally not recommended (*see* **Note 1**).

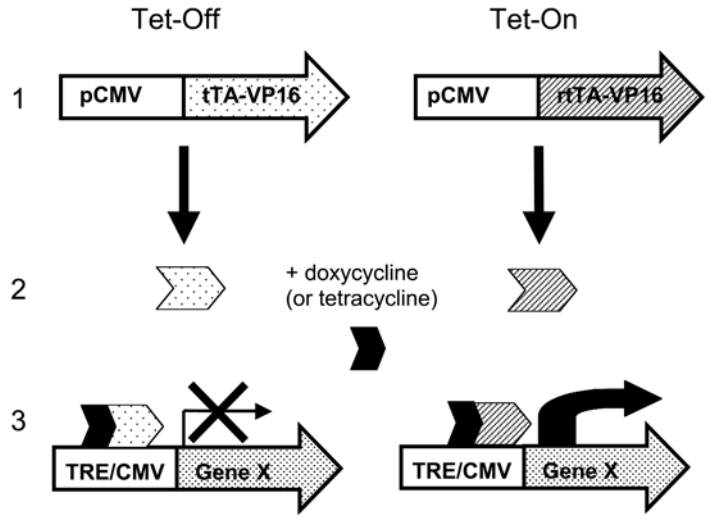


Fig. 1. Summary of the Tet-Off and Tet-On systems. In the first transfection **(1)**, cells are stably transfected with the TetR regulator plasmid that expresses either the tTA-VP16 (Tet-Off) or rtTA-VP16 (Tet-On) fusion proteins along with a neomycin resistance gene. Neomycin-resistant cell lines are then tested for TetR expression. Once the second transfection is performed to introduce the TRE-gene X response or target plasmid, expression of gene X can be regulated by tetracycline or doxycycline. The addition of tetracycline or doxycyline **(2)** to the Tet-Off cells shuts off expression of gene X, whereas the addition of antibiotic to Tet-On cells results in activation of gene X expression **(3)**.

Although many laboratories, including our own, obtained the plasmids from Professor H. Bujard from the University of Heidelberg in the 1990s, the technology is now available through two sources. Tet Systems® have licensed the technology to Clontech (the Tet-On™ and Tet-Off™ systems) (*see* **Note 2**). A second version is available through Invitrogen; the T-REx™ system uses a modified form of the system lacking the VP16 activation domain ***(16,17)***. Therefore, we will refer to the adopted commercial names and sources for these plasmids and other reagents required to establish the tetracycline-regulated system in keratinocytes.

2. Materials

2.1. Plasmids

2.1.1. TetR Expression Plasmids

1. pTet-On™ (cat. no. 631018) or pTet-Off™ (cat. no. 631017) plasmids (Clontech, Palo Alto, CA).
2. pcDNA™6/TR (Invitrogen, Carlsbad, CA; available with T-REx™ Complete (cat. no. K1020-01) or Core Kits (cat. no. K1020-02).

2.1.2. TRE Plasmids

There are a large number of tetO (TRE) plasmids containing reporter genes or polylinkers for gene cloning. A few examples follow.

2.1.2.1. Reporter Plasmids

1. pTRE-Tight-EGFP, positive control plasmid containing the green fluorescent protein (GFP) gene (Clontech; cat. no. 631060).
2. pTRE2-Tight-DsRed2, positive control plasmid containing the red fluorescent protein gene (Clontech; cat. no. 631061).
3. pcDNA™4/TO/*lacZ* and pcDNA™4/TO/*myc*-His/lac Z positive control plasmids containing β-galactosidase gene (Invitrogen, available with T-REx™ Complete, cat. no. K1020-01, or Core Kits, cat. no. K1020-02).

2.1.2.2. TRE Plasmids for Gene Cloning

Plasmids have been designed that contain the TREs upstream of the cytomegalovirus promoter with or without an enhancer, and cloning sites that contain one or more epitope tags for expressing tagged proteins, for example, with *c-myc*, HA, or hexahistidine (6xHN) tags. Some of these plasmids contain a drug selectable marker, while others do not. A partial list of available plasmids includes:

1. pTRE2hyg2-Myc,-HA, and -6xHN, tetO plasmids with multiple cloning site and N-terminal epitope tags as indicated, and encoding pyromycin resistance (Clontech, cat. nos. 631052, 631051, and 631053, respectively).
2. pTRE2pur-Myc,-HA, and -6xHN, tetO plasmids with multiple cloning site and N-terminal epitope tags as indicated, and encoding pyromycin resistance (Clontech, cat. nos. 631055, 631054, and 631056, respectively).
3. pTRE-Myc, pTRE-HA, and pTRE-6xHN, tetO plasmids with multiple cloning site and N-terminal epitope tags, as indicated. These plasmids lack an antibiotic selectable marker (Clontech; cat. no. 631010, 631012, and 631009).
4. pcDNA™4/TO, tetO plasmid for gene cloning with multiple cloning site, and encoding Zeocin™ resistance (Invitrogen, available with T-REx™ Complete, cat. no. K1020-01, or Core Kits, cat. no. K1020-02).
5. pcDNA™4/TO/*myc*-His A, B, and C, tetO plasmid with multiple cloning site, and *c-myc* and hexahistidine tags in all three reading frames. Also encodes Zeocin™ drug resistance (Invitrogen, available with T-REx™ Complete, cat. no. K1020-01, or Core Kits, cat. no. K1020-02).

2.1.3. Other Plasmids

pTK-Hyg and pPUR contains the hygromycin- and puromycin-resistance genes, respectively (Clontech, cat. no. 631750 and 631601). One of these plasmids, or an equivalent drug selectable marker, is needed if the TRE plasmid used in the second transfection (**Subheading 3.3.**) lacks such a selectable marker.

2.2. Keratinocyte Cell Lines

Like any cell line, all keratinocyte cell lines are a compromise. One trades the convenience of a relatively stable, often immortal line that is usually easily cultured and passaged with the departure from the limited lifespan and fastidious culture demands of more "normal" cell lines cultured directly from tissue. Which cell line one chooses depends on convenience (availability, culture conditions) and the properties of the line (e.g., which markers of keratinocyte differentiation does the line express). In the case of stable transfectants, the more "normal" cell lines are not practical. An adequate supply of any line that is chosen should be frozen in order to insure a reli-

able source of starting material. The thawed line should be well characterized and should serve as the starting material.

Some keratinocyte lines may be purchased from American Type Culture Collection (ATCC, Manassas, VA), whereas others can be obtained from individual laboratories. The following is a list of some available keratinocyte cell lines:

1. ATCC has the following human epidermal or epidermoid cell lines available as of October, 2003:
   A-431, cat. no. CRL-1555
   SCC-4, cat. no. CRL-1624
   SCC-9, cat. no. CRL-1629
   SCC-15, cat. no. CRL-1623
   SCC-25, cat. no. CRL-1628
2. The HaCaT human keratinocyte cell line ***(18)*** is available from Dr. Norbert Fusenig, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280, 69120 Heidelberg, Germany, e-mail: N.Fusenig@DKFZ-Heidelberg.de.
3. The rat epidermal keratinocyte (REK) cell line we have used ***(1,19,20)*** can be obtained from Dr. H. P. Baden, Massachusetts General Hospital, Cutaneous Biology Research Center, 13th Street Building 149, Charlestown, MA 02129, e-mail: howard.baden@cbrc2.mgh.harvard.edu ***(21)***.
4. The nontransformed (spontaneous) human keratinocyte line NM-1 may also be obtained from Dr. H. Baden ***(22,23)***.
5. The human keratinocyte line RHEK was obtained by transformation of primary human keratinocytes with viral oncogenes ***(24)***.

2.3. Other Key Reagents

1. Transfection Reagent, e.g. Lipofectamine(tm) (Invitrogen, cat. no. 18324-111; available in various sizes). Many other liposome- and nonliposome-based transfection reagents are available from a number of suppliers. We describe here a transfection protocol using Lipofectamine (**Subheading 3.1.2.**).
2. Doxycycline (Sigma Chemical Co., St. Louis, MO; cat. no. D9891 or Clontech; cat. no. 631311) is the tetracycline derivative used by most laboratories (*see* **Note 3**). Prepare a 1 mg/mL stock in sterile water, filter sterilize, aliquot, and store at –20°C in the dark. It is stable for at least 6 mo. Wear gloves when handling this chemical. Final concentration used in cell culture induction experiments is typically 0.2–1 µg/mL of culture medium ***(4)***.

2.4. Cell Culture

1. Geneticin® Selective Antibiotic (G418 sulfate) is available from several suppliers including Invitrogen (cat. no. 11811031) and Clontech (cat. no. 631307). It is stored in powder form at room temperature and is stable for up to 3 yr. Stock solutions (50 mg/mL) are prepared in type I endotoxin-free water, adjusted to pH 7.0 with NaOH, and filter sterilized. Stock solutions should be stored at 2–8°C. Wear gloves and mask when handling this chemical.
2. Hygromycin B is available from several suppliers including Calbiochem, San Diego, CA (cat. no. 400050) and Clontech (cat. no. 631309). Stock solutions (50 mg/mL) should be made up in type I endotoxin-free water and filter sterilized. Aqueous stocks can be stored at –20°C or 4°C for several years. Wear gloves and mask when handling this chemical.
3. HEPES buffered saline (HBS), pH 7.4, contains: 30 m*M* HEPES, 4 m*M* dextrose (D-glucose), 3 m*M* KCl, 130 m*M* NaCl, 1 m*M* Na_2HPO_4, and 0.0012 g Phenol Red per liter.

HBS is made up in endotoxin-free dH_2O, the pH adjusted to 7.4 with 1 *M* NaOH, and filter-sterilized (**ref. *25***, and Pirrone et al., chapter 1, this volume).

4. 0.05% Trypsin, 0.53 m*M* ethylenediamine tetraacetic acid (Invitrogen; cat. no. 25300-062). Store at –4°C in 50-mL aliquots. Stable to multiple freeze-thaws.
5. Dulbecco's modified Eagle medium (DMEM), low glucose (Invitrogen; cat. no. 31600-034). Add 3.7 g/L sodium bicarbonate, pH to 6.7, and filter-sterilize. Store at 4°C in the dark.
6. Fetal bovine serum (FBS) can be purchased from several suppliers (but *see* **Note 4**). We currently use FBS that is non-heat inactivated from Atlanta Biologicals, Norcross, GA (cat. no. S11150). Serum is tested for viability of cells and induction of profilaggrin and K1 expression after confluence ***(20,21)***. FBS should be stored at –80°C in the dark.
7. Penicillin–streptomycin (10,000 U penicillin, 10,000 µg streptomycin/mL; Invitrogen; cat. no. 15140-022).
8. Aminoguanidine nitrate (Sigma-Aldrich Chemical Co.; cat. no. A5610-8). Prepare 0.75 *M* stock solution in endotoxin-free water and sterile-filter; store stock solution at 4°C and make fresh every 6–8 wk. For REK culture, warm stock solution to 37°C and vortex for 10 s, then add 1 µL/mL of keratinocyte culture medium (final concentration of 0.75 m*M*).
9. 20% Culture medium for the REK line contains 20% FBS, 1% penicillin-streptomycin, 0.4 µg/mL hydrocortisone, 0.75 m*M* aminoguanidine nitrate in DMEM. Stable for 2 wk at 4°C in the dark with the exception of aminoguanidine, which is added fresh at each feeding (*see* **item 8**). 20% Culture medium is used for carrying cells, but cells are seeded into 10% medium for transfections.
10. Glass cloning rings are purchased from Bellco Glass Inc., Vineland, NJ (cat. no. 2090-00808). They should be autoclaved in glass petri dishes with silicone vacuum grease in the bottom of the dish.
11. Polyethylene terephtalate culture centrifuge tubes, for transfection (Corning, Harrodsburg, KY; cat. no. 430055).
12. VP16 polyclonal antibody for confirmation of tetR expression (Clontech; cat. no. 631209).
13. TRI Reagent® (Molecular Research Center, Inc., Cincinnati, OH; cat. no. TR 118) or RNAqueous™ kit (Ambion, Austin, TX; cat. no. 1914 or 1912), for extracting RNA.
14. β-galactosidase assay kits. Available from Stratagene, San Diego, CA (cat. no. 200383 or 200710) or Promega, Madison, WI (cat. no. E2000).

3. Methods

Each keratinocyte line has specific growth requirements. The methods detailed here relating to keratinocyte culture, in particular the growth conditions and transfection method, are optimized for the REK line. These protocols should be modified as needed for the specific cell line to be used.

3.1. Step 1: Generation of Keratinocyte Lines That Express TetR

This section describes the methodology for generating keratinocyte lines that express TetR. It involves three parts: transfection with TetR plasmid; selection with the cytotoxic drug G418 to kill untransfected cells; and isolation of neomycin-resistant keratinocyte lines.

3.1.1. Culture of REK Line

1. Rinse each dish of cultured keratinocytes twice using 5 mL of warm HBS per rinse.
2. Split culture with 5 mL warm trypsin solution.
3. Count cells in a hemacytometer. Seed keratinocytes at 4×10^4 cells per 60-mm dish in 5 mL 20% medium for subculture. If adding less than 100 µL of cell-trypsin solution per 5 mL of medium, then the cells do not need to be centrifuged.

4. For transfections, seed at the following densities in 10% medium. Omit aminoguanidine from the medium during transfections:
   a. 60-mm dish = 3×10^4 cells in 5 mL 10% medium.
   b. 12-well plate = 1×10^4 cells in 1.5 mL 10% medium per well.
   c. Cultures should be set up 5 d before transfecting, and the medium should be changed the day before the transfection. Cells should be no more than 60–70% confluent.
5. Cultures should be subcultured weekly and fed twice a week, that is, once between passages.

3.1.2. Transfection and Selection of Keratinocytes (REKs)

Numerous methods are available for transfecting keratinocytes ***(20,26)***. We describe here our method for transfection of rat epidermal keratinocytes using Lipofectamine™ reagent ***(2)***.

1. Rinse 60-mm keratinocyte culture dishes twice with warm HBS, then add 2 mL of warm, plain DMEM (containing no antibiotics or serum) per dish. Return dishes to the 37°C incubator.
2. Prepare DNA/Lipofectamine mixtures. For one 60-mm dish, add the following reagents to a sterile polyethylene terephtalate culture centrifuge tube (1.1 mL final volume): 1072.5 µL of plain DMEM; 5.5 µL of plasmid DNA (at 1 mg/mL in water; *see* **Note 5**); 22 µL of Lipofectamine (final concentration: 20 µL/mL DNA/DMEM mixture).
3. Mix the mixture well by tapping the tube and let sit for 10 min at room temperature. Mix again and let sit for an additional 10 min. Do not mix by vortexing.
4. Add 1 mL of the DNA/lipid mixture to the plain DMEM in the 60-mm dish (total volume 3 mL/dish). Incubate cells with DNA/lipid mixture for 4 h with occasional gentle mixing.
5. After 4 h, add an equal volume, that is, 3 mL, of freshly prepared 20% FBS/DMEM (containing no antibiotics, hydrocortisone, or aminoguanidine) to each dish. Gently swirl dishes and return to the incubator.
6. The next morning, aspirate the medium and feed cells with their normal medium (DMEM containing 10% serum, hydrocortisone, and antibiotics in the case of REKs).
7. After 48–72 h, change the medium and add fresh medium containing G418 (*see* **Note 6** about determining what drug concentration to use with your keratinocyte line). For the REK line, we used 200 µg G418/mL medium, experimentally determined to be the minimum concentration required to effectively kill untransfected cells. Change the medium twice weekly. After about 5 d, untransfected cells will begin to die.
8. Grow keratinocytes until isolated colonies appear, which will take 2–4 wk. These colonies will be derived from one or a few progenitor (transfected) cells that survived drug selection.

3.1.3. Isolation of Stable Cell Lines

1. Once the colonies have grown sufficiently, the cell lines are ready to be isolated. Mark location of colonies on the bottom of each tissue culture dish with a colored marker. These should represent cells derived from one or a few transfected progenitor cells.
2. Wash cells twice with warm HBS, and remove all fluid from the dish.
3. Place a sterile glass cloning ring firmly on the plate with forceps, surrounding the area containing the keratinocyte colony. Be careful not to slide the cloning ring across the plate, which can damage or destroy viable cells. This step should be done as quickly as possible to prevent the cells from drying out.
4. Trypsinize cells contained within the cloning ring (for the REK line, trypsinization takes between 5 and 8 min at 37°C). Wash cells gently with 10% medium using a sterile Pasteur pipet. Transfer cells into an appropriate size tissue culture dish for propagation

and expansion. Six-, 12-, or 24-well plates work well, depending on cell type, growth rate, and colony size. Once these cell lines have been passaged an additional one to two times, they are ready for testing for TetR expression and inducibility by transfection with a reporter gene (*see* **Subheading 3.2.**).

3.2. Step 2: Testing Stable Keratinocyte Lines for TetR Expression and Inducibility

3.2.1. Determine TetR Expression of Stable Cell Lines

Stable cell lines should be checked for tetR mRNA expression by Northern blot, reverse transcription polymerase chain reaction, or ribonuclease protection assay. Alternatively, TetR protein expression may be verified by Western analysis with a VP16 polyclonal antibody. We used Northern analysis to identify TetR expressing lines, using the complete tetR cDNA insert as probe. We obtained three stable cell lines, of which two expressed TetR mRNA **(Fig. 2A)**.

1. Plate TetR keratinocyte cell lines and grow until cells are 80–90% confluent.
2. Isolate total RNA from cell lines. We generally use Trizol® reagent or RNAqueous® kit which reproducibly gives high-quality RNA from cultured cells. Total RNA should be extracted as recommended by the manufacturer.
3. Identify TetR-expressing cell lines by Northern blot, reverse transcription polymerase chain reaction, or Ribonuclease Protection Assay using established molecular biology procedures (*see* **Note 7**).

3.2.2. Inducibility Test of TetR Lines by Transient Transfection With Reporter Plasmid

TetR-expressing cell lines should be tested for tetracycline inducibility by transient transfection of a reporter plasmid, typically TRE-β-galactosidase or TRE-EGFP (*see* **Subheading 2.1.2.1.**) as follows:

1. Transfect each G418 cell line to be tested with TRE reporter plasmid (1–2 μg/dish) as described in **Subheading 3.1.2.** Each cell line to be tested should be transfected in quadruplicate. Cells should be transfected when 60–70% confluent.
2. After transfecting cells, add 1 μg/mL doxycycline to two dishes of each cell line being tested (**step 6** of **Subheading 3.1.2.**). The other two dishes of each line should be fed with medium lacking doxycycline (–doxycycline control; *see* **Note 4**).
3. Incubate cells for 48–60 h and assay for inducibility. Harvest by scraping the cells off the dish with a sterile rubber policeman. Prepare cell lysates, and perform β-galactosidase assays according to the manufacturer's directions. Calculate the fold induction of reporter gene expression by determining:

$$\frac{\text{Total activity in the presence of doxycycline/mg protein (+ doxycycline)}}{\text{Total activity in the absence of doxycycline/mg protein (– doxycycline)}}$$

4. Expand keratinocyte (TetR positive) cell lines that exhibit good inducibility and prepare frozen stocks (*see* **Note 8**) of each cell line as soon as is practical (*see* Pirrone et al., Chapter 1, this volume for details on freezing of keratinocyte lines). In our studies, both cell lines that expressed TetR showed a good induction of β-galactosidase expression 48 h after transfection with reporter plasmid (**Fig. 2B**; **ref. *1***).

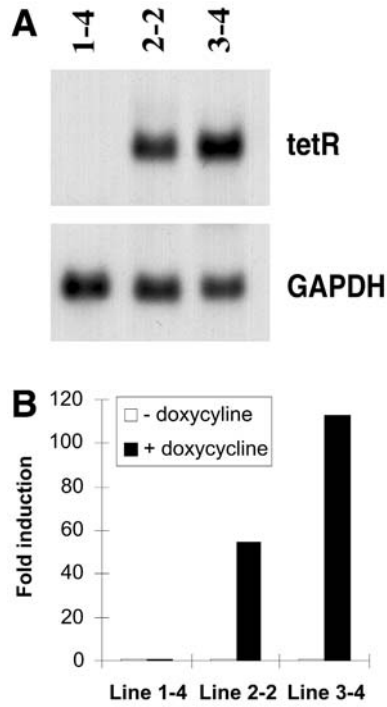


Fig. 2. Tetracycline regulation and expression of TetR in three keratinocyte stable cell lines. **A,** Expression of TetR mRNA in keratinocyte cell lines as measured by Northern blot. Total RNA was isolated from three neomycin-resistant keratinocyte lines, fractionated on glyoxal/agarose gels, and blotted to Genescreen Plus membrane. TetR mRNA levels were evaluated with a TetR cDNA probe. Note that lines 2-2 and 3-4, which showed a good induction response, expressed high levels of rtTA, whereas line 1-4 did not express TetR. The housekeeping gene GAPDH was used as a control probe to assess the uniformity of RNA loadings. **B,** Stable keratinocyte lines generated by transfection with TetR (rtTA-VP16) and selection with G418 were tested for doxycycline inducibility after transfection with TRE-β-galactosidase. Lines 2-2 and 3-4 showed good induction of β-galactosidase activity after incubation with 1 μg/mL doxycycline for 48 h compared with untreated (control) cells. Line 1-4 showed no induction of β-galactosidase expression caused by the absence of TetR expression in that line **(1)**.

3.3. Step 3: Preparation of Double-Stable Tet Cell Lines

In the second transfection experiment, one or more TetR-expressing lines that exhibit a good inducibility response (**Subheading 3.2.2.**; **Fig. 2B**) is transfected with the TRE-gene X or response plasmid and cell lines established expressing the gene(s) of interest.

1. Clone cDNA for gene to be studied (denoted gene X) into appropriate TRE vector. The TRE response plasmids are available in several forms, for example, with or without cytomegalovirus enhancer, or with or without epitope tags such as c-myc (*see* **Subheading 2.1.2.2.**). Investigators should consult the Clontech or Invitrogen catalogues or websites in order to choose the plasmids that best serve their needs.
2. Plate out TetR expressing lines to be transfected (3×10^4 cells/60-mm dish), allowing at least three dishes for each TetR expressing line (*see* **Note 8**).

3. Transfect TRE-gene X plasmid DNA into each cell line as described in **Subheading 3.1.2.** If the TRE plasmid does not contain a selectable marker such as hygromycin or Zeocin, a second plasmid, for example, pTK-hyg (0.5–1 μg/dish) will need to be transfected to allow selection of double-stable cell lines.
4. Proceed with selection of transfected keratinocytes as described in **Subheadings 3.1.2.** and **3.1.3.** Use the minimal concentration of cytotoxic drug, for example, hygromycin, that kills off keratinocytes not containing the transfected gene that confers antibiotic resistance. The kill curve experiment should be done with each TetR-expressing line that the investigator intends to use to generate double-stable cell lines from, as described in **Note 6**.
5. Isolate surviving colonies using the cloning ring as described in **Subheading 3.1.3.** Plate out and expand each line, passage and test for inducible expression of gene X as described below.

3.4. Step 4: Analysis of Double-Stable Keratinocyte Lines for Tetracycline-Inducible Gene Expression

1. Plate 2×10^4 cells into duplicate 60-mm dishes for each cell line to be tested.
2. Add 1 μg/mL doxycycline to one dish for each cell line (**step 6** of **Subheading 3.1.2.**). The other dish of each line should be fed with medium lacking doxycycline (–doxycycline control).
3. Incubate for 48 h and harvest the cells. Prepare cell lysates and test for expression of gene X-encoded protein by Western blot using an appropriate specific antibody, or for mRNA expression by Northern blot or ribonuclease protection assay.
4. Freeze cell lines that exhibit good inducibility of gene X expression for long-term storage.

3.5. Step 5: Characterization of Double-Stable Keratinocyte Lines

To optimize tetracycline-inducible expression of the response gene (gene X), double-stable cell lines should be plated out and grown in different concentrations of doxycycline, for example, 0–2 μg/mL, for varying times (12–72 h) and examined for inducible expression. In our studies with REK lines, filaggrin expression was maximal at 1 μg/mL doxcycline at 48–60 h (**Fig. 3**; **ref. *1***). Little or no filaggrin expression was seen in the absence of inducer, demonstrating the tight regulation of the TRE promoter function in our double-stable cell lines (**Figs. 2B** and **3**). Although most investigators using the Tet system use doxycycline, other tetracycline derivatives are available ***(4,27)***. You are now ready to begin functional studies with your double-stable Tet cell lines!

4. Notes

1. Gossen and Bujard recommend that the tetracycline-inducible cell lines be made as outlined in this chapter, first making lines expressing the TetR regulator and then introducing the response plasmid containing TRE gene X to generate the double-stable cell lines. This allows highly responsive TetR-expressing lines to be used to introduce a variety of different genes. If the regulatory and response plasmids are introduced together, the plasmids have a tendency to integrate at the same chromosomal locus resulting in an increased basal activity of the minimal TRE promoter (H. Bujard, personal communication). In addition, the inducibility of the response gene can vary widely in different lines depending on the ratio of regulator to response plasmid used in the transfection.
2. The Internet addresses and toll-free phone numbers (USA/Canada) for the companies listed in this article are as follows:
   a. Ambion, Inc., Austin, TX, 800-888-8804, http://www.ambion.com
   b. Atlanta Biologicals, Norcross, GA, 800-780-7788, http://www.atlantabio.com/default.htm

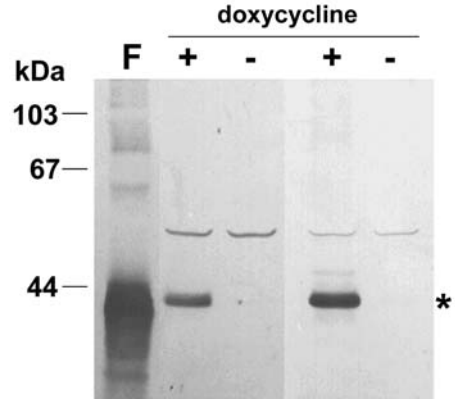


Fig. 3. Expression of human filaggrin in two double stable keratinocyte cell lines, 2F5 and 3F5, analyzed by Western blot with human filaggrin antibody 8959. Both lines express high levels of human filaggrin (approx 40-kDa protein, starred) in the presence of doxycycline inducer added to the media at 1 µg/mL for 48 h. hFG, human filaggrin; F, human foreskin used as a positive control for Western analysis (*see* **ref.** ***1*** for further discussion). Molecular weight standards (in kilodaltons) are shown at left.

c. American Type Culture Collection, Manassas, VA, (800) 638-6597, http://www.atcc.org
d. Bellco Glass, Inc., Vineland, NJ, (800) 257-7043, http://www.bellcoglass.com/us
e. Calbiochem, San Diego, CA, (800) 854-3417, http://www.calbiochem.com
f. Clontech (a division of BD Biosciences), Palo Alto, CA, 1-800-662-2566, ext. 1, http://www.clontech.com
g. Corning, Harrodsburg, KY, 1-607-974-9000 , http://www.corning.com
h. Invitrogen, Carlsbad, CA, (800) 955- 6288, Option 1, http://www.invitrogen.com
i. Molecular Research Center, Inc., Cincinnati, OH, 800-462-9868, http://www.mrc-gene.com
j. Promega Corp., Madison, WI, 800-356-9526, http://www.promega.com
k. Qiagen, Valencia, CA 800-426-8157, http://www.qiagen.com
l. Sigma Chemical Co., St. Louis, MO, 800-325-3010, http://www.sigmaaldrich.com
m. Stratagene, San Diego, CA, (800) 894-1304, http://www.stratagene.com

3. Doxycycline activates TetR at a significantly lower concentration than tetracycline (0.01–1 µg/mL vs 1–2 µg/mL, respectively). In addition, doxycycline has a significantly longer half-life than tetracycline in vitro (24 h vs 12 h), making it the inducer of choice for most experiments. Other tetracycline derivatives, such as anhydrotetracycline have also been successfully employed with this system ***(4,27)***.
4. It is important to use serum batches that are free or low in tetracycline or contain other reagents that will affect both expression of transgene and inducibility. The serum batches that are to be used to grow the keratinocyte lines should be tested for inducibility of a reporter gene such as TRE-β-galactosidase or TRE-EGFP. The serum batch that gives the greater induction of reporter gene activity should generally be used (*see* Clontech publication PT3001-1 for further discussion of this issue). A simpler alternative is to use serum that has been tested for tetracycline contamination, and is approved for use with the Tet system, for example, Tet System Approved FBS from Clontech, cat. no. 631101, or 631106. However the serum used must also support growth and differentiation of the cell line(s) studied.
5. The amount of plasmid transfected into a 60-mm dish of keratinocytes can vary from 0.5–10 µg of DNA depending on the experiment. For generation of TetR-expressing cell

lines, we would recommend starting with different DNA concentrations, for example, 1, 5, and 10 µg DNA. Some investigators linearize the plasmid DNA with a restriction enzyme prior to transfection. This does not improve transfection efficiency but it may help to prevent disruption of gene function, for example, TetR, during integration of the plasmid into the host cell chromosome. To linearize plasmid DNA:

a. Digest DNA with a restriction enzyme that cuts outside of the TetR or neomycin genes, for example, *Fsp*I for pcDNA4/TO or Sca I for pTet-On™ or pTet-Off™. Perform the restriction digestion according to the manufacturer's recommendation.
b. Check for linearization of the plasmid, that is, the presence of a single band, by running a small amount (0.2 µg) on an agarose gel.
c. If digestion is satisfactory, recover the linearized plasmid DNA by ethanol precipitation or by binding to a Qiagen Nucleotide Removal Column (Qiagen; cat. no. 28306) and store purified DNA at –20°C until needed.
d. Estimate the DNA concentration by running a small amount on an agarose gel next to known amounts of molecular weight DNA standards.

6. Antibiotic kill curve. The concentration required to kill untransfected cells will vary depending on the particular cell line and should be determined experimentally. There is also lot-to-lot variation in the potency of these drugs. Determine the minimum concentration of each drug required to kill your host cell line as follows:
   a. Plate 2×10^4 cells in each of ten 60-mm plates containing 5 mL of medium plus varying concentrations (0–800 µg/mL) of G418 or hygromycin.
   b. Incubate the cells for 10–14 d, replenishing the selective medium every 3–4 d and observe the percentage of surviving cells. This can be done by observing the number of adherent cells by phase contrast microscopy, or more precisely by trypsinizing and counting the number of adherent cells after approx 14 d. For selecting stable transformants, use the lowest concentration that begins to give massive cell death at 5–7 d and kills all the cells within 2 wk. For the REK line, we found 200 µg/mL G418 and 15 µg/mL hygromycin to be optimal concentrations.
7. The TetR cDNA can be used directly as a probe for Northerns. The Genbank accession numbers for the pTet-On and pTet-Off vectors are U89930 and U89929. The accession number for pTRE, the response plasmid, is U89931. All of these plasmids are available from Clontech. The Genbank web address is www.ncbi.nlm.nih.gov/entrez.
8. Working with frozen stocks of stable TetR lines allows one to prepare double-stable tetracycline-responsive cell lines from the same starting material.

Acknowledgments

We thank Ms. Anna Pirrone for helpful comments on the manuscript. The work from the authors' laboratories discussed in this review was supported by National Institutes of Health grants R29 AR45276 (to RBP), R01 AR49183 (to RBP), and P01 AR21557 (to PF), and by the Odland Endowed Research Fund of the Division of Dermatology, University of Washington.

References

1 Presland, R. B., Kuechle, M. K., Lewis, S. P., Fleckman, P., and Dale, B. A. (2001) Regulated expression of human filaggrin in keratinocytes results in cytoskeletal disruption, loss of cell-cell adhesion, and cell cycle arrest. *Exp. Cell Res.* **270,** 199–213.

2 Dale, B. A., Presland, R. B., Lewis, S. P., Underwood, R. A., and Fleckman, P. (1997) Transient expression of epidermal filaggrin in cultured cells causes collapse of intermediate filament networks with alteration of cell shape and nuclear integrity. *J. Invest. Dermatol.* **108,** 179–187.

3 Gossen, M. and Bujard, H. (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl. Acad. Sci. USA* **89,** 5547–5551.

4 Gossen, M., Freundlieb, S., Bender, G., Muller, G., Hillen, W., and Bujard, H. (1995) Transcriptional activation by tetracyclines in mammalian cells. *Science* **268,** 1766–1769.

5 Resnitzky, D., Gossen, M., Bujard, H., and Reed, S. I. (1994) Acceleration of the G1/S phase transition by expression of cyclins D1 and E with an inducible system. *Mol. Cell Biol.* **14,** 1669–1679.

6 Hofmann, A., Nolan, G. P., and Blau, H. M. (1996) Rapid retroviral delivery of tetracycline-inducible genes in a single autoregulatory cassette. *Proc. Natl. Acad. Sci. USA* **93,** 5185–5190.

7 Lindemann, D., Patriquin, E., Feng, S., and Mulligan, R. C. (1997) Versatile retrovirus vector systems for regulated gene expression in vitro and in vivo. *Mol. Med.* **3,** 466–476.

8 Neering, S. J., Hardy, S. F., Minamoto, D., Spratt, S. K., and Jordan, C. T. (1996) Transduction of primitive human hematopoietic cells with recombinant adenovirus vectors. *Blood* **88,** 1147–1155.

9 Shockett, P., Difilippantonio, M., Hellman, N., and Schatz, D. G. (1995) A modified tetracycline-regulated system provides autoregulatory, inducible gene expression in cultured cells and transgenic mice. *Proc. Natl. Acad. Sci. USA* **92,** 6522–6526.

10 Kistner, A., Gossen, M., Zimmermann, F., Jerecic, J., Ullmer, C., Lubbert, H., and Bujard, H. (1996). Doxycycline-mediated quantitative and tissue-specific control of gene expression in transgenic mice. *Proc. Natl. Acad. Sci. USA* **93,** 10,933–10,938.

11 Weinmann, P., Gossen, M., Hillen, W., Bujard, H., and Gatz, C. (1994). A chimeric transactivator allows tetracycline-responsive gene expression in whole plants. *Plant J.* **5,** 559–569.

12 Li, J. J., Cao, Y., Young, M. R., and Colburn, N. H. (2000) Induced expression of dominant-negative c-jun downregulates NFkappaB and AP-1 target genes and suppresses tumor phenotype in human keratinocytes. *Mol. Carcinog.* **29,** 159–169.

13 Diamond, I., Owolabi, T., Marco, M., Lam, C., and Glick, A. (2000) Conditional gene expression in the epidermis of transgenic mice using the tetracycline-regulated transactivators tTA and rTA linked to the keratin 5 promoter. *J. Invest. Dermatol.* **115,** 788–794.

14 Liu, X., Alexander, V., Vijayachandra, K., Bhogte, E., Diamond, I., and Glick, A. (2001) Conditional epidermal expression of TGFbeta 1 blocks neonatal lethality but causes a reversible hyperplasia and alopecia. *Proc. Natl. Acad. Sci. USA* **98,** 9139–9144.

15 Jaubert, J., Cheng, J., and Segre, J. A. (2003) Ectopic expression of Kruppel like factor 4 (Klf4) accelerates formation of the epidermal permeability barrier. *Development* **130,** 2767–2777.

16 Yao, F., Svensjo, T., Winkler, T., Lu, M., Eriksson, C., and Eriksson, E. (1998) Tetracycline repressor, tetR, rather than the tetR-mammalian cell transcription factor fusion derivatives, regulates inducible gene expression in mammalian cells. *Hum. Gene Ther.* **9,** 1939–1950.

17. T-REx™ System (1999), in *Expressions*, Vol. 6, Invitrogen Newsletter, pp. 2–3.

18 Boukamp, P., Petrussevska, R. T., Breitkreutz, D., Hornung, J., Markham, A., and Fusenig, N. E. (1988) Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J. Cell Biol.* **106,** 761–771.

19 Kuechle, M. K., Presland, R. B., Lewis, S. P., Fleckman, P., and Dale, B. A. (2000) Inducible expression of filaggrin increases keratinocyte susceptibility to apoptotic cell death. *Cell Death Differ.* **7,** 566–573.

20 Haydock, P. V., Blomquist, C., Brumbaugh, S., Dale, B. A., Holbrook, K. A., and Fleckman, P. (1993) Antisense profilaggrin RNA delays and decreases profilaggrin expression and alters in vitro differentiation of rat epidermal keratinocytes. *J. Invest. Dermatol.* **101,** 118–126.

21 Baden, H. P. and Kubilus, J. (1983) The growth and differentiation of cultured newborn rat keratinocytes. *J. Invest. Dermatol.* **80,** 124–130.

22 Baden, H. P., Kubilus, J., Wolman, S. R., Steinberg, M. L., Phillips, S. B., and Kvedar, J. C. (1987) NM1 keratinocyte line is cytogenetically and biologically stable and exhibits a unique structural protein. *J. Invest. Dermatol.* **89,** 574–579.

23 Baden, H. P., Kubilus, J., Kvedar, J. C., Steinberg, M. L., and Wolman, S. R. (1987) Isolation and characterization of a spontaneously arising long-lived line of human keratinocytes (NM 1). *In Vitro Cell Dev. Biol.* **23,** 205–213.

24 Rhim, J. S., Jay, G., Arnstein, P., Price, F. M., Sanford, K. K., and Aaronson, S. A. (1985) Neoplastic transformation of human epidermal keratinocytes by AD12-SV40 and Kirsten sarcoma viruses. *Science* **227,** 1250–1252.

25 Tsao, M. C., Walthall, B. J., and Ham, R. G. (1982) Clonal growth of normal human epidermal keratinocytes in a defined medium. *J. Cell Physiol.* **110,** 219–229.

26 Jiang, C. K., Connolly, D., and Blumenberg, M. (1991) Comparison of methods for transfection of human epidermal keratinocytes. *J. Invest. Dermatol.* **97,** 969–973.

27. Gossen, M. and Bujard, H. (1993) Anhydrotetracycline, a novel effector for tetracycline controlled gene expression systems in eukaryotic cells. *Nucleic Acids Res.* **21,** 4411–4412.

30

Gene Targeting by Oligonucleotides in Keratinocytes

Olga Igoucheva and Kyonggeun Yoon

Summary

Oligonucleotide-directed gene alteration produces a targeted deoxyribonucleic acid (DNA) sequence change in the genome of mammalian cells at low frequency that is only detectable by highly sensitive methods. To measure the low frequency, we have established an assay using the mutant *lacZ* vector that contains a single point mutation in the *lacZ* gene, which results in a loss of enzymatic activity. When cells containing this mutant reporter gene are corrected by gene targeting, the mutant β-galactosidase enzymatic activity is restored, and corrected cells can be visualized by histochemical staining. Using this method, we detected a low level of gene correction in the primary human keratinocytes, in spite of highly efficient nuclear uptake of oligonucleotide. Therefore, it is important to consider many other factors for successful gene repair, including DNA repair and recombination activities, status of replication and transcription, in addition to the well-known requirements like the quality and delivery of oligodeoxynucleotides to cells. Available methods to manipulate epidermal stem cells and the accessibility of the tissue make the epidermis attractive for gene targeting. Given the low frequency, however, general selection procedures and amplification of corrected cells via epidermal stem cells are ultimately needed to make the gene repair technology practical.

Key Words:

Keratinocytes; gene targeting; correction frequency; oligodeoxynucleotides; reporter gene.

1. Introduction

Gene therapy is a rapidly growing field that holds much promise for treatment of inherited and acquired diseases. For a successful therapy, genes must be efficiently delivered to the target cells, and gene products must be expressed for a prolonged period without harmful effects to a host. Currently, two approaches are taken for this purpose: an in vivo strategy where genes are transferred directly into host organs, and an ex vivo approach through which cells are removed, cultured for gene delivery and selection, and returned back to the host. Several obstacles continue to delay safe and effective application of gene therapy. Transient expression of transferred genes, limited survival of transplanted cells, and difficulties in gene transfer to stem cells constitute major technical challenges requiring further investigation.

The epidermis is an ideal system for testing and developing different modalities of gene therapy because it is readily available for local or systematic delivery of therapeutic agents. The treated area can be closely monitored and compared with untreated areas within the same organism, and the genetically modified tissue can easily be removed if termination of the treatment is desired. Stratified squamous epithelia are con-

From: *Methods in Molecular Biology, vol. 289:*
Epidermal Cells: Methods and Protocols
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

tinuously renewing tissues that are maintained by the division of keratinocytes in the proliferative basal layer that replace differentiated keratinocytes in the upper layer that are sloughed ***(1)***. The long-term success of cultured epithelial autographs in treating burn patients indicates that a stem cell therapy may be possible ***(2,3)***. Keratinocytes have a high turnover rate, approx 7 to 10 d in mice and 30 d in humans ***(4)***. Thus, in renewing tissues such as the epidermis of skin, a gene therapy approach must be targeted toward the stem cell population and should require identification, enrichment, and targeting of stem cells to ensure the continued presence of the transferred gene. The epidermis is also well suited to an ex vivo approach because keratinocytes can be cultured, selected for the expression of transferred genes, and returned to the donor by grafting. Furthermore, if the transferred gene encodes a secreted protein, cultured keratinocytes have been shown to secrete the protein for a long period of time after grafting ***(5)***. Therefore, optimal gene therapy strategies are being tested in epidermal keratinocytes to treat dominant and recessive genetic disorders as well as acquired skin diseases. However, epidermal gene therapy is in an early stage and yet holds great promise for its ultimate clinical application.

Many different viral vectors were used for efficient gene delivery. To replace a defective gene, viral vectors must contain a good copy of the entire gene or the cDNA as well as some of the regulatory regions important for gene expression. Gene delivery by integrating viral particles (retrovirus, lentivirus, and adeno-associated virus) provides a long-term expression because the transgene can integrate permanently into the host chromosome ***(6)***. Although the viral gene delivery has made an impressive advancement toward clinical applications, several drawbacks exist. For example, retroviral transfer requires cell division for infection, thereby limiting infections of slowly cycling stem cells ***(7)***. It has also been difficult to generate a stable high-titer virus effective for an in vivo infection ***(8)***. Moreover, several studies have documented gradual inactivation of transferred gene expression driven by the viral promoter, even though the vector DNA clearly remains present ***(9)***. Also, there is a safety concern of a possible generation of replication competent retrovirus ***(10)***. In addition, a random integration of virus could activate an oncogene or inactivate a tumor suppressor gene ***(11)***. The adenoviral delivery system has advantages because it does not require the target cell division for infection and high-titer virus can be generated ***(12)***. By the same token, adenoviral vectors are likely to infect nonreplicating, terminally differentiated cells, thus limiting expression to the transit times of these cells. Moreover, infected cells become the target for the immune system and are eliminated from the body ***(12)***. In addition, high-titer adenovirus used in clinical situations cause acute immune responses and toxicity to the host.

Considering the difficulties encountered in the use of viral vectors, it would be useful to develop other modalities of gene therapy for skin. Several gene-targeting strategies have been developed to correct mutations through a homologous recombination process. This approach has a potential to correct the desired mutation while maintaining a complex genomic organization important for the appropriate expression and regulation of genes. Through the use of traditional gene-targeting strategies via double-stranded DNA, it has become possible to replace or delete genetic information in chromosomes. However, the application of this strategy to embryonic stem cells has been possible by selecting rare successful targeting products ***(13)***. An alternative approach involves targeted mutagenesis facilitated by triple-helix-forming oligo-

nucleotides coupled to reactive chemical groups. A triple-helix-forming oligonucleotide recognizes the sequence surrounding a targeted base and a reactive group chemically modifies the targeted nucleotide or elicits DNA repair ***(14)***. A small-fragment homologous replacement strategy using a 300- to 400-base single-stranded DNA was used to generate homologous replacement in mammalian cells at approx 1% ***(15)***. Another strategy involves a bifunctional oligonucleotide that consists of two different domains, a triple-helix domain for binding to the sequence nearby the targeted base and a repair domain containing a mismatch to the targeted base ***(16)***. Recently, adeno-associated virus vectors were used to modify homologous chromosomal sequences, and targeting rates close to 1% were observed at the HPRT locus in normal human cells ***(17)***. Although each of these methods were shown to work in limited cases, the low absolute frequency of homologous recombination remains a serious limitation.

Currently, our laboratory has focused on the development of an experimental strategy that centers on the site-specific correction of single-point mutations by using relatively short single-stranded oligodeoxynucleotides (ODNs). These studies include establishing sensitive and reproducible assays to score the frequencies of gene correction in mammalian cells and mechanistic studies to improve the targeting frequency. Toward these goals, two assay systems have been established in which phenotypic changes can be detected upon gene correction, the mutant *lacZ* and mutant tyrosinase. Both systems have provided clear evidence of gene correction by ODN in the episome and chromosome of several mammalian cells: CHO-K1 ***(18)***, primary human keratinocytes ***(19)***, melanocytes ***(20)***, and mouse embryonic stem cells ***(21)***. In another recent study, we showed that two ODNs designed to produce alterations in tyrosinase and c-kit genes, respectively, can be used to cause simultaneous modifications of both genes in a single albino mouse melanocyte ***(20)***. Our results indicate that if two ODNs are present within the nucleus of a "repair-competent" cell, then dual targeting events can occur at relatively high frequency. This strategy may allow the use of a selection procedure to overcome the low frequency of gene correction, a limitation of the current oligonucleotide-based gene targeting.

We will specifically address each critical issue for efficient gene targeting in this chapter: the design of the targeting ODN, the efficiency of ODN delivery, the stability of ODN inside the cells, the culture conditions of the cells before, during, and after transfection, and the assessment of gene alteration.

1.1. Design of ODN

Our recent studies showed that short ODNs exhibit a similar or higher activity than chimeric RNA–DNA oligonucleotide (RDo) in the targeted alteration of single base point mutations in mammalian cells ***(18,20)***. The use of ODN in gene alteration may be preferable to other synthetic oligonucleotides because ODN can be synthesized and purified relatively easily. The ODN with the highest activity was 45-nt long and composed of a sequence complementary to the coding sequence, except for a single mismatch to the targeted base **(Fig. 1)**. To increase the stability of ODN in mammalian cells, ODN has been protected at both 5′ and 3′ ends by incorporation of four residues of 2′-*O*-methyl uridine. Incorporation of phosphorothioates at both ends in our experience, showed less efficient gene targeting. These ODNs are relatively stable in mammalian

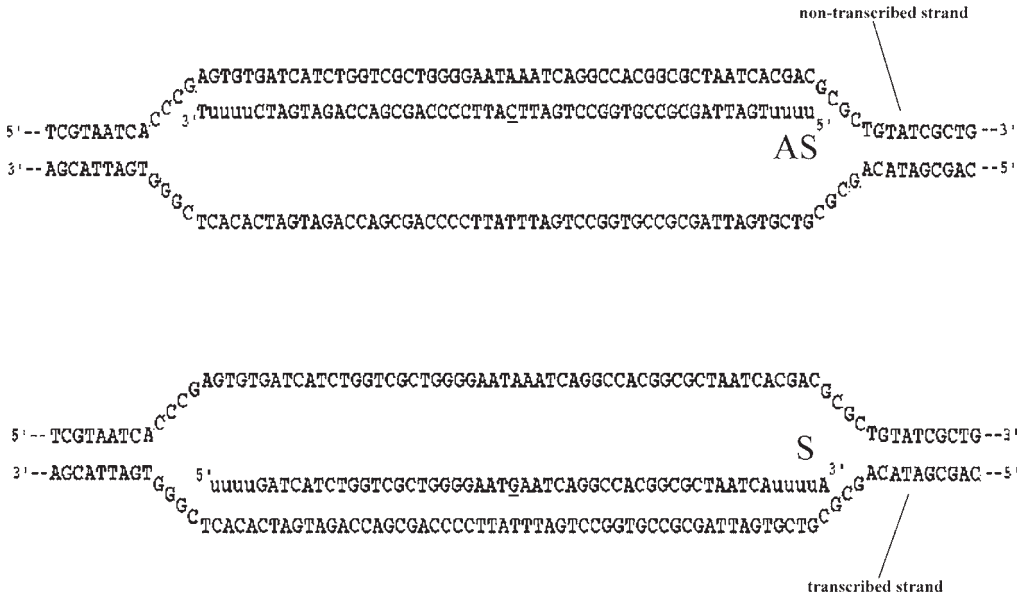


Fig. 1. A hypotetical intermediates between antisense (AS) and sense (S) ODNs and mutant *LacZ* sequence. Both ODNs were designed to restore an enzymatic activity of the mutant β-galactosidase by incorporation of a single mismatch to the targeted base (underlined). AQ double-stranded target sequence is postulated to be paired with AS and S ODNs generating a mismatch AC: and G:T, respectively.

cells and a minor fraction of ODNs can be recovered from the cells 24 h after transfection as an intact full-length ODN ***(18)***.

1.2. Delivery of ODN

In recent years a series of highly effective cationic liposome agents have been introduced. The use of positively charged liposomes reduces the negative charges of the DNA molecule facilitating its delivery through the cell membrane. DNA also induces cationic liposome fusion, perhaps by bridging two liposomes, drawing them closer and destabilizing the bilayer. This fusogenic property provides opportunity for the DNA to enter the new liposome or to associate with the net negative charge on a cell membrane to deliver their DNA ***(22)***. The efficiency of liposome appears to depend on many variables: cell type, lipid membrane composition, relative ratio of the lipid to DNA, endocytosis of ODN, release of ODN from endosome, nuclear transfer, and the stability of the DNA–lipid complex during these processes. Delivery of ODN requires optimization for each cell type by testing many available liposomes. It has been difficult to deliver ODN by liposome to the majority of human primary cells. In contrast, highly efficient delivery of ODN was achieved in human primary keratinocytes, even in the absence of liposome ***(23)***. We have been able to achieve consistently high efficiency of nuclear ODN delivery ***(19,24)***. Keratinocytes have the ability to take up ODN probably by receptor-mediated endocytosis or macropinocytosis. The exposure of normal human keratinocytes to ODN induced several genes, including interleukin-1α and follistatin in a nonsequence-specific manner ***(25)***. The mechanism of this efficient ODN delivery in keratinocytes has not yet been fully elucidated although internalized

ODN has been speculated to bind to an intracellular receptor (e.g., Toll-like receptor 9), which mediates signaling.

1.3. Requirements for Successful Gene Targeting by ODN

Initially, we expected an efficient ODN-directed gene alteration in keratinocytes since large amounts of ODN can be delivered to the nucleus. However, keratinocytes in tissue culture exhibited a very low level of gene correction by ODN that cannot be detected by the conventional polymerase chain reaction (PCR)-based RFLP analysis ***(24)*** nor by the assay based on immunofluorescence ***(26)***. RFLP analysis cannot detect frequencies less than 5% and is plagued by PCR artifacts caused by priming of the degraded RDO during amplification. Hence, it was necessary to develop a highly sensitive system to measure low frequency. In 1999, we established the mutant *lacZ* vector that contains a single point mutation in the *lacZ* gene (G1651A), which results in a loss of enzymatic activity caused by an amino acid substitution (E523K). When cells containing this mutant reporter gene are corrected by gene targeting, the enzymatic activity of the mutant β-galactosidase is restored and corrected cells can be visualized by histochemical staining. This mutant *lacZ* system has been instrumental in developing gene repair strategy by providing an easily detectable and measurable marker for gene correction in biochemical studies using nuclear extracts, episome and chromosome of mammalian cells ***(18,27)***. Using the mutant *lacZ* vector, we have developed an in vitro reaction where one can measure the gene repair activity in a given cell type. This in vitro reaction by nuclear extracts has been valuable in predicting the feasibility of gene targeting in a given cell type because there is a good correlation between the in vitro gene repair activity and the chromosomal gene repair activity ***(18,27)***. The nuclear extracts isolated from human primary keratinocytes exhibited a much lower level of gene correction in comparison to CHO-K1 and DT40 cells, indicating that keratinocytes may not have robust homologous recombination or DNA repair activities required for ODN-directed gene alteration.

To measure the low frequency, it is essential to develop sensitive assay systems where targeted gene alteration can be scored accurately without backgrounds. We have utilized the mutant *lacZ* system for this purpose, but other genes encoding drug-resistant markers (neomycin, puromycin) or green fluorescent protein can also be targeted. What is important is that a reporter gene has to be mutated by a site-directed mutagenesis to introduce a single-point mutation that inactivates the enzymatic activity completely. In addition, the vector must contain an additional selectable marker to enrich the cells containing the mutated reporter gene. This mutated reporter gene can be introduced to cells either by DNA transfection or retrovirus. Retroviral infection is preferable because it is more efficient than transfection and results in the integration of a single or a low copy of transgene to the chromosome. We have generated human primary keratinocytes transduced by a retrovirus containing the mutant *lacZ* in collaboration with Dr. Lorne Taichman at University of New York, Stony Brook. Using these keratinocytes, we observed a low level of gene correction, 5–10 blue cells per 10^6 cells, despite the fact that 80–90% of keratinocytes exhibited efficient nuclear uptake **(Fig. 2)**, in agreement with our previous published data ***(24)***. Therefore, it is very important to consider many other factors for successful gene repair, including DNA repair and recombination activities, status of replication and transcription, in addition to the well-known requirements like the quality and delivery of ODN to cells.

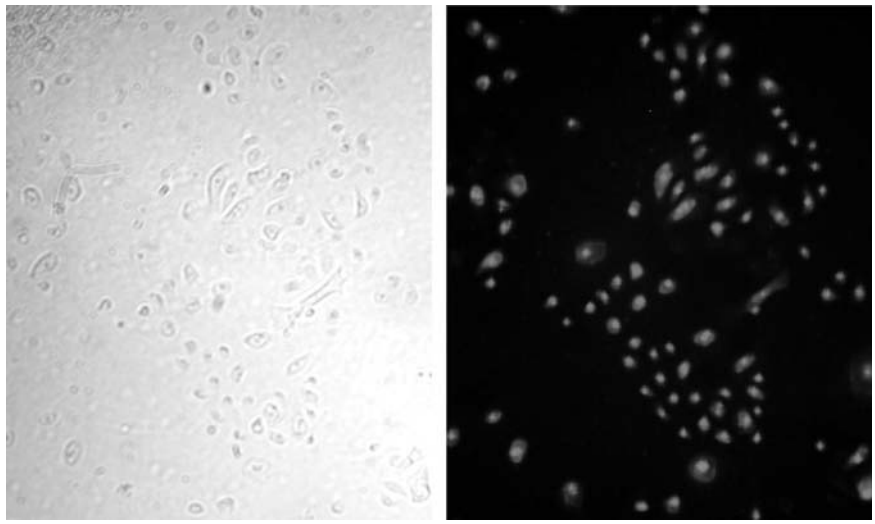


Fig. 2. Uptake of the fluorescein-conjugated oligonucleotide in the mutant *LacZ* retrovirus-transduced human primary keratinocytes.

2. Materials

2.1. Keratinocytes Isolation and Cell Culture

1. Foreskin tissue.
2. Primary human keratinocyte culture.
3. Frozen stocks of the cells stored in liquid nitrogen. These stocks should be made from cells of the lowest passage number.
4. Appropriate cell culture medium including necessary growth factors and supplements. EpiLife (Cascade Biologics, Portland, OR) calcium-free media (cat. no. M-EPI-500) and human keratinocyte growth supplement (cat. no. S-001-5) from Cascade Biologics (Portland, OR) are recommended for growing keratinocytes.
5. Phosphate-buffered saline (DPBS) without calcium or magnesium (Cambrex; cat. no. 17-512F).
6. Trypsin-Versene mixture 0.25% (Cambrex; cat. no. 17-161E).
7. Antibiotics: 100 U/mL penicillin G, 100 µg/mL streptomycin, 2.5 µg/mL amphotericin B made up in 1X DPBS.
8. 100% Ethanol.
9. Trypan blue solution: 0.4% (Cambrex; cat. no. 17-942E).
10. 10-cm Tissue culture dishes, six-well plates, and pipets of all sizes.
11. Hemacytometer.
12. Tissue culture hood.
13. Sterile cryogenic vials (Nalgene; cat. no. 5000-0020) and dimethyl sulphoxide (Sigma; cat. no. D2650).
14. Inverted microscope.
15. Sorval centrifuge.
16. Sterile 15-mL disposable centrifuge tubes.
17. Pipets for dispensing small and large volumes and sterile pipet tips.

2.2. Retroviral Infection of Keratinocytes and Characterization of Transduced Cells

1. Retroviral vectors listed below are available from Clontech (Palo Alto, CA). Constitutive retroviral vectors containing the target gene: pLNCX (cat. no. K 1060-C), pLXSN (cat. no. K 1060-B).
2. Tetracycline (Tc) regulatable vectors: *RevTet*-On (Clontech; cat. no. K 1659-1), *RevTet*-Off (Clontech; cat. no. K 1640-1).
3. Self-inactivating vector: pSIR (Clontech; cat. no. 6003-1).
4. 293-Based packaging cells: *ϕNX*-eco (ATCC; cat. no. SD-3444) and *ϕNX*-ampho (ATCC; cat. no. SD-3443).
5. QIAGEN Maxi Prep kit (cat. no. 12163).
6. CalPos Maximizer™ Transfection kit (Clontech; cat. no. K 2050-1), ProFection mammalian Transfection System Calcium Phosphate (Promega; cat. no. E 12).
7. Mammalian cell selection reagents: Neomycin (FisherBiotech; cat. no. BP673-1), Puromycin (Sigma; cat. no. 82595).
8. Phosphate-buffered saline (DPBS).
9. Polybrene solution (Sigma; cat. no. H9268).
10. Phase Lock Gel tube (PLG, Eppendorf; cat. no. E0032005.152).
11. Staining solution for the assessment of the reporter gene activity if applicable.
12. Sterile 1.5-mL disposable centrifuge tubes.
13. Gel electrophoresis equipment and reagents such as agarose, 0.5X TBE running buffer, 0.25 *M* HCl, 0.5 *N* NaOH, 50 m*M* NaOH, 10X saline sodium citrate solution, and ethidium bromide.
14. UV Stratalinker 2400 (Stratagene, La Jolla, CA).
15. Thermocycler.
16. Restriction enzymes.
17. Reagents for Southern and Northern blot assays: cell lysis buffer (100 m*M* Tris-HCl at pH 8.3–8.5, 5 m*M* EDTA, 200 m*M* NaCl, 0.2% sodium dodecyl sulfate [SDS]), phenol/chloroform/isomyl alcohol; isopropanol; Zeta-Probe GT blotting membrane (Bio-Rad; cat. no. 163754A); hybridization solution (0.25 *M* sodium phosphate, pH 7.2, and 7% SDS); washing solutions (20 m*M* sodium phosphate, pH 7.2, and 5% SDS and 20 m*M* sodium phosphate, pH 7.2, and 1% SDS); X-ray film (Kodak; cat. no. 165 1579); and hybridization apparatus Robbins Scientific, model 310).
18. Megaprime DNA labeling system (Amersham; cat. no. RPN 1606/7).
19. RNeasy Kit (Qiagen; cat. no. 74104).
20. Reagents for Western blot assay: SDS-PAGE gel containing 7% resolving gel (30% acrylamide/8% bis-acrylamide; 1.5 *M* Tris-HCl, pH 8.85–8.9; 10% SDS; 10% APs; and 5 µL TEMED) and 4% stacking gel (30% acrylamide/8% bis-acrylamide, 0.5 *M* Tris-HCl, pH 6.7; 10% SDS; 10% APS; and 5 µL TEMED).
21. Cell lysis buffer: 50 m*M* Tris-HCl at pH 7.4, 1% Na-deoxycholate, 1% Triton X-100, 0.1% SDS, 0.15 m*M* NaCl, 1.5 m*M* EDTA, and protease inhibitors (1 m*M* PMSF, 10 µg/mL leupeptine, 10 µg/mL aprotinine).
22. Laemli buffer: 50% 0.5 *M* Tris-HCl, pH 6.8; 4% SDS; 10% β-mercaptoethanol; and 40% glycerol.
23. Electro-blotting apparatus (Pharmacia Biotech).
24. TTBS buffer: 10 m*M* Tris-HCl, pH 7.4, 0.1% Tween-20, 150 m*M* NaCl.
25. Blocking solution: 3–5% bovine serum albumin (BSA) in 1X TTBS buffer.
26. BCIP/NTB tablets (Sigma; cat. no. B-5655).

2.3. Gene-Targeting Procedure

1. Several materials required for culturing keratinocytes listed previously.
2. Spectrophotometer.
3. Refrigerated tabletop centrifuge.
4. Gel electrophoresis equipment and reagents.
5. Inverted microscope.
6. Fluorescent microscope.
7. Sterile-distilled water.
8. Targeting oligonucleotides that have been purified by high-performance liquid chromatography and desalted: ODN should be diluted with sterile water or TE solution. For gene targeting experiments, the final ODN concentration should be approx 1 µg/µL.
9. Transfection reagents: FuGENE 6 (Roche; cat. no. 1 814 443).
10. Opti-MEM I medium (Gibco; cat. no. 51985-034).
11. Cover slip and glass slides.
12. Denaturing 12% acrylamide gel: 0.6 g acrylamide, 0.03 g bis-acrylamide, 2.1 g of urea, 0.5 mL 10X TBE, 2.0 mL formamide, and 0.8 mL water. This will make 5 mL of 12% acrylamide gel.
13. Acid wash solution: 1 *M* NaCl, HAc, pH 2.5.
14. Fixing solution: 1% glytaraldehyde solution (Sigma; cat. no. G-6257).
15. 3.7% Paraformaldehyde solution.
16. Vectashield mounting medium (Vector Laboratories; cat. no. H-1000).

2.4. Assessment of Correction Events

1. Several materials required for the keratinocyte cell culture and characterization of transduced keratinocytes listed are also required for assessment of correction events.
2. Appropriate selective media if selection is desired.
3. Fixing solution: 1% gluteraldehyde (Sigma; cat. no. G-6257).
4. Staining solution for assessment of reporter activity: 2 m*M* $MgCl_2$, 5 m*M* $K_3Fe(CN)_6$, 5 m*M* $K_4Fe(CN)_6$ $3H_2O$, 0.5 m*M* X-gal.
5. Materials required for the preparation of genomic DNA.
6. Material and reagents required for PCR-RFLP analysis: PCR buffer, appropriate primers, AmpliTaq Gold with GeneAmp (Roche; cat. no. N808-0247), restriction enzymes.
7. Stripping solution: 0.1X saline sodium citrate (SSC) and 0.5% SDS.
8. Monoclonal anti-β-galactosidase antibody from Promega (Madison, WI; cat. no. Z3781).
9. Anti-mouse secondary antibodies labeled with the alkaline phosphatase from Promega (Madison, WI; cat. no. S3721).

3. Methods

3.1. Keratinocytes Isolation and Cell Culture

3.1.1. Isolation of Primary Human Keratinocytes From Foreskin

1. Place the foreskin tissue in the EpiLife + HKGS medium on ice until it is processed. All procedures are performed under a laminar flow hood using sterile instruments.
2. Wash the tissue in a 5-mL antibiotic solution.
3. Remove the tissue from the antibiotic solution and place it in 100% ethanol for 1 min on ice, then wash the tissue in the antibiotic solution for an additional 10 min.
4. Mince the tissue into small pieces (as fine as possible) and transfer them to 5 mL of digestion solution.
5. Shake the tube vigorously for 30 min at 37°C.
6. Let the tube sit at room temperature for 3 min without shaking to allow tissue pieces to settle.

7. Remove the digestion solution containing keratinocytes from the tube without disturbing the tissue pieces. Transfer the cell suspension/digestion solution to a new tube placed in ice.
8. Add a 5 mL fresh digestion solution to the tissue pieces and incubate another 30 min, shaking vigorously at 37°C.
9. Repeat **steps 5–7** three times for each tissue by adding the cell suspension/digestion solution containing keratinocytes to the previously released cells kept on ice to make a total 15 mL of cell suspension.
10. Pellet keratinocytes (325*g*, 10 min, 4°C).
11. Transfer digested cells to a 10-cm plate containing the EpiLife + HKGS medium and continue to grow cells until confluent.

3.1.2. Culturing of Keratinocytes

To maintain an undifferentiated state of keratinocyte cells, cells should be kept in suspension for a minimum time. When splitting keratinocytes, work as fast as possible. If trypsinization takes less that indicated, then proceed to the next step.

1. Warm up trypsin to 37°C.
2. Aspirate all media from the 10-cm tissue culture dish.
3. Optional: wash cells with DPBS at room temperature.
4. Add 3 mL trypsin. Make sure that it covers the plate completely.
5. Incubate the plate at 37°C for exactly 5 min.
6. Look under the microscope to make sure that more than 95% of cells are round.
7. Bang the plate against the palm of you hand (or the microscope) to make sure all cells are detached from the surface.
8. Quickly add 7 mL 10% serum in 1X DPBS to the plate with cells to quench the trypsin. Pipet up and down to break any clumps of cells and resuspend cells in the plate. Work fast.
9. Transfer all 10 mL to a 15-mL Falcon tube. If more than 50% of the cells are still attached, wash cells twice with DPBS, and repeat the **steps 1–9**.
10. Centrifuge the cells for 4 min at 100*g*.
11. Aspirate the supernatant and wash cells by resuspending the pellet in a 10 mL 1X DPBS.
12. Centrifuge the cells again for 4 min at 100*g*.
13. Aspirate the supernatant and resuspend the cell pellet in the EpiLife + HKGS media. Ten milliliters is a good volume to use for resuspension. If cell counting is required, take 100 µL of cell suspension with an equal volume of Trypan blue dye. Transfer 10 µL of this mixture to the hemacytometer and count the cells present within the two big squares. To estimate the cell number, use the following equation: $X/2 \times 2 \times 10^4 = Y$ cells/mL: X is number of counted cells, 2 is the dilution factor (number of big squares used for counting), and Y is number of cells per mL.
14. Generally, cells are split 1 : 3 (also 1 : 2 or 1 : 4 are commonly used). If this is the case, transfer approx 3.3 mL of resuspended cells from the previous step to a new 10-cm plate and add approx 6 mL of the EpiLife + HKGS media.
15. Usually, cells are ready to split once a week and fed every other day. Always split them before they are completely confluent. Do not leave cells on the same plate more than 10 d.

3.1.3. Freezing of Keratinocytes

1. When cells are 80–90% confluent, trypsinize them according to the procedure described above (**Subheading 3.1.2., steps 1–12**).
2. While cells are being centrifuged, place a sterile 2-mL freezing tubes on ice.
3. Prepare a solution containing 5–10% DMSO in the EpiLife + HKGS media.

4. Resuspend cell pellets in the DMSO-containing media at the cell density of 10^6 cells/mL.
5. Transfer a 1–1.5 mL cell suspension to each tube and keep them at –20°C for about 2 h.
6. Place the tubes overnight at –80°C. The next day, store them in liquid nitrogen.

3.2. Retroviral Infection of Keratinocytes and Characterization of Transduced Cells

3.2.1. Construction of Retroviral Vector With a Reporter Gene and Production of Recombinant Virus

Recently, we published a detailed description of "Retroviral delivery of extracellular matrix genes to mammalian cells" ***(28)***. There you can find specific details regarding retroviral vectors, packaging cells, virus production, and infection; however, some important features will be briefly described here. We have used the mutant *lacZ* system, but other drug-resistant genes (neomycin, puromycin) or gene encoding green fluorescent protein can be used instead. These genes have to be mutated by a site-directed mutagenesis to introduce a single-point mutation that inactivates the enzymatic activity completely. In addition, the retroviral vector should contain an additional selectable marker to enrich the cells containing the mutated reporter gene.

1. Construct a retroviral vector containing the mutant reporter gene of choice and a selectable marker by conventional cloning procedure.
2. Perform various diagnostic restriction enzyme digests to verify the plasmid construction.
3. Perform a large-scale preparation of a recombinant retroviral vector using the QIAGEN Maxi Prep kit.
4. The *ϕNX* packaging cells are maintained in a growth medium containing Dulbecco's modified Eagle's medium/10% fetal bovine serum, 1% glutamine and splitted 1:4 or 1:5 for every 3–4 d. Good transfection efficiency is achieved when cells are 40–50% confluent at the time of transfection.
5. Prior to transfection, feed cells with fresh media.
6. Prepare the transfection mixture according to the manufacturer's instruction.
7. Add the transfection mixture dropwise onto the cells with media, gently and quickly. Then, shake the plates back and forth to distribute DNA/calcium phosphate particles evenly.
8. The next day, add 5 mL of fresh media.
9. After 24–48 h, collect the virus that has been secreted in the media.
10. Centrifuge the viral supernatant at 1000*g* for 5 min to pellet the cell debris and filter the supernatant through a 0.45-μm Millipore filter to remove cells as well. Now, the virus is ready for use. Viral supernatant can be frozen at –80°C for later use but the viral titer will decrease upon freezing and thawing.

3.2.2. Infection of Keratinocytes by Recombinant Retrovirus

1. Seed keratinocytes at 2.0×10^5 cells per 60-mm plate with 2 mL of the EpiLife + HKGS media. Keratinocytes should be in a log phase at the time of infection.
2. Add the polybrene solution at final concentration of 4 μg/mL to each plate containing 1 mL growth media.
3. Add 1 mL viral suspension to cells and continue incubation at 37°C. If necessary, titer the virus according to the procedure outlined ***(28)***. Usually, the multiplicity of infection (number of viral particles/number of cells) of 5–10 is recommended for infection.
4. After 24 h of infection, remove the virus and feed cells with 5 mL of fresh medium.
5. After 24–48 h, cells are ready to assay for viral transduction. If selection is required, add or remove antibiotics depending on the viral vector.

3.2.3. Characterization of Transduced Keratinocytes

Usually, several clones will need to be isolated and analyzed for the presence and expression of the mutant reporter gene and protein prior to the targeting experiment. Southern blot is used to determine the copy number of transgene and Northern blot and Western blot are used to detect gene expression. However, primary human keratinocytes have a limited life span (usually up to 10–15 passages when cultured in the EpiLife media), which makes the clonal analysis of keratinocytes difficult. In this case, one can pool the selected cells and characterize the gene expression by Northern blot and Western blot analysis.

3.2.3.1. Southern Blot Analysis

1. Scrape cells from the plate using a rubber policeman in a 1 mL of 1X DPBS, collect, and spin down cells at 100*g* for 7 min.
2. Lyse the cell pellets in a digestion buffer containing proteinase K (200 µg/mL final concentration) for 2–4 h at 56°C.
3. Transfer the cell lysate to the Phase Lock Gel tube, add an equal volume of equilibrated phenol/chloroform/isoamyl alcohol (25 : 24 : 1), and place the PLG tube on the shaker for 5 min with gentle rocking motion.
4. Centrifuge the tube at 16,000*g* in a tabletop centrifuge for 5 min at room temperature to separate two phases. The PLG will form the barrier between the aqueous and organic phases.
5. Add an equal volume of chloroform/isoamyl alcohol (24 : 1) to the same tube and shake gently for 5 min.
6. Centrifuge the tubes as before.
7. Carefully remove the aqueous phase and precipitate the genomic DNA by adding an equal volume of isopropanol. Mix the solution until threads form a tight knot or clump.
8. Centrifuge the tube for 1 min at maximum speed at room temperature.
9. Wash the pellet once with 70% ethanol and centrifuge it again for about 1–2 min.
10. Pour off ethanol and touch the open tube to a paper towel. Leave the tube inverted and let it air dry in this position.
11. Resuspend the DNA pellet in 50 µL dH_2O. Let it sit for 5–10 min. Pipet the sample up and down until it becomes viscous. Genomic DNA can be stored at 4°C for no longer than 2–4 mo.
12. For Southern blot, digest a 10 µg of the genomic DNA with appropriate restriction enzymes, which generate a fragment residing within the introduced reporter gene. To measure the copy number, digest the genomic DNA with the restriction enzyme (usually *Hin*dIII), which has only one site within the coding region of the mutant reporter gene.
13. Run the digested genomic DNA on a 0.7% agarose gel in a 0.5X TBE buffer at 100 volts for 1.5–2 h.
14. Depurinate the gel by soaking it in a solution containing 0.25 *M* HCl for 15 min.
15. Rinse the gel twice with the Milli-Q water.
16. Denature the gel in a solution containing 0.5 *N* NaOH for 30 min.
17. Neutralize the gel in a 10X SSC solution for 90 min.
18. Transfer DNA to the Zeta-Probe blotting membrane. Before the transfer, prewet the membrane in the Milli-Q water for 2 min and then in a 10X SSC transfer buffer. Transfer DNA to the membrane by using a vacuum transfer.
19. Crosslink the transferred DNA to the membrane (both sides) by using a UV Stratalinker 2400.
20. Prehybridize the membrane with immobilized nucleic acids in a buffer containing 0.25 *M* sodium phosphate, pH 7.2, and 7% SDS at 65°C for 30–45 min.

21. Remove the prehybridization solution and replace it with the same buffer containing the denatured radiolabeled probe (95°C for 5 min). The radiolabeled DNA fragment can be prepared using Megaprime DNA labeling system from Amersham. Usually, hybridization is carried out in a solution containing 1×10^6 cpm/mL of hybridization solution.
22. Hybridize the membrane for 16–18 h at 65°C with gentle rotation using a hybridization incubator.
23. Wash the membrane two times, 30–60 min each, in a buffer containing 20 m*M* sodium phosphate, pH 7.2, and 5% SDS at 65°C. Adjust the length of wash time according to the hybridization signal.
24. Wash the membrane once for 30–60 min in a buffer containing 20 m*M* sodium phosphate, pH 7.2, and 1% SDS at 65°C.
25. Detect the hybridization signal by autoradiography using X-OMAT AR film (*see* **Note 1**).

3.2.3.2. Northern Blot Analysis

1. Total RNA can be isolated with RNeasy Kit from QIAGEN. This kit provides a fast and simple method for the preparation of up to 100 µg of high-quality total RNA from mammalian cells.
2. For Northern blot, electrophorese 20 µg of total RNA through a 1.0% denaturing agarose gel containing formamide.
3. Rinse the gel twice with the Milli-Q water.
4. Soak the RNA gel in 50 m*M* NaOH for 5–15 min depending on the mRNA size.
5. Perform the **steps 19–25** outlined in the **Subheading 3.2.3.1.** for Southern blot analysis.

3.2.3.3. Western Blot Analysis

1. Begin by preparing a SDS-PAGE gel.
2. While preparing the gel, digest the cells in lysis buffer.
3. Collect the cell lysate and centrifuge it for 10 min at 8000–10,000*g*. Supernatant contains proteins.
4. Once the stacking gel is polymerized, prepare samples by mixing three parts lysate with one part of Laemli buffer.
5. Heat samples for 3–5 min at 95°C and cool them to room temperature.
6. Load the samples and run the gel at 15 mA. When proteins reach the resolving gel, then turn up the current to 25 mA. The slower you run the gel, the better resolution will be.
7. Transfer the proteins in the gel to the nitrocellulose membrane by using an electroblotting apparatus. The transfer is run at constant current depending on the size of the gel (approx 0.8 mA/cm) and the size of the protein of interest. For low-molecular weight (MW) proteins (10–50 kDa) 40–50 min is enough. For high-MW proteins (60–200 kDa), 1 h 20 min is enough.
8. Once the transfer is complete, put the nitrocellulose membrane into 1X TTBS buffer for 1–2 min for washing. To prevent nonspecific binding, incubate the membrane in the blocking solution for 30 min at room temperature.
9. Pour off the blocking solution and add fresh TTBS solution containing 3–5% bovine serum albumin with a proper dilution of primary antibodies. Incubation time can vary from 1 h to overnight at 4°C (*see* **Note 2**).
10. After incubation, pour off the primary antibodies and add enough amount of TTBS solution to cover the membrane. Rock the solution for 5 min at room temperature. Repeat the wash twice.
11. Add the blocking solution containing a proper dilution of secondary antibodies and incubate for 1 h at room temperature (*see* **Note 3**).
12. Wash the membrane in the TTBS buffer three times 5 min each.
13. Detect secondary antibodies with a proper substrate, such as BCIP/NTB tablets.

3.3. Gene-Targeting Procedure

3.3.1. Characterization of ODNs

The ODNs to be used in the targeting experiments are perhaps the most essential reagents, so it is very important to check ODNs for their size and purity. Usually, the oligonucleotides are synthesized by the standard phosphoramidite procedure and purified by HPLC. The quality of ODN should be checked by electrophoresis on a denaturing gel prior to the targeting experiment. A minigel (5 mL) is convenient for a quick check.

1. Prepare 5 mL of 12% denaturing acrylamide gel.
2. Stir the solution at low heat until all components are completely dissolved.
3. Cool the solution to room temperature by stirring in a cool water bath.
4. Add a 21.4 µL of 25% ammonium persulfate and a 7.2 µL TEMED.
5. Stir and pour into a minigel unit. Allow the gel to solidify about 1 h.
6. Measure the concentration of ODN that is going to be tested. Prepare a 20 ng/µL stock solution.
7. Take 5 µL of sample and combine with 5 µL of the formamide loading dye.
8. Heat the sample at 95°C for 5 min and quickly cool it on ice for at least 2 min.
9. Wash out the wells to remove excess acrylamide prior to loading of the sample.
10. Load a 10-bp ladder DNA first and then load the sample.
11. Run the gel at 200 V until the Bromophenol dye runs off. This takes about 1.5 h.
12. Stain with the ethidium bromide solution for 5 min and take a picture.

3.3.2. Transfection of Primary Human Keratinocytes With ODN

1. The day prior to transfection, split cells (**Subheading 3.1.2.**) and plate them into a 1 mL/well into a 12-well plate (24- and 6-well plates can also be used) at cell density of 1.0×10^5 cells/well. For efficient transfection, it is very important to grow cells to 70% confluency.
2. Add 2–3 µg ODN to Opti-MEM I media, enough to make a 100 µL total volume. Set aside.
3. In a separate tube, add 6 µL of FuGENE 6 reagent to Opti-MEM I media to make a final volume of 100 µL. Add the FuGENE 6 reagent directly to the media not touching the wall of the tube. It is important that the ratio of DNA to FuGENE 6 should not exceed 2:3 (µg:µL).
4. Add the diluted liposome solution dropwise to the ODN solution. Mix it gently and allow it to stand for 15 min at room temperature.
5. During incubation, replace the media of cell-containing wells with a 1 mL of fresh media.
6. Add the FuGENE 6–ODN complex dropwise to the plate. Start to add the mixture from the middle of the plate and move outwards in a spiral fashion. Evenly distribute the mixture by shaking the plate side to side and back and forth.
7. After 6 h, aspirate the solution from the transfected cells and refeed cells with 1 mL fresh media. FuGENE 6 is not toxic for keratinocytes, so it can remain on cells for 24 h.
8. Forty-eight hours later, cells are ready for the analysis of gene correction (*see* **Subheading 3.4.**).

3.3.3. ODN Uptake in Primary Human Keratinocytes

1. The day before the transfection, trypsinize keratinocytes as described in the **Subheading 3.1.2.** and seed them in a flame-sterilized cover slip placed inside of a six-well plate.
2. For the visualization of ODN uptake, use the ODN that has been synthesized to incorporate a fluorescent group at the 5′ end of ODN (fluorescein, Cy5, Cy3, or rhodamine derivatives of phosphoramidite). Transfect an appropriate amount of fluorescent ODN and liposomes to cells (*see* **Subheading 3.3.2.**).

3. Incubate at 37°C for 3–6 h. Make sure that the solution covers the cover slip completely during the incubation time.
4. Wash the cover slip twice with 1X DPBS, once with the acid wash solution and once with 1X DPBS.
5. Fix the cells in a 1% glytaraldehyde solution in 1X DPBS for 5 min at 4°C or in a 3.7% paraformaldehyde solution for 20 min at room temperature.
6. Aspirate the fixing solution and wash the slides twice with 1X DPBS.
7. Take the cover slips out of the plate and let them dry.
8. Put one drop of Vectashield mounting medium in the center of a slide.
9. Place the cover slip on the slide with cells in between and make sure that no air bubbles are trapped.
10. Seal the edges of the cover slip with nail enamel and let it dry.
11. Slides are ready for visualization under the florescent microscope. Store slides at –20°C.

3.4. Assessment of Targeted Events

3.4.1. Histochemical Staining of Targeted Keratinocytes for the Presence of Active β-Galactosidase

The following procedure is used to detect the ODN-directed gene correction in keratinocytes, which have integrated copies of the mutant *lacZ* in their chromosome by retroviral transduction (*see* **Subheading 3.2.**). When the ODN corrects this mutant *lacZ* in keratinocytes, the enzymatic activity of the mutant β-galactosidase is restored and corrected cells are detected by the X-gal staining. If the mutated drug resistant gene ($puro^S$ or neo^S) was used instead, then the total number of surviving clones in the appropriate antibiotic selection media will represent the successful targeting events.

1. When cells are ready for analysis, remove the plate from the incubator and aspirate the media and wash cells three times with 1X DPBS.
2. Add 1 mL fixing solution (1% gluteraldehyde in 1X DPBS) gently and incubate for 5 min at 4°C.
3. Aspirate the fixing solution and wash cells three times with 1 mL of 1X DPBS at room temperature.
4. Depending on the size of the plate, add 1 mL (for 12-well plate) or 2 mL (for six-well plate) of a staining solution.
5. Incubate overnight at 37°C in a bacterial incubator.
6. Rinse the wells of plate with distilled water several times.
7. After the last rinse, place just enough water on cells to view under a light microscope. Corrected cells are stained blue.

3.4.2. Analysis of Genomic DNA by RCP-RFLP Analysis

If the mutated drug resistant gene ($puro^S$ or neo^S) was used for targeting, the surviving clones ($puro^r$ or neo^r) can be selected in the medium containing an appropriate antibiotic. Each clone must be picked individually by using cloning cylinders. Genomic DNA can be isolated from these clones and used to verify for the gene correction by the PCR-RFLP based assay, Southern Blot (*see* **Subheading 3.2.3.1.**) and DNA sequencing.

1. Perform the **steps 1–19** outlined in **Subheading 3.2.3.1.** to isolate the genomic DNA from cloned cell lines.
2. Prepare the PCR as follows: genomic DNA lysate (from 1 to 5 µL), 10X PCR buffer (5 µL), dNTPs mix (1 µL), 10 µ*M* Primer 1 and Primer 2 (1 µL each), AmpliTaq Gold polymerase

(0.25 µL). Adjust the reaction volume with dH_2O up to 50 µL. To increase the sensitivity of detection, the PCR product can also be radio labeled. In this case, add a 1 µL of [α-^{32}P] dCTP (3000 Ci/mmol) to the PCR reaction mixture.

3. Perform the PCR using an appropriate cycle regime.
4. After the amplification is finished, take out an 8 µL of the PCR product for the restriction digestion with the appropriate restriction enzyme in a total volume of 10 µL.
5. Incubate the reaction for 3–5 h at appropriate temperature.
6. Prepare a mini-acrylamide gel (5 mL) and run the digested samples together with uncut controls. Load the entire digested samples.
7. Stain gel with the ethidium bromide solution and take a picture.
8. If a radioactive PCR was used, transfer a 1–2 µL of the radiolabeled product to the scintillation vial and count the radioactivity with a scintillation counter.
9. Usually a 1 µL of the PCR mixture is sufficient for the restriction enzyme digestion.
10. Prepare a mini-acrylamide gel and run the digested samples as before. Load 2–5 µL per well.
11. Fix the gel in a 30% methanol/7% acetic acid solution for 20 min at room temperature.
12. Dry the acrylamide gel in a gel dryer (3–5 h at 50°C) and expose it to an X-ray film for about 10 min.

4. Notes

1. If reprobing is desired, do not allow the Zeta-Probe GT membrane to dry between hybridization. The membrane should be stripped as soon as possible after autoradiography in large volume of solution containing 0.1X SSC/0.5% SDS at 95°C for 30 min.
2. To detect the β-galactosidase, we recommend a monoclonal anti-β-galactosidase antibody from Promega and anti-mouse secondary antibodies labeled with the alkaline phosphatase from Promega with dilution 1 : 5000 and 1 : 7500, respectively.
3. Incubation conditions are important, too long incubation can give nonspecific binding. One hour incubation at room temperature is typically enough.

References

1 Fuchs, E., Merrill, B. J., Jamora, C., and Das Gupta, R. (2001) At the roots of a never-ending cycle. *Dev. Cell* **1,** 3–25.
2 Gallico, G., O'Connor, N. E., Compton, C. C., Kehinde, O., and Green, H. (1984) Permanent coverage of large burn wounds with autologous cultured human epithelium. *N. Engl. J. Med.* **311,** 448–451.
3 Watt, F. M. (2000) Epidermal stem cells as targets for gene transfer. *Hum. Gene Ther.* **11,** 2261–2266.
4 Lavker, R. M. and Sun, T. T. (2000) Epidermal stem cells: properties, markers, and location. *Proc. Natl. Acad. Sci. USA* **97,** 13,473–13,475.
5 Morgan, J. R., Barrandon, Y., Green, H., and Mulligan, R. C. (1987) Expression of an exogenous growth hormone gene by transplantable human epidermal cells. *Science* **237,** 1476–1479.
6 Mulligan, R. C. (1993) The basic science of gene therapy. *Science* **260,** 926–932.
7 Miller, D. G., Adam, M. H., and Miller, A. D. (1990) Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. *Mol. Cell. Biol.* **10,** 4239–4242.
8 Miller, A. D. (1990) Retrovirus packaging cells. *Hum. Gene Ther.* **1,** 5–11.
9 Palmer, T. D., Roseman, G. J., Osborne, W. R. A., and Miller, A. D. (1991) Genetically modified skin fibroblasts persist long after transplantation but gradually inactivate introduced genes. *Proc. Natl. Acad. Sci. USA* **88,** 1330–1334.

10 Temin, H. M. (1990) Safety considerations in somatic gene therapy of human disease with retrovirus vectors. *Hum. Gene Ther.* **1,** 111–123.
11 Donahue, R. E., Kessler, S. W., Bodine, D., McKonaugh, K., Dunbar, C., Goodman, S., et al. (1992) Helper virus induced T cell lymphoma in non-human primates after retroviral mediated gene transfer. *J. Exp. Med.* **176,** 1125–1135.
12 Kozarsky, K. F. and Wilson, J. M. (1993) Gene therapy: adenovirus vectors. *Curr. Opin. Gene Dev.* **3,** 499–503.
13 Capecchi, M. R. (1989) Altering the genome by homologous recombination. *Science* **244,** 1288–1292.
14 Wang, G., Seidman, M. M., and Glazer, P. M. (1996). Mutagenesis in mammalian cells induced by triple helix formation and transcription-coupled repair. *Science* **271,** 802–805.
15 Goncz, K. K., Kunzelmann, K., Xu, Z., and Gruenert, D. C. (1998) Targeted replacement of normal and mutant CFTR sequences in human airway epithelial cells using DNA fragments. *Hum. Mol. Genet.* **7,** 1913–1919.
16 Culver, K. W., Hsieh, W. T., Huyen, Y., Chen, V., Liu, J., Khripine, Y., et al. (1999) Correction of chromosomal point mutations in human cells with bifunctional oligonucleotides. *Nat. Biotechnol.* **17,** 989–993.
17 Russell, D. W. and Hirata, R. K. (1998) Human gene targeting by viral vectors. *Nat. Genet.* **18,** 325–330.
18 Igoucheva, O., Alexeev, V., and Yoon, K. (2001) Targeted gene correction by small single-stranded oligonucleotides in mammalian cells. *Gene Ther.* **8,** 391–399.
19 Yoon, K., Igoucheva, O., and Alexeev, V. (2002) Expectations and reality in gene repair. *Nat. Biotechnol.* **20,** 1197–1198.
20 Alexeev, V., Igoucheva, O., and Yoon,K. (2002) Simultaneous targeted alteration of the tyrosinase and c-kit genes by single-stranded oligonucleotides. *Gene Ther.* **9,** 1667–1675.
21 Pierce, E. A., Liu, Q., Igoucheva, O., Omarrudin, R., Ma, H. C., Diamond, S., et al. (2002) Oligonucleotide-directed single-base DNA alterations in mouse embryonic stem cells. *Gene Ther.* **10,** 24–33.
22 Felgner, P. L. and Ringold, G. M. (1989) Cationic liposome-mediated transfection. *Nature* (London) **337,** 387–388.
23 Nestle, F. O., Mitra, R. S., Bennett, C. F., Chan, H., and Nickoloff, B. J. (1994) Cationic lipid is not required for uptake and selective inhibitory activity of ICAM-1 phosphorothioate antisense oligonucleotides in keratinocytes. *J. Invest. Dermatol.* **103,** 569–575.
24 Santana, E., Peritz, A. E., Iyer, S., Uitto, J., and Yon, K. (1998) Different frequency of gene targeting events by the RNA-DNA oligonucleotide among epithelial cells. *J. Invest. Dermatol.* **111,** 1172–1177.
25 Mirmohammadsadegh, A., Maschke, J., Basner-Tschakarjan, E., Bar, A., and Hengge, U. R. (2002) Reaction of keratinocytes to exogenous DNA. *Cells Tissues Organs* **172,** 86–95.
26 van der Steege G., Schuilenga-Hut, P. H., Buys, C. H., Scheffer, H., Pas, H. H., and Jonkman, M. F. (2001) Persistent failures in gene repair. *Nat. Biotechnol.* **19,** 305–306.
27 Igoucheva, O., Peritz, A. E., Levy, D., and Yoon, K. (1999) A sequence-specific gene correction by an RNA-DNA oligonucleotide in mammalian cells characterized by transfection and nuclear extract using a lacZ shuttle system. *Gene Ther.* **6,** 1960–1971.
28. Yoon, K. and Alexeev, V. (2000) Retroviral delivery of EMC genes to cells. *Methods Mol. Med.* **139,** 197–208.

31

Promoter Analysis in the Human *SPRR* Gene Family

David F. Fischer and Claude Backendorf

Summary

Protocols to study the regulation of a conserved multigene family (*SPRR* genes) during calcium-induced differentiation of cultured normal human keratinocytes (NHKs) are provided. Transfection of promoter-reporter (CAT or luciferase) constructs, combined with promoter truncation, can be used to study the expression of individual *SPRR* genes and to identify specific transcription factor binding sites. Interaction of regulatory factors with these control elements can be visualized and quantified by electrophoretic mobility shift analysis. Inclusion of specific antibodies in these experiments will identify the transcription factors involved in the observed mobility shift (supershift). A competitive electrophoretic mobility shift analysis, that is well suited to study the differential regulation of various *SPRR* members, is also described. These methods should be applicable to the study of other multigene families regulated during keratinocyte terminal differentiation.

Key Words:

SPRR gene family; CAT; luciferase; competitive EMSA; supershift; differential regulation; regulatory promoter elements; keratinocyte; terminal differentiation; epidermal barrier adaptation.

1. Introduction

The cornified cell envelope (CE), synthesized at late stages of keratinocyte terminal differentiation, constitutes the major determinant of the protective barrier, which is provided by stratified squamous epithelia to the organism. It is composed of structural proteins, including involucrin, loricrin, late envelope precursors (LEPs), and small proline-rich proteins (*SPRR*s), all encoded by genes localized on human chromosome 1q21 ***(1,2)***. *SPRR*s constitute a gene family of 11 individual members. Although the proteins are highly conserved, the corresponding genes are differentially regulated in various epithelia and respond to various physiological and environmental stimuli. *SPRR* protein dosage appears to be important for modulating the biomechanical properties of squamous epithelia and for establishing an adaptive barrier function, which is in constant balance with the outside milieu ***(3)***. The protocols provided in this chapter are especially well suited to study the differential regulation of highly conserved members of large multigene families.

From: *Methods in Molecular Biology, vol. 289:*
Epidermal Cells: Methods and Protocols
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

2. Materials

2.1. Cultures of Normal Human Keratinocytes

Primary human keratinocytes and keratinocyte serum-free culture medium can be purchased from Gibco™ (Invitrogen Corporation, San Diego, CA):

1. Primary human keratinocytes (Gibco™: cat. no. 12332-011).
2. Defined keratinocyte–SFM (Gibco™; cat. no. 10744-019) (storage 4°C; 3 mo).
3. Dulbecco's modified Eagle's medium (DMEM; Gibco™; cat. no. 12800). Can be stored at 4°C for 6 mo.
4. Bovine calf serum (defined/iron-supplemented) (Hyclone, Logan, UT; cat. no. SH30072.03). Can be stored at –20°C for several years).
5. HiCal medium: DMEM + 8% bovine calf serum (can be stored at 4°C for 3 wk).

2.3. Plasmid Preparations

Qiagen Plasmid Midi Kit (Qiagen N.V., Venlo, The Netherlands; cat. no. 121143).

2.3. Transfection of Reporter Plasmids

1. CAT basic vectors: pBA-cat, pBLcat5 (available from C. Backendorf, University of Leiden).
2. luciferase basic vector: pGL3-basic vector (Promega, Madison, WI; cat. no. E1751).
3. SPRR-reporter fusion plasmids as described in the following publications (**refs. *7–9***; available from C. Backendorf, University of Leiden).
4. Polystyrene tubes with cap (Greiner Bio-One Inc., Longwood, FL; cat. no. 155180).
5. DMEM (w/o calcium) (Gibco™; cat. no. 21068). Can be stored at 4°C for 6 mo).
6. DOTAP liposomal transfection reagent (Roche Applied Science, Basel, Switzerland; cat. no. 1811177), Can be storaed at 4°C for 1 yr).
7. Phosphate-buffered saline (PBS; Gibco™; cat. no. 14190). Can be stored at room temperature (RT) for 1 yr).

2.4. CAT Assay

1. 20X TBS: 500 m*M* Tris-HCl, pH 7.4, 2.74 *M* NaCl, 100 m*M* KCl, 14 m*M* $CaCl_2$, 10 m*M* $MgCl_2$. Can be stored at RT for 1 mo.
2. 0.25 *M* TRIS-HCl, pH 7.8. Can be stored at 4°C for several months.
3. CAT assay buffer: 400 m*M* Tris-HCl, pH 7.8, 100 m*M* KCl, 24 m*M* $MgCl_2$ (can be stored at –20°C for 1 yr).
4. 5 m*M* Coenzyme A in 0.25 m*M* Tris-HCl, pH 7.8 (Sigma-Aldrich, St. Louis, MO; cat. no. C3144). Aliquot and store at –80°C for up to 1 yr.
5. 100 m*M* ATP (Roche Applied Science; cat. no. 519979), dissolve in 0.1 *M* Tris-base, aliquot, and store at –20°C for at least 1 yr.
6. 100 mg/mL chloramphenicol (Sigma; cat. no. C0378) (in ethanol). Can be stored at –20°C for several years.
7. ^{3}H Na-acetate (5 mCi/mL) (ICN Radiochemicals, Irvine, CA; cat. no. 012200405). Use as 1 : 10 dilution in ethanol. Can be stored at 4°C for 1 yr.
8. *S*-acetyl coenzyme A synthetase (2.5 mg/mL; Sigma; cat. no. A1763). Store at –20°C for up to 1 yr.
9. Econofluor-2 (Perkin Elmer Life Science, Boston, MA; cat. no. 6NE9699).

2.5. Luciferase Assay

1. 5X Luciferase lysis buffer (Promega; cat. no. E1531): 125 m*M* Tris-HCl, pH 7.8; 10 m*M* DTT, 10 m*M* 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′,-tetraacetic acid (CDTA), 50% glycerol, 5% Triton X-100 (storage at –20°C for up to 1 yr).

2. CDTA (Sigma; cat. no. D0922). Can be stored at RT for several years.
3. Luciferase assay reagent (Promega; cat. no. E1483): 20 m*M* Tricine, 1.07 m*M* $(MgCO_3)_4$ $Mg(OH)_2 \cdot 5H_2O$, 2.67 m*M* $MgSO_4$, 0.1 m*M* ethylene diamine tetraacetic acid (EDTA), 33.3 m*M* DTT, 270 µ*M* coenzyme A, 470 µ*M* luciferin, 530 µ*M* ATP. Aliquot and store at –80°C for up to 1 yr.
4. Coenzyme A (Sigma; cat. no. C3144). Store at –20°C for up to 1 yr).
5. D-luciferin synthetic (Sigma; cat. no. L9504). Store at –20°C for up to 1 yr).
6. Recombinant firefly luciferase (Promega; cat. no. E1701 or Sigma; cat. no. L9506). Store at –20°C for up to 1 yr.
7. Bio-Rad Protein Assay Dye Reagent Concentrate (Biorad Laboratories Inc., Hercules, CA; cat. no. 500-0006). Store at 4°C for several years).

2.6. Nuclear Extracts

1. Buffer A: 10 m*M* HEPES, pH 7.9, 10 m*M* KCl, 0.1 m*M* EDTA, 0.1 m*M* EGTA, 1 m*M* DTT, 0.5 m*M* PMSF. Can be stored at –20°C for up to 1 yr).
2. Buffer C: 20 m*M* HEPES, pH 7.9, 0.4 *M* NaCl, 1 m*M* EDTA, 1 m*M* EGTA, 1 m*M* DTT, 1 m*M* PMSF.
3. Nonidet P-40 (Sigma; cat. no. N6507, recently replaced by Igepal Ca-630, cat. no. I 3021). Can be stored at RT for several years.

2.7. Electrophoretic Mobility Shift Assay (EMSA; Bandshift Analysis)

1. 10X Kinase buffer: 500 m*M* Tris-HCl, pH 7.5, 100 m*M* $MgCl_2$, 50 m*M* DTT (storage conditions: –20°C for up to 1 yr).
2. Urea loading buffer: 10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, 6 *M* urea, 15% Ficoll 400, 0.25% Bromophenol blue, 0.25% xylene cyanol FF (can be stored at RT for up to 1 yr).
3. Ficoll 400 (Sigma; cat. no. F4375). Store at RT.
4. T4 polynucleotide kinase (10 U/µL, Invitrogen; cat. no. 18004). Store at –20°C for up to 1 yr.
5. Redivue γ-^{32}P-ATP (10 mCi/mL, 5000 Ci/mmol, Amersham Biosciences Corp., Piscataway, NJ; cat. no. AA0018). Store at 4°C for up to 1 mo.
6. Criterion™ TBE-Urea gels (15% acrylamide; Bio-Rad; cat. no. 345-0091). Store at RT for several months.
7. Elution buffer: 0.1 *M* Na–acetate, pH 8.0. Can be stored for 6 mo at 4°C.
8. Eppendorf micropestles (Eppendorf AG, Hamburg, Germany; cat. no. 022365622).
9. 4.5 mL Conical PS-tubes (Greiner, cat. no. 116101).
10. Octadecyl (C_{18}) Zorbax LP 100/40 C_{18} (Sigma; cat. no. S7421). Can be stored at RT for several years.
11. Actonitrile (J.T. Baker Chemicals B.V., Phillipsburg, NJ; cat. no. 8004). Can be stored at RT for several years.
12. Oligo-annealing buffer: 10 m*M* Tris-HCl, pH 7.5, 100 m*M* KCl, 1 m*M* $MgCl_2$. Can be stored at –20°C for up to 1 y).
13. 30% Acrylamide/bis (Bio-Rad; cat. no. 161-0154). Can be stored at 4°C for several months.
14. 5X TBE buffer: 0.45 *M* Tris-borate, 10 m*M* EDTA. Can be stored at RT for several months.
15. 5X TGE buffer: 125 m*M* Tris, 950 m*M* glycine, 5 m*M* EDTA. Can be stored at RT for several months.
16. Poly(dI-dC)•poly(dI-dC) (Amersham; cat. no. 27-7880) stock solution: 1 µg/µL in 10 m*M* Tris-HCl, pH 7.5, 0.1 m*M* EDTA, 50 m*M* NaCl; heat 5 min at 45°C. Store at –20°C for up to 1 yr).

2.8. Commercial Antibodies Used in Supershift Analysis

All antibodies are from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA):

1. c-Jun (N) (cat. no. sc-45).
2. JunB (N-17) (cat. no. sc-46).
3. JunD (329) (cat. no. sc-74).
4. c-Fos (4) Cat. (No. sc-52).
5. FosB (102) (cat. no. sc-48).
6. Fra-1 (R-20) (cat. no. sc-605).
7. Fra-2 (Q-20) (cat. no. sc-604).
8. ATF-2 (C-19) (cat. no. sc-187).

3. Methods

3.1. Transfection of Cultured Human Keratinocytes With Promoter-Reporter Fusion Plasmids

1. Primary human keratinocytes can be cultured to confluency in 6-cm dishes in defined keratinocyte serum-free medium according to the instructions of Gibco™. Promoter CAT or promoter luciferase reporter plasmid (*see* **Note 1**) can be purified with the Qiagen plasmid purification kits, which provide good-quality DNA for subsequent transfections.
2. Assemble (per 6-cm culture dish to be transfected) the following in a sterile 12-mL polystyrene tube (*see* **Note 2**): 0.5 mL DMEM (w/o calcium) and 5 µg of reporter plasmid.
3. Mix and add dropwise 25 µL DOTAP transfection reagent.
4. Mix gently (do not vortex) and incubate at room temperature for 20 min.
5. Wash the keratinocyte dishes twice with 5 mL of DMEM (w/o calcium) and add 0.5 mL of the same medium to each dish.
6. Distribute the transfection mix (±0.5 mL) over each dish and incubate for 2 h in a humidified CO_2 incubator, while occasionally rocking the culture dishes (*see* **Note 3**).
7. Discard the transfection mix and wash each dish twice with PBS.
8. Add 5 mL HiCal medium (calcium concentration: 1.8 m*M*) and induce keratinocyte differentiation for 24 to 48 h in a CO_2 incubator.

3.2. Preparation of CAT Extracts

1. Wash the transfected cells once with cold TBS (1X) and add 0.5 mL TBS to each dish.
2. Scrape the cells with a rubber policeman and transfer to a microfuge tube. Centrifuge at 3300*g* for 5 min at 4°C.
3. Discard the supernatant and resuspend the cell pellet in 100 µL 0.25 *M* Tris-HCl, pH 7.8.
4. Place the tube on ice and sonicate with a total energy of 35 Joules (~10 s; *see* **Note 4**).
5. Incubate 10 min at 65°C (*see* **Note 5**), microfuge at 16,000*g* for 10 min at RT.
6. Transfer the supernatant to a new Microfuge tube.
7. Determination of protein concentration ***(7–9)***: dilute 5 µL of lysate in 95 µL H_2O and measure the OD at 230 and 260 nm. The protein concentration in the lysate can than be deduced from the following formula:

$$c\ (\mu g/mL) = (187\ OD_{230} - 81.7\ OD_{260}) \times 20$$

8. Store the lysate in aliquots at –20°C. Do not freeze/defreeze repeatedly.

3.3. CAT Quantification (see Note 6)

1. Assemble in a Microfuge tube the following labeling mix (sufficient for 20 reactions): 200 µL H_2O, 125 µL CAT assay buffer, 40 µL Coenzyme A, 15 µL ATP, 10 µL NaAc, 7 µL chloramphenicol, 2 µL ^3H NaAc, and 5 µL *S*-acetyl CoA synthetase.

2. Incubate 30 min at 37°C.
3. Add 5 µg of cell lysate to a scintillation vial and adjust the total volume to 40 µL with 0.25 *M* Tris-HCl, pH 7.8.
4. Add 20 µL of preincubated labeling-mix. Mix well by pipetting several times.
5. Add carefully 1.5 mL Econofluor Scintillation fluid (the phases should not mix).
6. Count the radioactivity overnight in a scintillation counter on the ^{3}H channel. Each vial should be counted overnight approx 15–20 times for 1 min to obtain a good reaction curve.
7. The CAT activity from each sample can be determined by calculating the slope in the linear range of the reaction curve.

3.4. Preparation of Luciferase Extracts

1. Wash the transfected cells twice with PBS.
2. Add 500 µL Luciferase Lysis buffer, incubate at room temperature for 1 min, and scrape-off the cells with a rubber policeman. Homogenize the lysate by pipetting several times up and down, and transfer to a microfuge tube.
3. Sonicate the cells with a total energy of 35 J (*see* **Note 5**), centrifuge at 16,000*g* for 5 min, and collect the supernatant.
4. Perform the luciferase assay immediately after collecting the cell lysates (**Subheading 3.6.**; *see* **Note 7**). Measure the protein concentration with the Bradford method ***(8)*** by using a 1 : 5 dilution of the Bio-Rad Protein Assay Dye reagent. Read optical density at 595 nm.

3.5. Combined CAT–Luciferase Extracts

If both CAT and luciferase activity have to be determined from the same lysate (one reporter can for instance function as an internal control; *see also* **Note 1**), the following procedure should be used:

1. Collect the cells in 100 µL 0.25 *M* Tris-HCl, pH 7.8, as described in the CAT procedure (**Subheading 3.2.**).
2. After sonification (35 J; **Note 5**), distribute the sample over two Microfuge tubes.
3. The CAT sample is incubated 10 min at 65°C (**Note 4**) and further processed as described in **Subheading 3.2.**
4. The luciferase sample is supplemented with 1 volume Luciferase Lysis buffer, homogenized by pipetting several times and centrifuged at 16,000*g* for 5 min, and the supernatant is collected (**Note 7**).

3.6. Luciferase Quantification

Add 10 µL lysate (in general 10–50 µg total protein) to 100 µL Luciferase Assay Reagent, mix, and read light emission in luminometer (e.g., Berthold Lumat luminometer). Perform several successive readings (at 10-s intervals) and use the average value for calculating luciferase activity. Commercially available recombinant firefly luciferase can be used as a standard.

3.7. Preparation of Nuclear Extracts (According to ref. 9)

1. Confluent cultures of proliferating or differentiating keratinocytes (*see* **Subheading 3.1.**), grown on 10-cm dishes, are washed twice with ice-cold PBS (or Tris-buffered saline).
2. Add 1 mL ice-cold PBS (or Tris-buffered saline) to the dish, scrape the cells with a rubber policeman, and transfer to a microfuge tube.
3. Spin 15 s at 16,000*g* at 4°C and discard the supernatant. The following handlings should be performed in a cold room at 4°C.

4. Add 400 µL cold buffer A to the pellet and resuspend by gently pipetting in a yellow tip.
5. Allow the cells to swell for 15 min on ice.
6. Add 25 µL of a 10% (vol/vol) solution of NP40, vortex vigourously for 10 s, and centrifuge at 16,000*g* for 20 s. The supernatant can be either discarded or stored at –20°C for RNA isolation (refer to **ref. *9***).
7. Wash the pellet once with 200 µL buffer A and resuspend in 100 µL buffer C.
8. Rock the tube vigourously for 15 min at 4°C by using an orbital microtube mixer.
9. Centrifuge for 5 min at 16.000*g*.
10. Transfer the supernatant to a new Microfuge tube (do not disturb the pellet). Aliquot in portions of 10 µL and store at –80°C.
11. The protein concentration is determined with the 230/260 spectrophotometric approach described in **Subheading 3.2.**

3.8. EMSA

3.8.1. Preparation of Radiolabeled Double-Stranded DNA Probes (see ***Note 8***)

1. Synthetic single-stranded oligonucleotides that contain the consensus sequence of a specific transcription recognition site (refer to **Table 1**) flanked on each side by at least three additional nucleotides are 5′-labeled with T4 polynucleotide kinase and γ-^{32}P-ATP, purified by denaturing gel electrophoresis and reverse-phase chromatography, and finally annealed to unlabelled complementary strand.
2. Assemble the following labeling mixture: 5 µL H_2O, 2 µL oligonucleotide (1 pmol/µL), 1.5 µL 10X kinase buffer, 6 µL γ-^{32}P-ATP, and 0.5 µL T4 polynucleotide kinase.
3. Incubate at 37°C for 30 min, add 5 µL urea loading buffer, and run on a 15% TBE-Urea slab gel until the bromophenol dye has run approx half of the gel.
4. Expose the gel to autoradiographic film (10–20 s) and excise the radioactive band with a scalpel and transfer to microfuge tube.
5. Break up carefully the gel band with a dispensable micropestle (Eppendorf) and suspend in 200 µL elution buffer (0.1 *M* Na–acetate, pH 8). Incubate overnight at 60°C.
6. Prepare an octadecyl (C18) column in a blue pipet tip as follows: push a small plug of glasswool into the tip with a Pasteur pipet and add approx 2-mm^3 C_{18} silica gel on top of the glasswool. Put the pipet tip in an appropriate centrifuge tube (the conical 4.5-mL PS tubes from Greiner work well with most blue tip brands). Wash the column with 200 µL ethanol and twice with 200 µL H_2O by centrifuging at 100*g* for 1 min.
7. After an overnight incubation at 60°C, centrifuge the Microfuge tube with the labeled oligonucleotide for 10 min at 16,000*g* and transfer the supernatant to the C_{18} column. Add 100 µL elution buffer to the gel-band pellet, centrifuge, and add the supernatant also to the C_{18} column.
8. Centrifuge the C_{18} column (1 min, 100*g*), discard the flow-through and wash the column three times with 200 µL H_2O.
9. Elute the labeled oligonucleotide with 200 µL of a 25% aqueous solution of acetonitrile (freshly prepared).
10. Evaporate the acetonitrile in a vacuum centrifuge (e.g., Speed-Vac, Savant), add 100 µL of H_2O, evaporate again, and dissolve the labeled oligonucleotide in 100 µL oligo-annealing buffer.
11. Add 4 pmol of unlabeled complementary strand, put the tube for 1 min in a 90°C water bath (make sure that the tube cannot open), shut off the heating and slowly allow the water to cool to room temperature.
12. Store the annealed double-stranded oligonucleotide at –20°C (final concentration 20 fmol/µL).

Table 1
Sequence of the Various Double-Stranded Oligomers Used in Bandshift Experiments (only one strand is shown)

Binding site	Gene	Sequence
POU	*SPRR2A*	5′ GGATAAATTTGCATCTGGCT 3′
AP-1	*SPRR1A*	5′ GTAGTGTGAGTCATGTGTG 3′
ATF	*SPRR3*	5′ GCCCAGGTGACATCACTGTC 3′
KLF	*SPRR2A*	5′ CCTGCTGGGTGGGGTAGCAGGCTCTA 3′
ETS	*SPRR1A*	5′ CTTCTATTTCCTTGAGGC 3′
IRF	*SPRR2A*	5′ GGGTAGTTTCACTTtCaGCTG 3′ **(Note 14)**

Consensus sequences are underlined and correspond to the binding sites of the following transcription factor families: POU, POU-domain family; AP-1, fos/jun family; ATF, Atf/Creb family; KLF, Krueppel-like factor family; ETS, ets transcription factors; IRF, interferon responsive factor.

3.8.2. EMSA

1. Prepare a 200 × 200 × 1.5-mm slab gel with the following composition: 4 or 6% acrylamide/bis **(Table 2)**, 2% glycerol (*see* **Note 9**), 0.25X TBE (or 1X TGE) **(Table 2)**, 0.1% (w/v) ammonium persulfate, 0.1% (w/v) TEMED.
2. Allow to polymerize for 30 min at RT and position the gel in an horizontal electrophoresis apparatus with 0.25X TBE (or 1X TGE; *see* **Table 2**) as the running buffer.
3. Prerun the gel at least 30 min at 200 V (with buffer circulation) before use.
4. Prepare the following binding mixture (20 µL final volume): 10 µL 2X binding-buffer (*see* **Table 2**); 2 µL poly(dI-dC)•poly(dI-dC) (1 µg/µL); 10 µg nuclear extract; and H_2O until a final volume of 19 µL.
5. Preincubate this mixture at room temperature for 5 min (*see* **Note 10** and **Subheadings 3.8.3.** and **3.8.4.**).
6. Add 1 µL (20 fmol) of labeled double-stranded oligonucleotide.
7. Incubate at room temperature for 30 min.
8. Clean the slots of the prerun slab gel with running buffer and apply the incubation mixtures (*see* **Note 11**).
9. Run the gel at 200 V for 1.5 h at (RT (with buffer circulation; *see* **Note 12**).
10. Remove one glass plate and carefully put a sheet of Whatman 3MM filter paper on top of the gel; remove the second glass plate and dry the gel on a vacuum slab gel dryer.
11. Expose the dry gel to X-ray film for 1 h or overnight depending on the binding affinity.

3.8.3. Supershift Analysis

To identify the transcription factor responsible for the mobility shift on the gel, specific antibodies are included in the binding reaction before the addition of the labelled probe and are incubated at 4°C for 2 h followed by a 5-min incubation step at RT. The labeled oligonucleotide is than added, and the binding reaction is incubated for 30 min at room temperature (as described in **Subheading 3.8.2.**). Binding of an antibody to the transactivator/DNA complex will induce a larger shift in mobility (supershift) than binding of the transcription factor alone. In order to obtain a good supershift it is essential that the antibody does not interfere with complex formation. Conversely, inhibition of complex formation by a specific antibody can also be used as an indication for the presence of a given transcription factor, at least if an unrelated complex is not affected

Table 2
Optimal Binding–Buffer Conditions for Various Transcription Factors Involved in the Regulation of *SPRR* Genes

	POU	AP-1	ATF	KLF	ETS	IRF	PROX
Tris-HCL, pH 6.8						20 m*M*	
HEPES-KOH, pH 7.9	10 m*M*	10 m*M*	10 m*M*	5 m*M*	20 m*M*		10 m*M*
NaCl				25 m*M*		50 m*M*	50 m*M*
KCl	60 m*M*	60 m*M*	60 m*M*		50 m*M*		
Ficoll-400	4%	4%	4%		3%		
Glycerol				10%	20%	20%	10%
EDTA	0.5 m*M*	0.5 m*M*	0.5 m*M*	0.5 m*M*	0.2 m*M*	0.2 m*M*	0.5 m*M*
$MgCl_2$			5 m*M*				
$ZnCl_2$				0.5 m*M*			0.5 m*M*
DTT	1 m*M*	1 m*M*	1 m*M*	1 m*M*	2.5 m*M*	1 m*M*	1 m*M*
p(dI-dC)•p(dI-dC)	2 µg	2 µg	2 µg	1 µg	1 µg	1 µg	1 µg
BSA						0.25 µg/µL	0.25 µg/µL
% polyacrylamide/bis	4%	4%	4%	4%	4%	6%	4%
Gel/running buffer	0.25X TBE	0.25X TBE	1X TGE	1X TGE	1X TGE	1X TGE	1X TGE

POU, POU domain family; AP-1, fos/jun family; ATF, Atf/Creb family; KLF, Krueppel-like factor family; ETS, ets transcription factors; IRF, interferon responsive factors.

PROX represents the conditions used for the competetive EMSA using the proximal promoter region as a probe (*see* **Fig. 1**).

by the same antibody. In **Subheading 2.8.**, several commercial antibodies (Santa Cruz Biotechnology) that we have used in SPRR EMSAs ***(4)*** are indicated.

3.8.4. Competitive Promoter EMSA

This technique, which we have recently developed, is especially well suited to compare transcription factor binding profiles between various genes in a gene family. In the example depicted in **Fig. 1**, a 40-mer corresponding to the proximal promoter region of *SPRR1A* (just upstream of the TATA box) was labeled and used as a radioactive probe in EMSA. Competition experiments were conducted by including a 100-fold molar excess of unlabelled fragments of either *SPRR1A* (self-competition), or fragments corresponding to the same promoter regions in the related *SPRR2A* and *SPRR3* genes, in the 5-min preincubation step before the addition of the labeled probe (*see* **Subheading 3.8.2.**). The binding buffer conditions for this experiment are represented in **Table 2** (column PROX). The various *SPRR1A* complexes (bands 1–6) that are observed in lane 1 (no competition) are specific because they are all competed by an excess of unlabeled *SPRR1A* probe (lane 2). However, complex 2 is not competed by the *SPRR2A* fragment and complexes 3 and 6 are not competed by the *SPRR3* fragment. Lack of competition indicates that these binding sites are not present in the competing oligonucleotides. Complexes 3 and 6 correspond to the Ets binding site (*see* **Note 13**), and we have indeed previously shown that *SPRR3* does not contain a proximal Ets binding site ***(5)***. Complexes 1, 4, and 5 correspond to the Klf binding site, which is found in each promoter and is indeed competed by *SPRR2A* and *SPRR3* promoter fragments.

4. Notes

1. The decision whether to use CAT or luciferase reporters is mainly dictated by the different half-lifes of these two proteins: whereas CAT is very stable (half-life in cells: approx 50 h), the luciferase activity is more sensitive to degradation (half-life of 2–3 h). As transient transfection experiments are conducted in general for 24 to 48 h, it follows that the values obtained with the CAT reporter are cumulative values that represent the total amount of gene activity during the entire duration of the experiment. The high stability of the CAT protein can be disadvantageous for the study of inducible gene regulation, especially when efficient induction of a gene occurs relatively late after stimulation: this induction can be masked by the accumulation of constitutive CAT activity during earlier stages of the experiment. In these cases the use of a reporter with a much shorter half-life (such as luciferase), that measures gene activity in a more restricted time window, is desirable. One should also take into account this feature when using one of the reporters as internal control.
2. The use of polypropylene tubes should be avoided as liposomes stick to these tubes.
3. The culture dishes must not dry out, and should be rocked approximately each 20 min. Alternatively, an automatic plate rocking device (Gyro-rocker) can be used.
4. This heating step inactivates cellular enzymes consuming acyl CoA but does not affect CAT activity.
5. The sonification step is especially needed for differentiated cells (because of partial cornification). For proliferating keratinocytes (grown in KSFM), or when nondifferentiating cells such as HeLa or HaCaT cells are used, this step can be skipped.
6. This assay uses tritium-labeled radiochemicals. Work with extreme care and follow the local regulations as established by your radiation protection adviser. The use of protective gloves is strongly recommended.
7. Luciferase cell lysates can be stored at –80°C, but they lose part of their activity.

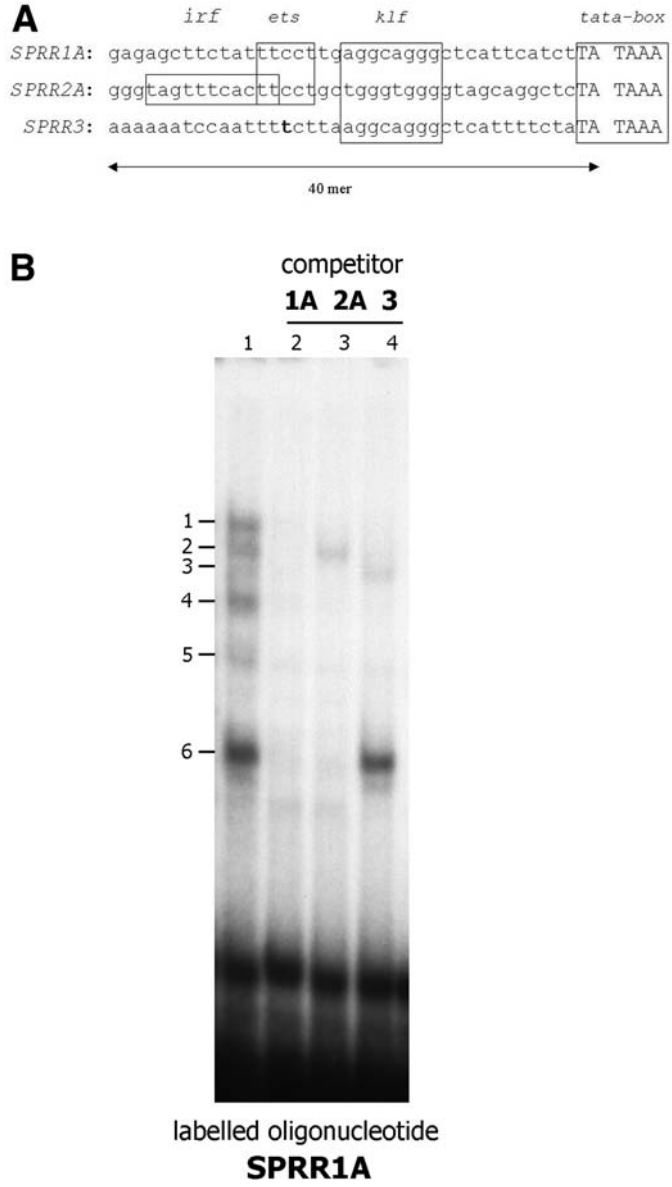


Fig. 1. Example of a competitive EMSA using 40-mer oligonucleotides covering the proximal regions of *SPRR1A*, *SPRR2A*, and *SPRR3*. **A,** Sequence of the three oligonucleotides used in the experiment: the localization of the TATA box and binding sites for Irf, Ets, and KLF are indicated. The mutation in *SPRR3* responsible for the disappearance of the Ets site is in bold lettertype. **B,** Mobility shift assay using a 4% polyacrylamide gel as described in **Subheading 3.8.2.** The *SPRR1A* probe was labeled and competed with a 100-fold molar excess of unlabeled fragments corresponding to the same promoter region in *SPRR2A* or *SPRR3*.

8. The assay uses ^{32}P-labeled radiochemicals. Work with extreme care and follow the local regulations as established by your radiation protection adviser. The use of protective gloves, Perspex/Plexiglas shields and continous monitoring (Geiger counter) of the working area and used apparatus are strongly recommended.

9. Addition of glycerol prevents cracking of the gel during drying.
10. To test the specificity of the interaction, unlabeled double-stranded oligonucleotides with either the same, a mutant or an unrelated sequence can be included in the preincubation step. These oligonucleotides should be added at different concentrations varying from 2- to 100-fold molar excess over the labeled oligonucleotide. From such data, conclusions can be drawn on both the nature of the interaction (e.g., which nucleotides in the binding site are important) and on the affinity of a transcription factor for a given binding site (refer for examples to **refs. *4–6***).
11. The samples are applied on the gel in binding buffer without dye markers. We have observed that both Bromophenol Blue and Xylene Cyanol dyes can affect gel migration
12. Buffer circulation is especially important when 0.25X TBE is used as gel/running buffer to prevent pH changes in buffer tanks and gel.
13. The identity of the various retarded bands can be determined by competion with the single binding sites depicted in **Table 1** (*see* also **Note 10**).
14. The small letter types represent bases which were mutated in order to destroy the overlapping Ets binding site ***(9)***.

Acknowledgments

The authors would like to thank all present and former members of the keratinocyte group at Molecular Genetics (Leiden Institute of Chemistry), who have contributed to the establishment and testing of the protocols described here.

References

1 South, A. P., Cabral, A., Ives, J. H., James, C. H., Mirza, G., Marenholz, I., et al. (1999) Human epidermal differentiation complex in a single 2.5 Mbp long continuum of overlapping DNA cloned in bacteria integrating physical and transcript maps. *J. Invest. Dermatol.* **112,** 910–908.

2. Marshall, D., Hardman, M. J., Nield, K. M., and Byrne, C. (2001) Differentially expressed late constituents of the epidermal cornified envelope. *Proc. Natl. Acad. Sci. USA* **98,** 13,031–13,036.

3 Cabral, A., Voskamp, P., Cleton-Jansen, A. M., South, A., Nizetic, D., and Backendorf, C. (2001) Structural organization and regulation of the small proline-rich family of cornified envelope precursors suggest a role in adaptive barrier function. *J. Biol. Chem.* **276,** 19,231–19,237.

4 Sark, M. W., Fischer, D. F., de Meijer, E., van de Putte, P., and Backendorf, C. (1998) AP-1 and ets transcription factors regulate the expression of the human SPRR1A keratinocyte terminal differentiation marker. *J. Biol. Chem.* **273,** 24,683–24,692.

5 Fischer, D. F., Sark, M. W., Lehtola, M. M., Gibbs, S., van de Putte, P., and Backendorf, C. (1999) Structure and evolution of the human SPRR3 gene: implications for function and regulation. *Genomics* **55,** 88–99.

6 Fischer, D. F., Gibbs, S., van De Putte, P., and Backendorf, C. (1996) Interdependent transcription control elements regulate the expression of the SPRR2A gene during keratinocyte terminal differentiation. *Mol. Cell Biol.* **16,** 5365–5374.

7 Kalb, V. F., Jr. and Bernlohr R. W. (1977) A new spectrophotometric assay for protein in cell extracts. *Anal. Biochem.* **82,** 362–371.

8 Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72,** 248–254.

9. Schreiber, E., Matthias, P., Muller, M. M., and Schaffner, W. (1989) Rapid detection of octamer binding proteins with “mini-extracts,” prepared from a small number of cells. *Nucleic Acids Res.* **17,** 6419.

32

Stable Integration of Large PAC Constructs in Keratinocytes

Sarah H. Williams and Alain Hovnanian

Summary

Transfer of P1-derived artificial chromosome (PAC) deoxyribonucleic acid (DNA) into keratinocytes is an extremely important technique that enables functional studies of keratinocyte-specific genes to be performed and genomic gene therapy for inherited and acquired diseases to be attempted. Ex vivo gene therapy approaches are possible using well-established conditions for keratinocyte culture and grafting, whilst the skin is the most accessible organ for administering in vivo therapy. PAC vectors lack relevant reporter genes to distinguish transfected mammalian cells from the non-transfected background, or to select clones in which the PAC construct has stably integrated into the genome. In this chapter, protocols to retrofit a reporter gene cassette will be described, together with techniques for transfecting large PAC constructs into keratinocytes without breakage. Protocols to select for stable integrants and to assess the integration event(s) within the keratinocyte genome will also be provided.

Key Words:

PAC; genomic DNA transfer; stable integration; integrin-targeting peptide; keratinocytes.

1. Introduction

Large deoxyribonucleic acid (DNA) fragments were initially required for physical mapping studies of complex genomes but have been used extensively in more recent times for functional gene expression studies and to deliver genomic transgenes. A genomic approach to gene transfer confers many advantages and offers the potential for sustained expression of the transgene. Transcriptional silencing of many complimentary DNA-based expression vectors with heterologous promoters has been observed, whereas several studies suggest that the use of genomic loci containing flanking and intragenic regulatory regions and native promoters provides tissue-specific, physiological levels of transgene expression ***(1,2)***.

Yeast artificial chromosomes were the first large DNA vector to be developed ***(3)***, carrying up to 2 Mb DNA, but their high chimaerism, insert instability, and difficulty in preparation has prompted the development of alternative systems such as the P1-derived artificial chromosome (PAC). PACs are based on the plasmid replicon of bacteriophage P1 and contain the negatively selectable marker *Sac*BII, the kanamycin resistance gene, and a *lox*P site and can carry genomic DNA inserts up to 300 kb that show no evidence of rearrangement or chimaerism ***(4)***. Several techniques have been

From: *Methods in Molecular Biology, vol. 289:*
Epidermal Cells: Methods and Protocols
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

developed for the modification of large *Escherichia coli*-based DNA vectors for expression in eukaryotic cells ***(1,5)***. This chapter describes in detail a highly efficient protocol for the retrofitting of PACs with markers to enable their selection in mammalian cells and the monitoring of transfected cells ***(6)***. The protocol details the partial digestion of the PAC construct with *Not*I to linearize it prior to ligation of a purified retrofitting cassette containing the *EGFP* gene for visualisation of live transfected cells, and puromycin and ampicillin resistance genes for antibiotic selection in mammalian and bacterial cells respectively.

One of the challenges of genomic gene therapy or functional genomics is the mode of delivery. Most viral methods that package DNA are limited in their capacity to approx 8 kb. Viruses are also highly immunogenic, even with the majority of viral-coding sequences removed—so-called high-capacity or gutless viruses. Nonviral delivery methods avoid these problems but are often low in transfection efficiency. In this chapter, protocols are presented describing the formation of transfection complexes containing $\alpha_5\beta_1$ integrin-targeting peptide and lipofectin ***(7)***, and their use in delivering PAC DNA to keratinocytes. These complexes exploit the integrin-mediated internalization pathway of receptor-mediated endocytosis used by many intracellular pathogens, and deliver large PAC constructs to keratinocytes with relatively high efficiency ***(8)***.

Maintenance of large DNA after delivery is essential for sustained transgene expression and is achieved by stable integration or persistence as a stable episome. In the latter case, many studies have reported episomal rearrangement and a 2–10% loss per cell generation of the episome in the absence of selection ***(9,10)***. In contrast, PAC constructs that have integrated into the genome of a cell are maintained even without selection. Protocols are described in this chapter for applying selection to a transfected keratinocyte population, with the aim of identifying, isolating and expanding stably transfected clones. Also presented are the complementary techniques of quantitative Southern blotting and fluorescent *in situ* hybridisation (FISH) to analyze PAC integration events in terms of copy number and location of integration.

2. Materials

2.1. Equipment

1. CHEF mapper pulsed field gel electrophoresis apparatus (Bio-Rad, Hemel Hempstead, UK, CHEF-DR III chiller system; cat. no. 170-3702).
2. Gene Pulser II electroporator (Bio-Rad; cat. no. 165-2106).
3. Phosphorimager cassette and scanner (Molecular Dynamics, Chesham, UK).
4. Inverted fluorescence microscope, for example, Nikon Diaphot 300.
5. Laser scanning confocal microscope, for example, Bio-Rad MRC 1024.

2.2. PAC Modification

1. Midiprep kit Tip-100 (Qiagen, Crawley, UK; cat. no. 10043).
2. 2xYT medium: 16 g bacto-tryptone, 10 g bacto-yeast extract, 5 g NaCl, to 1 L.
3. Terrific broth: 12 g bacto-tryptone, 24 g bacto-yeast extract, 4 mL glycerol, 100 mL KPB (17 m*M* KH_2PO_4, 72 m*M* K_2HPO_4) to 1 L.
4. Alkaline SDS: 0.2 *N* NaOH, 1% SDS; make fresh each time.
5. 7.5 *M* Ammonium acetate (filter sterilized, store room temperature).
6. pPBKG plasmid ***(8)*** (**Fig. 1**; request from alain.hovnanian@well.ox.ac.uk).
7. QIAquick gel extraction kit (Qiagen; cat. no. 28704).

8. Millipore V series membranes (0.025-μm pore size, Millipore, Watford, UK; cat. no. VSWP 01333).
9. 0.1-cm electroporation cuvets (Bio-Rad; cat. no. 165-2089).
10. ElectroMAX DH10β electrocompetent cells (Gibco-BRL, Paisley, UK; cat. no. 18290-015, store at –80°C).
11. Primers (5 pmol/μL):
    PAC2 5′-CGCCGTACGTTTCTTTGTAT-3′
    PAC4 5′-TGACTGGGTTGAAGGCTCTC-3′
    AMP1 5′-TGCGCGTAATCTGCTGCTTG-3′
    EGFP1 5′-TCCTCGCCCTTGCTCACCAT-3′

2.3. Keratinocyte Culture

1. Keratinocytes, for example, HaCaT cells ***(11)***.
2. Keratinocyte media (defined basal media with L-glutamine and supplements of bovine pituitary extract and epidermal growth factor, Gibco-BRL; cat. no. 17005-042. Store media at 4°C and supplements at –20°C until required, then mix well, store at 4°C and use within 4 wk).
3. Trypsin/ethylenediamine tetraacetic acid (EDTA) (Sigma, Poole, UK; cat. no. T3924).
4. Lipofectin (Invitrogen, Paisley, UK; cat. no. 18292-011).
5. 150 m*M* NaCl.
6. OptiMem (Gibco-BRL; cat. no. 31985-039, store in the dark at 4°C).
7. Integrin-targeting peptide ***(7)*** ($[K]_{16}$GACRRETAWACG). Store lyophilized at –80°C until needed. Before use, dissolve in OptiMem to 0.1 mg/mL and cyclize by oxidizing overnight at 4°C in a conical flask covered with perforated Parafilm. Once resuspended, the peptide is stable at –80°C for several years, and stable at –20°C for up to 6 wk.
8. Puromycin solution (Sigma; cat. no. P8833) concentrations ranging from 0.5 to 3 μg/mL dissolved in water, filter-sterilized, and stored at –20°C. Stable for up to 6 wk.
9. Cloning rings (made by cutting 0.8-cm lengths of the widest part of 200-μL tips. Autoclave before use. Used with autoclaved Vaseline).

2.4. Analysis of Integration

1. Colcemid (Gibco-BRL; cat. no. 15210-040).
2. 75 m*M* KCl.
3. Methanol : acetic acid (3 : 1; make fresh each time).
4. Nick translation kit (Vysis, Richmond, UK; cat. no. 32-801-300).
5. Spectrum Red dUTP (Vysis; cat. no. 30-803-400, store in the dark at 4°C).
6. Biotin dUTP (Roche, Lewes, UK; cat. no. 1093070).
7. *E. coli* tRNA (Roche; cat. no. 109541).
8. Sonicated salmon sperm DNA (Sigma; cat. no. D-7656).
9. 50% Formamide, 10% dextran sulphate, 2X SSC.
10. Human Cot-1 DNA (Gibco-BRL; cat. no. 15279-011).
11. Blocking solution and antibody dilution solution: 5% nonfat dried milk in 1X PBS. Dissolve 0.5 g milk in 10 mL 1X PBS–Tween. Spin 1.5-mL tubes at 16,000*g* for 20 min. Remove the top 1 mL without disturbing the pellet and use this as the blocking and antibody dilution solution. Dilute the antibody to the correct dilution on the day of use. Keep on ice and in the dark until required. Just before use, spin at 16,000*g* for 1 min. Take care not to disturb the pellet when using the antibody.
12. FITC-conjugated streptavidin (Vector Labs, Peterborough, UK; cat. no. SA5001), store in the dark at 4°C).

13. Biotinylated antistreptavidin (Vector Labs; cat. no. BA-0500).
14. To-Pro-3 (Molecular Probes Europe BV, Leiden, The Netherlands; cat. no. T-3605); store in the dark at 4°C).
15. Yo-Pro-1 (Molecular Probes; cat. no. Y-3603); store in the dark at 4°C).
16. Cy-3-conjugated chromosome paint (Cambio, Cambridge, UK; cat. no. depends on chromosome, for example, chromosome 3 paint 1153-303; store in the dark at 4°C).
17. Vectorshield mounting medium (Vector Labs; cat. no. H-1000).
18. Hybridization buffer: 50% formamide, 4X SSC, 50 m*M* sodium phosphate buffer, pH 7.2, 1 m*M* EDTA, 8% dextran sulfate, 1% SDS, 200 mg/mL degraded salmon sperm DNA, 10X Denhardt's (20 g Ficoll, 20 g BSA, 20 g PVP-40/L).
19. Megaprime labeling kit (Amersham Pharmacia, Little Chalfont, UK; cat. no. RPN1606).

3. Methods

3.1. PAC Preparation and Retrofitting

This section describes the preparation of microgram quantities of PAC DNA of a pure enough quality for transfection and the modification of PAC clones for use in stable transfection experiments by retrofitting a cassette containing reporter genes.

3.1.1. Maxi-Preparations of PAC DNA

1. Qiagen Tip-100 midi plasmid columns are used with some modifications to the manufacturer's instructions.
2. Use a 5-mL preculture to inoculate 400 mL of terrific broth.
3. Resuspend the pellet thoroughly in 40 mL P1, incubating at 37°C for 15 min.
4. Add 80 mL fresh alkaline SDS, then 60 mL ammonium acetate in the place of solutions P2 and P3 to lyse and neutralise the bacterial cells, respectively.
5. Repeat the centrifugation step after lysis and neutralization after first filtering the supernatant through sterile gauze. This removes bacterial debris and prevents the column from becoming blocked (*see* **Note 1**).
6. Elute the DNA using 5X 1 mL buffer QF warmed to 70°C. The heated buffer elutes more of the DNA than buffer at room temperature.
7. If the DNA is to be used for transfection, resuspend it in sterile TE 10/1.
8. Using cut-off tips (*see* **Note 2**), digest 2 µL PAC DNA with 10 U *Not*I (New England Biolabs, Hitchin, UK) in a 20-µL reaction to release the insert from the 16-kb vector.
9. Run on a 1% pulsed-field gel made with 0.5X TBE and electrophoresed for 14 h at 6V/cm with a switch time of 2–15 s. This enables the quality and quantity of the DNA preparation to be assessed. The concentration should also be measured by a spectrophotometer.
10. Store the DNA at 4°C for frequent use or at –20°C for longer storage.

3.1.2. Retrofitting the PAC Construct **(6)**

3.1.2.1. Preparing the Cassette

1. Digest 10 µg plasmid pPBKG (**Fig. 1**) with 50 U *Not*I in a 50-µL reaction at 37°C for 3 h.
2. Check 2 µL on a 1% agarose gel for completion of the digest.
3. Heat inactivate the digest at 65°C for 20 min and run on a 1% low-melt agarose gel without ethidium bromide in 1X Tris–acetate–EDTA (TAE) buffer.
4. Cut off a strip of the gel comprising the marker and a small fraction of the pPBKG digestion, and stain it for 20 min in TAE buffer containing 0.2 µg/mL ethidium bromide.
5. Locate the position of the 6.9-kb fragment under UV transillumination by cutting a notch in the gel spanning the fragment, and use this marker to excise the corresponding band from the unstained part of the gel (do not expose this to UV light).

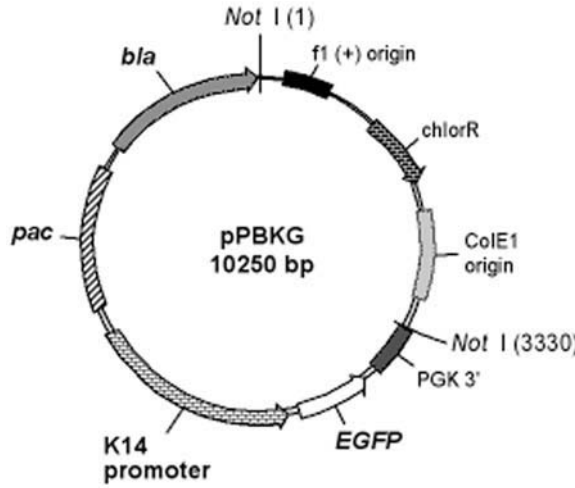


Fig. 1. Plasmid pPBKG. This construct is based on a pBS KS backbone and contains the *EGFP* gene under the control of the keratin 14 (K14) promoter with a murine phosphoglycerate kinase (*pgk*-1) 3′ UTR. Bacterial resistance to ampicillin is provided by the b-lactamase gene *(bla)*, whereas the puromycin *N*-acetyl transferase gene *(pac)* confers puromycin resistance to mammalian cells.

6. Gel-purify using the Qiagen QIAquick gel extraction kit according to the manufacturer's instructions.
7. Store the purified fragment at –20°C for up to 2 wk.

3.1.2.2. Partially Digesting the PAC

1. To linearize the PAC at one of the *Not*I sites, label 12 Eppendorf tubes 1–12 and prepare two master mixes using cut off tips for pipetting PAC DNA:
   a. 39 µg PAC DNA
      52 µL 10X buffer 3 (NEB)
      52 µL 10X BSA (NEB)
      dH_2O to 520 µL
   b. 35 µL 10X buffer 3
      35 µL 10X BSA
      dH_2O to 350 µL
2. Place 36.8 µL mix b in tube 1 and 20 µL of mix b in tubes 2–12 inclusively.
3. Add 3.2 µL *Not*I (10U/µL, NEB) to tube 1 and mix thoroughly.
4. Carry out a serial dilution: Transfer 20 µL from tube 1 to tube 2, mixing well. Then, transfer 20 µL from tube 2 to tube 3, from tube 3 to tube 4 and so on. Use a new tip and mix thoroughly each time.
5. Store tubes on ice until **step 7**.
6. Label 12 clean Eppendorf tubes 1–12 and add 40 µL mix a to each tube.
7. Transfer 10 µL enzyme mix from tubes 1–12 stored on ice to the corresponding tubes containing DNA. The total volume of digest is 50 µL and each tube contains half as much *Not*I per µg DNA as the previous tube: 2.67 U/µg in tube 1, 1.33 U/µg DNA in tube 2, and so on.
8. Incubate the samples at 37°C for 2 h and heat-inactivate at 65°C for 20 min.

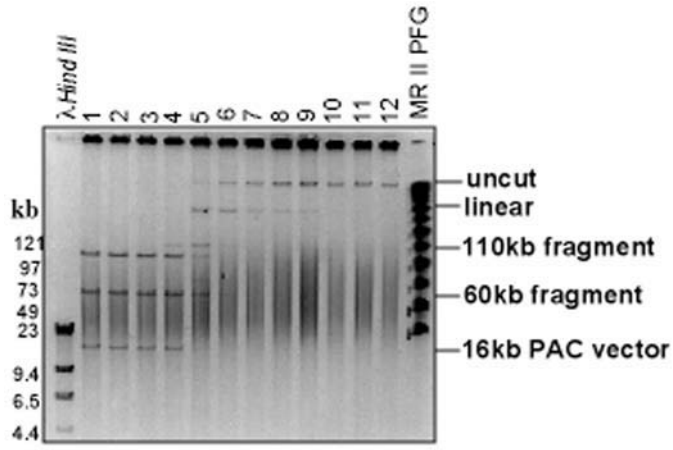


Fig. 2. PFGE analysis of a *Not*I partial digest of a PAC construct containing a 170-kb insert with an internal *Not*I site. The samples in the first few lanes (lanes 1–4) are completely digested and the 16-kb vector fragment and 110-kb + 60-kb insert fragments (deriving from the internal *Not*I site) are clearly resolved. As the enzyme concentration decreases, fewer sites are digested and more DNA molecules are linearised (lanes 5–9). The final few lanes show uncut PAC DNA. Samples containing linear DNA (lanes 5–9) and as little free vector as possible are chosen for retrofitting to avoid vector-only background.

9. Analyze 10 µL of each digest on a 1% pulsed-field gel electrophoresed for 14 h at 6 V/cm with a switch time of 2–15 s. This enables the extent of digestion to be determined. Stain the gel with ethidium bromide for photography. Store the remaining 40 µL of digest at 4°C.
10. Drop-dialyze those 40-µL digests containing mainly linearized PAC molecules **(Fig. 2)** and little free vector on Millipore V membranes to concentrate the DNA: Carefully place the droplet in the center of the membrane, floating shiny side up, on top of a 50-to 100-mL beaker of 5 m*M* Tris–HCl, pH 8.0, 0.5 m*M* EDTA for 2 h at room temperature. Pipet up the droplet after dialysis and store at 4°C.

3.1.2.3. Ligating the Cassette to the Linearized PAC

1. For each sample selected, assemble the following using the highest possible amount of PAC DNA up to 0.6 µg: 2 µL of T4 DNA ligase buffer; 1.5 µL of T4 DNA ligase (400 U/µL; NEB); purified retrofitting cassette to achieve a cassette : vector molar ratio of 1 : 3 (for mg of a PAC or BAC clone n kb in size, the amount of cassette DNA [6.9kb] to use is: 3 m/n µg); and dH_2O to 20 µL.
2. Incubate at 16°C for 16 h then heat-inactivate the ligase at 65°C for 20 min.
3. Drop-dialyze the total volume of ligation against dH_2O for 2 h at room temperature. This reduces the salt concentration in the sample, preventing arcing in the electroporation cuvets.
4. Place a vial of ElectroMAX electrocompetent DH10β cells on ice and when just defrosted add the ligated PAC/cassette sample and flick very gently to mix.
5. Transfer to an electroporation cuvet (0.1 cm) and electroporate at 1.8 kV, 200 Ω, and 25 µF using a Bio-Rad Gene Pulser.
6. Immediately add 500 µL SOC medium, transfer to an Eppendorf tube, and incubate at 37°C for 1 h.

7. Plate 400 µL, 40 µL, and 4 µL onto 2X YT agar with 30 µg/mL kanamycin and 50 µg/mL ampicillin.
8. Pick colonies and carry out minipreps based on an alkaline lysis protocol with some modifications:

   Inoculate 5 mL 2XYT media (containing 30 µg/ml kanamycin and 50 µg/mL ampicillin) and culture overnight at 37°C in a shaking incubator. Pellet 2 mL of this (reserve the remainder for glycerol stocks) and resuspend the pellet in 200 µL TE 50/10. Lyse the bacteria using 600 µL of alkaline SDS and neutralize with 450 µL of 7.5 *M* ammonium acetate. Invert to mix and incubate on ice for 10 min. Centrifuge at 16,000*g* for 15 min and transfer the supernatant to a fresh tube containing 700 µL of isopropanol. Mix and recentrifuge at 16,000*g* for 10 min. Resuspend the pellet in 200 µL of TE 50/10. Precipitate the proteins with 100 µL 7.5 *M* ammonium acetate on ice for 15 min and centrifuge at 16,000*g* for 10 min. Wash the pellet with 70% ethanol and resuspend in 50 µL TE 10/1 containing 50 µg/mL RNase A.
9. Analyze 5 µL by *Not*I digestion and pulsed-field gel electrophoresis to determine whether the cassette has been inserted and if any genomic insert has been lost.
10. If any internal *Not*I sites are present in the PAC insert, perform colony polymerase chain reaction (PCR) amplification to ensure the cassette has been inserted adjacent to the vector and not within the genomic insert:

    Dilute a colony in 500 µL dH_2O, vortex, and denature at 100°C for 5 min. Pellet cell debris by centrifuging at 16,000*g* for 5 min. Use 5-µL supernatant as template for PCR using primers specific for the ampicillin (AMP1) or *EGFP* (EGFP1) regions of the cassette and primers specific for the P1 lytic replicon (PAC4) or *sacB* region (PAC2) of the PAC vector.

    Set up the PCR reaction on ice in thin-wall polycarbonate 96-well plates: 5 µL supernatant template; 1 µL forward primer (AMP1 or EGFP1) 5 pmol/µL; 1 µL reverse primer (PAC4 or PAC2) 5 pmol/µL; and 3 µL dH_2O.

    Overlay with a drop of mineral oil. Make a master mix on ice with the following (multiply by n + 1, where n is the number of PCR reactions): 2 µL of 2 m*M* dNTPs (Pharmacia); 0.4 U AmpliTaq polymerase (Perkin-Elmer); 2 µL 10X PCR buffer containing 15 m*M* $MgCl_2$; and dH_2O to 10 µL. Incubate the 96-well plate at 94°C for 2 min in the PCR thermal cycler, then add 10 µL of the buffer/dNTP/Taq mix to each well and perform a touchdown cycling amplification:

    Five cycles of 94°C for 30 s, 55°C for 40 s (decreasing the annealing temperature by 1 degree per cycle to 51°C over 5 cycles), 72°C 30 s, then 35 cycles of 94°C for 30 s, 50°C for 40 s, and 72°C for 30 s (adding 1 s elongation time per cycle).
11. Analyze 2 µL on a 2% agarose gel. Choose colonies in which the cassette has been retrofitted adjacent to the PAC vector in the desired orientation for future work and transfections.

3.2. Keratinocyte Culture and Transfection

This section describes the routine culturing of keratinocyte cell lines in a low-calcium medium to prevent cell differentiation, and details the procedure used to stably transfect large PAC molecules into keratinocytes using an integrin-targeting peptide.

3.2.1. Keratinocyte Subculture

Keratinocytes are maintained in serum-free keratinocyte media supplemented with bovine pituitary extract and epidermal growth factor. Grow cells in 75-cm^2 flasks until confluent in a humidified 37°C incubator with 6% CO_2.

1. Aspirate media, wash cells very briefly with trypsin–EDTA and aspirate the wash solution. PBS should not be used to wash keratinocytes as they do not tolerate high salt concentrations.
2. Dissociate cells using 4 mL trypsin–EDTA, inactivating the trypsin using 4 mL keratinocyte media containing 10% fetal calf serum.
3. Centrifuge the cells at 200*g* for 5 min, aspirate the supernatant, and resuspend the pellet in 2 mL keratinocyte media.
4. Count the cells using a hemocytometer if required for transfection.
5. Alternatively, for routine culture, passage the cells at a 1 : 3 dilution into 75-cm^2 flasks.
6. Determine the concentration of puromycin required to select for stably transfected cells by applying puromycin to the cells within complete keratinocyte media at different concentrations ranging from 0.5–3 µg/mL. The optimal concentration is lethal to 100% nontransfected cells after 2–3 wk of selection.

3.2.2. Transfection of Keratinocytes

The day before transfection, seed 1×10^6 keratinocytes in a 10-cm dish. The next day, ensure the cells are approx 70% confluent.

1. Prepare four separate premixes of the following in 15-mL Falcon tubes (*see* **Note 3**): 3 µL lipofectin; 240 µL 0.1 mg/mL peptide; and 4µg PAC DNA (prepared using Tip-100 columns, in sterile TE, linearized if required—*see* **Note 4**) mixed with 600 µL 150 m*M* NaCl.
2. Add the components in this order, mixing with each addition by gently flicking the tube.
3. Incubate the premixes for 2 h at room temperature.
4. Add OptiMem (prewarmed to 37°C) to each premix, making the volume up to 2.5 mL.
5. Aspirate the media from the 10-cm dish of cells and add the four transfection premixes (4×2.5 mL) dropwise while swirling the dish.
6. Return the dish to the 37°C incubator for 5 h.
7. Aspirate the transfection mix, replace with keratinocyte media and return to the incubator for a further 19–31 h.
8. Visualize the live cells using an inverted fluorescence microscope with a mercury bulb shone through a FITC filter set (excitation 488 nm, emission 512 nm). Cells expressing EGFP fluoresce green and the percentage of expressing cells under several fields of view can be used as an approximate indicator of transfection efficiency.

3.2.3. Selection and Expansion of Transfected Cells

1. Apply puromycin selection to transfected cells at the previously determined optimal concentration 48–72 h after transfection to allow one or more cell divisions to have occurred.
2. Grow the cells under selection for 2–3 wk, replacing the selection media with fresh every 3–4 d.
3. Fluorescent colonies will develop on this dish. When these are large enough for picking, but not so large that they grow into other colonies, they can be picked for expansion of individual clones (*see* **Note 5**).
4. Mark the position of the colonies to be selected by ringing in ink on the underside of the plastic dish.
5. Aspirate the media and, using forceps, pick up a sterile cloning ring and dip one end in autoclaved Vaseline.
6. Place the ring around the colony, applying gentle but firm pressure with a finger so that it provides a good seal with the Vaseline.
7. Add 50 µL of trypsin–EDTA into the ring, return the cells to the incubator for 2–3 min, then detach the colony by pipetting the trypsin up and down within the ring.

8. Transfer the cells to one well of a 24-well plate containing keratinocyte media without puromycin selection. Repeat for each ringed colony.
9. After 2–3 d of growth, puromycin selection can be reapplied, and the cells grown until confluent within the well. At this point, transfer them to a 12- or 6-well plate and continue the expansion until sufficient cells are available for analysis.
10. Freeze some cells of the clone(s) at the earliest opportunity to provide a stock of newly-transfected cells. This will be of use should the expanding cells become contaminated.

3.3. Analyzing PAC Integration Event(s) in the Keratinocyte Genome

This section describes the complementary techniques of FISH and Southern blotting to analyze PAC integration. The chromosomal location of the PAC integration site(s) is determined using FISH, which also gives an indication of PAC copy number. A quantitative measurement of integrated PAC copy number is carried out using Southern blotting. The integrity of the integrated PAC(s) can also be assessed by hybridising probes for the PAC vector/gene(s) of interest carried on the PAC to Southern blots of genomic DNA digested with restriction enzymes that cut infrequently within the PAC construct (*see* **Note 6**).

3.3.1. Metaphase FISH

Two probes are used to hybridize metaphase chromosome spreads of keratinocyte clones: the PAC construct that has not been retrofitted and the plasmid pPBKG. Labeling the probes with different colors enables the endogenous signal (PAC only) to be distinguished from the integrated signal (PAC and plasmid).

3.3.1.1. Preparation of Metaphase Spreads

1. Add 100 µL of 10 µg/mL colcemid to an actively growing 25-cm^2 flask of keratinocytes, containing 5 mL media.
2. Incubate at 37°C for 1 h, trypsinize, centrifuge at 250*g* for 5 min, and discard the supernatant.
3. Resuspend the pellet in 7 mL of 75 m*M* KCl (prewarmed to 37°C) and incubate at 37°C for 10–20 min (*see* **Note 7**).
4. Centrifuge at 250*g* for 5 min, discard the supernatant, and resuspend the pellet in the remaining tiny volume by flicking the tube.
5. Add one drop of methanol:acetic acid fixative (3 : 1), gently flick the tube to mix, add two drops of fixative, flick and so on up to five drops of fixative. This gentle addition of fixative prevents irreversible clumping of cells.
6. Then make up the volume to 10 mL with fixative, centrifuge at 250*g* for 5 min, and discard the first fixative.
7. Conduct a second and third fix (it is not necessary to add these dropwise) and store the final 10-mL fixed cell suspension at –20°C overnight.
8. Clean a microscope slide with freshly made fixative, then add one drop of fixed cell suspension onto the slide and allow to dry.
9. Store the slide overnight at –20°C with desiccant.

3.3.1.2. Probe Labeling

1. For labeling of the probes, assemble the following on ice (one tube per probe):
   a. 500 ng PAC or plasmid DNA
   b. 1.25 µL 0.2 m*M* Spectrum Red dUTP for PAC labeling or Biotin dUTP for plasmid labeling (diluted in H_2O, kept in the dark)

c. 2.5 µL 0.1 m*M* dTTP (diluted in H_2O)
d. 5 µL 0.1 m*M* dNTP mix (dATP, dCTP + dGTP diluted in H_2O)
e. 2.5 µL nick translation buffer
f. 5 µL nick translation enzyme
g. H_2O to 25 µL

} from nick translation kit

2. Mix well and incubate at 16°C for 4 h. Ensure the labeling is complete by loading 100 ng on a 2% agarose gel and determining the size of the smear, which should be 200–600 bp. If the smear is too large, continue the incubation for 2–4 h more (PACs may need 8 h total incubation time).

3.3.1.3. Probe Purification

1. Remove the unincorporated nucleotides from the probe mix by ethanol precipitation.
2. Add the following to the 25-µL labeled probe: 6.25 µL of 10 mg/mL tRNA : 10 mg/mL salmon sperm DNA (1 : 1); 0.1X volume 3 *M* NaAc, pH 5.2; and 2X volume ice-cold 100% EtOH.
3. Mix by inversion and store at –20°C overnight.
4. Centrifuge at 16,000*g* for 20 min at 4°C, wash the pellet twice with 50 µL 70% EtOH, air-dry, and resuspend in TE at 20 ng/µL.
5. Store at –20°C.

3.3.1.4. Slide Preparation

The target DNA needs to be made single-stranded before it can be hybridized with the probe DNA. By using 70% formamide, the denaturing temperature can be reduced to 70°C, thus preserving the morphology of the chromosomes.

As the denaturation time depends on the age of the slides, a new batch of slides should be tested over a range of denaturation times for signal intensity and chromosome morphology. The optimal denaturation time will preserve some G banding and give a bright signal.

1. Warm the slide box to room temperature before opening.
2. Place 50 mL 70% formamide, 0.6X SSC in a Coplin jar and place the jar in a 71°C water bath, ensuring the water level reaches the collars of the Coplin jar to maintain a constant formamide temperature.
3. Incubate the slides (up to three at a time) for 2–3 min in formamide, followed by 5 min in ice-cold 2X SSC in a second Coplin jar. Ensure slides are removed from the denaturing solution in the same order they went in.
4. Dehydrate the slides through an alcohol series of 70%, 95%, 100%, and 100% EtOH in Coplin jars, incubating for at least 30 s in each alcohol.
5. Air-dry the slides.

3.3.1.5. Probe Preparation

Repetitive sequences within the large PAC probe are preannealed with unlabeled Cot-1.

1. Assemble the following in a 0.2-mL PCR tube: 4 µL labeled PAC probe (20 ng/µL; 5 µL 50% formamide, 10% dextran sulphate, 2X SSC; and 1 µL Cot-1 (2 µg/µL).
2. Denature at 80°C for 5 min, then incubate at 37°C for 20 min to allow repeat sequences to preanneal.
3. Plasmid probes do not require competition with Cot-1. Mix 4 µL labeled pPBKG probe with 5 µL 50% formamide, 10% dextran sulfate, 2X SSC.

4. Denature at 80°C for 5 min, then place on ice for 5 min.
5. Gently "huff" on the metaphase spread slides to reveal the cells and ring this area on the underside of the slide using a waterproof marker.
6. Pool the probe DNA, then apply 1 µL probe mix to the spot of cells.
7. Cover with a 6-mm circular cover slip, seal with a rubber seal and incubate at 37°C for three nights in a moist chamber.

3.3.1.6. Probe Detection

1. Remove cover slip sealant with forceps and float off the cover slips by standing the slides in 2X SSC.
2. Conduct stringency washes by incubating the slides in: 50% formamide, 1X SSC for 10 min at 37°C; 2X SSC for 5 min at 43°; and 1X PBS for 5 min at room temperature.
3. Drain the slides for a few seconds on tissue paper but do not allow to dry out.
4. Place 80 µL blocking solution onto the slide and cover with a 22- × 50-mm cover slip.
5. Incubate horizontally at room temperature for 10 min.
6. Remove cover slips by standing the slides in a coplin jar of PBS/0.1% Tween-20.
7. Transfer the slides to a slide box containing PBS/Tween and place on a shaking table for 3 min.
8. Replace with fresh PBS–Tween and gently shake for a further 3 min.
9. Drain the slides briefly, place 80 µL streptavidin–FITC antibody dilution on the slides, cover slip and incubate in the dark for 30 min at 37°C.
10. Remove the cover slips as before, wash twice with PBS/Tween as before on a shaking table and add 80 µL biotinylated goat anti-Streptavidin antibody dilution to the slides.
11. Repeat the addition of cover slips, incubation, cover slip removal and washes as before prior to the final antibody incubation of 80 µL of streptavidin-FITC.
12. Remove cover slips and wash as before, air-dry (protecting from light), add 25 µL To-Pro-3 as a mountant and cover slip.
13. Leave for 15 min before viewing under a confocal microscope.
14. Take note of vernier references of metaphases showing strong signals and capture images using appropriate software.
15. Determine the average number of chromosomes per metaphase for later use in quantifying copy number (**Subheading 3.2.2.2.**).

3.3.1.7. Chromosome Painting

1. Remove cover slips by soaking in PBS–Tween for 15 min, fresh PBS/Tween for a further 15 min, 1X PBS for 15 min, then air-dry the slides.
2. Denature chromosome paint for 10 min at 72°C and preanneal for 60–90 min at 37°C.
3. Add 1 µL denatured paint to slides, cover slip, seal with a rubber seal and hybridize at 37°C overnight in a moist chamber.
4. Remove the cover slips and carry out stringency washes as before.
5. Air-dry, add 25 µL Yo-Pro-1 to slides and cover slip.
6. Leave for 15 min and analyze metaphase spreads using vernier references to locate the same metaphases as before.

3.3.2. Quantitative Southern Blotting

3.3.2.1. Probe Preparation

Digest 2 µg pPBKG with 15 U *Hin*dIII in a 30-µL reaction for 3 h at 37°C, run on a 1% low-melt gel in 1X TAE buffer and gel-purify the 3-kb band using the QIAquick gel extraction kit according to the manufacturer's instructions.

3.3.2.2. Digestion and Membrane Preparation

1. Prepare genomic DNA from transfected keratinocyte clones and nontransfected keratinocytes (negative control) using standard methods.
2. Digest 5 µg genomic DNA from transfected keratinocyte clones and 5 µg from nontransfected keratinocytes with 50 U *Not*I in a 150-µL reaction for 3 h at 37°C.
3. Perform a dilution series of pPBKG DNA over 10 Eppendorf tubes ranging from 2000 pg to 0.1 pg.
4. Add 5 µg nontransfected keratinocyte DNA (*see* **Note 8**) to each dilution and digest with 50 U *Not*I in 150 µL for 3 h at 37°C.
5. Precipitate all digests at –20°C overnight, centrifuge at full-speed for 30 min at 4°C, and resuspend in 20 µL.
6. Load the pPBKG dilution series alongside digests of transfected and nontransfected keratinocyte DNA on a 1% agarose gel.
7. Conduct Southern blotting using standard methods, transferring the DNA to a positively charged Hybond N^+ nylon membrane.
8. Prehybridize this membrane in a formamide hybridization buffer at 42°C for at least 4 h.
9. Hybridize with 100 ng pPBKG *Hin*dIII fragment labeled with α-^{32}P (using the Megaprime labeling system) in fresh hybridization buffer at 42°C for 16 h.
10. Wash the membrane under agitation in 0.5% SDS, 0.5X SSC for 30 min at room temperature, then in fresh 0.5% SDS, 0.5X SSC for 30 min at 65°C. The stringency of washes may be increased if necessary.
11. Wrap in plastic wrap and expose to a PhosphorImager cassette.
12. Use densitometric analysis, for example, ImageQuant software to quantify the intensity of the 7-kb bands in lanes of transfected keratinocyte DNA and compare with the intensities of the control pPBKG dilution series to calculate the effective mass of the integrated PAC construct.
13. The copy number is calculated using the equation:

$$\text{Copy number} = \frac{\dfrac{\text{no. chromosomes in cell line}}{23} \times \dfrac{\text{effective mass of construct}}{5\ \mu\text{g}}}{\text{control plasmid size}} \times \text{haploid genome size}$$

The chromosome number has been determined earlier (**Subheading 3.3.1.6.**), the haploid genome size is approx 3.3×10^9 bp and plasmid pPBKG is 10258-bp long.

4. Notes

1. After the first centrifugation, the supernatant is filtered through sterile gauze into a clean centrifuge bottle for a second centrifugation. The bacterial debris within the first centrifugation bottle, however, often adheres fairly loosely to the sides of the bottle and may be released on inversion of the bottle. This is usually unavoidable, as longer centrifugations do not improve adherence and centrifugations at a higher resolution can be damaging to the bottle. To overcome this problem, the gauze can be supported on a funnel and squeezed very gently to allow the supernatant to pass through whilst preventing the highly viscous debris from entering the second bottle. Repeating the centrifugation should enable rapid flow-through of the supernatant/wash buffer in Tip-100 columns at a later stage, but blockage of the columns sometimes still occurs. A sterile 10-mL syringe plunger can be used to speed up the flow-through by inserting it into the Tip-100 column and pressing gently to push the solution through the column. This has no adverse effect on yield or quality of the PAC DNA recovered at the end of the protocol.

2. Large DNA molecules are highly susceptible to breakage. Therefore, cut off tips (made using a scalpel blade to remove the narrowest 3–5 mm of yellow tips) should be used at all times when manipulating PACs to prevent shearing. Maxiprep and miniprep protocols for PACs should never use vortexing to resuspend or mix. Gentle flicking or inversion of the tube should suffice.
3. Optimize the ratio of lipid:peptide:DNA by delivering smaller marker constructs, for example, pPBKG to the keratinocytes before performing scaled-up stable transfections. The optimal ratio for HaCaT and MRC5 cell transfection was determined as 0.75 : 6 : 1 ***(8)*** but this may vary depending on the keratinocyte culture. A premix containing more than 4 µg PAC DNA was observed to precipitate when the peptide is added, so four separate premixes (4×4 µg) are made to deliver a total of 16 µg PAC DNA.
4. PAC DNA can be linearized prior to transfection at a unique site that may already be present in the construct or could be engineered when retrofitting. This is not essential and may decrease transfection efficiency, as the DNA will be less condensed. However, as the construct breaks in at least one place before integrating into the genome, it confers the advantage of choosing the breakage point, perhaps avoiding the disruption of the gene of interest or a known regulatory region (assuming no additional breaks during the transfection process).
5. If the colonies grow too large and merge into each other, carry out a dilution series of the cells within a well, transferring them to several wells of a 12-well plate. This enables colonies of different sizes to be picked over the forthcoming weeks. Some keratinocytes do not respond well to picking of colonies as cell-cell contact is insufficient for growth due to the small number of cells within a colony. If this is the case, selection of whole populations can be carried out. FISH analysis of the population at a later stage will determine the number of integration events and thus the number of founder cells within a population.
6. With the advent of the Human Genome Project, it is now straightforward to ascertain the full sequence of a PAC construct. A restriction map of the construct can be used to choose enzymes such as *Not*I that will cut to reveal a diagnostic pattern and used to prepare Southern blots. These blots can be hybridized with fragments of the 5′/3′-ends of the gene(s) of interest within the PAC construct, or regions of the PAC vector. Several enzymes should be chosen to provide coverage of the entire construct and to give a more accurate representation of the integrity of the integrated PAC(s).
7. The time needed for treatment with the hypotonic KCl solution varies according to the cells, so this must first be optimised on non-transfected keratinocytes. After too short a treatment, cytoplasm will trap the metaphase chromosomes and prevent them spreading properly whilst too long a treatment will cause the cells to burst and the chromosomes to be lost in suspension.
8. The dilution series of pPBKG DNA is used to quantify the PAC copy number by comparing band intensities, and acts as a size reference for the *Not*I pPBKG fragment expected in keratinocyte genomes containing integrated PAC(s). As pure plasmid DNA often migrates faster than genomic DNA through an agarose gel, a background of non-transfected keratinocyte DNA in the pPBKG digests ensures that all DNA samples migrate at the same rate, enabling more accurate sizing of bands.

Acknowledgments

The authors would like to thank Dr José Mejía for help in developing the protocols described in this chapter and are grateful to Andrew Jefferson for his assistance.

References

1 Yang, X. W., Model, P., and Heintz, N. (1997) Homologous recombination based modification in *Escherichia coli* and germline transmission in transgenic mice of a bacterial artificial chromosome. *Nat. Biotechnol.* **15,** 859–865.

2 Antoch, M. P. (1997) Functional identification of the mouse circadian clock gene by transgenic BAC rescue. *Cell* **89,** 655–667.
3 Burke, D. T., Carle, G. F., and Olsen, M. V. (1987) Cloning of large segments of exogenous DNA into yeast by means of artificial chromosome vectors. *Science* **236,** 806–812.
4 Ioannou, P. A., Amemiya, C. T., Garnes, J., Kroisel, P. M., Shizuya, H., Chen, C., et al. (1994) A new bacteriophage P1-derived vector for the propagation of large human DNA fragments. *Nat. Genet.* **6,** 84–89.
5 Zhang, Y., Buchholz, F., Muyrers, J. P. P., and Stewart, A. F. (1998) A new logic for DNA engineering using recombination in *Escherichia coli*. *Nat. Genet.* **20,** 123–128.
6. Meja, J. E. and Monaco, A. P. (1997) Retrofitting vectors for *Escherichia coli* based artificial chromosomes (PACs and BACs) with markers for transfection studies. *Genome Res.* **7,** 179–186.
7 Hart, S. L., Arancibia-Carcamo, C. V., Wolfert, M. A., Mailhos, C., O'Reilly, N. J., Ali, R. R., et al. (1998) Lipid-mediated enhancement of transfection by a non-viral integrin-targeting vector. *Hum. Gene Ther.* **9,** 575–585.
8 Compton, S. H., Mecklenbeck, S., Meja, J. E., Hart, S. L., Rice, M., Cervini, R., et al. (2000) Stable integration of large (>100 kb) PAC constructs in HaCaT keratinocytes using an integrin-targeting peptide. *Gene Ther.* **7,** 1600–1605.
9 Wohlgemuth, J. G., Kang, S. H., Bulboaca, G. H., Nawotka, K. A., and Calos, M. P. (1996) Long-term gene expression from autonomously replicating vectors in mammalian cells. *Gene Ther.* **3,** 503–512.
10 Huertas, D., Howe, S., McGuigan, A., and Huxley, C. (2000) Expression of the human CFTR gene from episomal *oriP-EBNA-1*-YACs in mouse cells. *Hum. Mol. Genet.* **9,** 617–629.
11. Boukamp, P., Petrussevska, R. T., Breitkreutz, D., Hornung, J., Markham, A., and Fusenig, N. E. (1998) Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J. Cell Biol.* **106,** 761–771.

33

Targeted Somatic Mutagenesis in the Mouse Epidermis

Daniel Metzger, Mei Li, and Pierre Chambon

Summary

The efficient introduction of somatic mutations in a given gene at a given time and in specific cell types of the skin will greatly facilitate the studies of a number of genes expressed in the skin and the production of animal models for skin diseases. We describe here strategies and techniques to create spatiotemporally controlled somatic mutations of target genes in the skin using a conditional Cre/LoxP system. They are based on cell-specific expression of the chimeric Cre recombinase Cre-ER^{T2}, whose activity is induced by antiestrogens such as Tamoxifen (Tam), and which is obtained by fusing the Cre recombinase with a mutated ligand binding domain of the human estrogen receptor ERα. As an example, we present ablation of the retinoid receptor RXRα in epidermal basal keratinocytes of adult mice.

Key Words:

Skin; Cre; RXRα; keratinocyte; knockout; tamoxifen.

1. Introduction

Gene targeting in the mouse has yielded remarkable advances in understanding the roles played by specific gene products in mammalian development and in adult pathophysiology. However, targeting the mutation in the germ line has some inherent limitations, such as problems associated with embryonic lethality, the occurrence of developmental aberrations, or compensatory effects by functionally redundant genes. In many instances, these limitations prevent the determination of the function of a given gene product in a defined subset of cells at any given time during the animal's life and do not allow the engineering of mouse models for human diseases that are caused by somatic mutations, particularly when these diseases result from a combination of somatic mutations, such as in most forms of cancer ***(1,2)***. Thus, methods to achieve conditional gene inactivation have been developed, based mainly on the properties of the bacteriophage P1 site-specific Cre recombinase. Because the Cre recombinase can efficiently excise a DNA segment flanked by two loxP sites (floxed DNA) in animal cells, spatially or temporally controlled somatic mutations can be obtained by placing the Cre gene under the control of either a cell-specific or an inducible promoter, respectively ***(3)***. However, these conditional gene-targeting systems also have a number of limitations because they are either only spatially or temporally controlled. Recently, two systems that allow the generation of somatic mutations in a defined gene at a given time in the life of the animal and in a specific cell type have been developed.

From: *Methods in Molecular Biology, vol. 289: Epidermal Cells: Methods and Protocols*
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

First, the Cre/loxP system has been combined with tetracycline-dependent regulatory systems. Spatiotemporally controlled Cre recombinase expression was obtained by doxycycline (a tetracycline analog) treatment of transgenic mice harboring two transgenes encoding 1) a tetracycline-controlled transactivator under the control of a tissue-specific promoter and 2) the Cre recombinase under the control of a minimal promoter that contains the operator sites of the tet operon. However, the production of mice bearing both transgenes and the two LoxP-flanked alleles of a given gene requires complex breedings, and further improvements are required to eliminate basal levels of Cre expression ***(4,5)***. Second, fusion of the Cre recombinase to mutated ligand binding domains of steroid receptors results in in vivo ligand-inducibility of the recombinase activity ***(6,7)***. One of such fusion proteins, Cre-ER^{T2} (obtained by fusing Cre to a mutated ligand binding domain of the human estrogen receptor ERα) is particularly potent. Cell-specific expression of Cre-ER^{T2} in transgenic mice allows efficient tamoxifen-dependent Cre-mediated recombination at loci flanked by LoxP sites, without background activity, thus generating site-specific somatic mutations in a spatio-temporally controlled manner ***(8)***.

The skin is a complex organ composed of the epidermis, its appendages (hair follicles), and the dermis. The epidermis is a highly dynamic stratified epithelium made principally of keratinocytes ***(9)***. The innermost basal cells that are attached to the basement membrane form a proliferative layer, from which keratinocytes periodically withdraw from the cell cycle and commit to terminally differentiate while migrating into the suprabasal layers. Terminally differentiated keratinocytes that form the cornified layer are lost daily from the surface of the skin and are continuously replaced by newly differentiating cells. Hair follicles that develop through a series of mesenchymal–epithelial interactions during embryogenesis are also dynamic structures. They are also mainly composed of keratinocytes, and their outer root sheath is contiguous with the basal layer of the epidermis. Once formed, hair follicles periodically undergo cycles of regression, rest and growth, through which old hairs are eventually replaced by new ones ***(10,11)***.

Temporally controlled targeted somatic mutagenesis in the various compartments of the skin should allow to define the function of a number of genes expressed in the skin, and produce animal models for skin diseases. In this chapter, we report strategies and techniques to create spatiotemporally controlled somatic mutations of target genes in the skin, that we illustrate by selective ablation of RXRα in basal keratinocytes.

2. Materials

2.1. Antibodies

Biotinylated mouse monoclonal 2CRE 2D8-1-2 antibody (Euromedex, Mundolsheim, France; cat. no. MAB3120).

2.2. Transgenic Mice

1. RXRαaf2(I) (**ref. *12***; e-mail: chambon@titus.u-strasbg.fr)
2. RosaR26R (**ref. *13***; to be requested from P. Soriano; e-mail: psoriano@fhcrc.org)
3. RXRα$^{L2/L2}$ mice (**ref. *14***; e-mail: chambon@titus.u-strasbg.fr)

2.3. Reagents

2.3.1. Genomic DNA Extraction

1. Proteinase K stock solution at 10 mg/mL. Dissolve 100 mg proteinase K (Sigma Chimie, St. Quentin Fallavier, France; cat. no. P-6556) in 10 mL distilled H_2O and store as 0.5-mL aliquots at –20°C.

2. Proteinase K digestion buffer (50 m*M* Tris, pH 8.0; 5 m*M* ethylene diamine tetraacetic acid; 1% sodium dodecyl sulfate; 0.2 *M* NaCl). For 800 mL, assemble in the following order: 40 mL 1 *M* Tris-HCl, pH 8.0; 500 mL distilled water; 8 mL 0.5 *M* ethylene diamine tetraacetic acid; 40 mL 20% sodium dodecyl sulfate; 32 mL 5 *M* NaCl; and 180 mL distilled water.
3. Stabilized phenol (Tris-saturated, pH 7.5-8; Eurobio, Les Ulis, France; cat. no. 018335).

2.4. Genotyping

1. 10X PCR buffer: 500 m*M* KCl, 100 m*M* Tris-HCl, pH 8.8; 15 m*M* $MgCl_2$. For 100 mL, add: 50 mL 1 *M* KCl; 10 mL 1 *M* Tris-HCl, pH 8.8; 1.5 mL 1 *M* $MgCl_2$; 38.5 mL distilled water; and filter on a 0.22-µm stericup (Millipore, Bedford, MA; cat. no. SCGPUOIRE).
2. *Taq* DNA Polymerase (5 U/µL, Sigma, St. Louis; cat. no. D4545).
3. dNTP (10 m*M* each). For 1 mL, add: 100 µL 100 m*M* dATP (Amersham, Freiburg, Germany; cat. no. 27-2050-03); 100 µL 100 m*M* dCTP (Amersham; cat. no. 27-2060-03); 100 µL 100 m*M* dGTP (Amersham; cat. no. 27-2070-03); 100 µL 100 m*M* dTTP (Amersham; cat. no. 27-2080-03); 600 µL sterile-distilled water. Store as 100-µL aliquots at –20°C.
4. Primers: oligonucleotides (1 µg/µL in H_2O) stored at –20°C. 301-bp Cre PCR product: TK139 (5′-ATTTGCCTGCATTACCGGTC-3′) and TK 141 (5′-ATCAACGTTTTC TTTTCGGA-3′). 194-bp RXRα af2(I) PCR product: UN126 (5′-CAAGGAGCCTC CTTTCTCTA-3′) and RR189 (5′-AAGCGCATGCTCCAGACTGC-3′). 183-bp RXRαaf2(II) and 138-bp wild-type (+) PCR products: UN126 and UN127 (5′-CCTGCTCTACCTGGT GACTT-3′). 701-bp RXRα L2 and 669-bp RXRα wild-type (+) PCR products: ZO243 (5′-TCCTTCACCAAGCACATCTG-3′) and ZO244 (5′-TGCAGCCCTCACAACTGTAT-3′). 426-bp RXRα L-PCR product: ZO243 and UD196 (5′-TCACCTGGACTTGTCACC TAG-3′). 301-bp ROSA PCR product: VD23 (5′-CGCCGACGGCACGCTGATTG-3′) and VD24 (5′-GTTTCAATATTGGCTTCATC-3′).
5. Dispase (Invitrogen, Karlsruhe, Germany; cat. no. 17105-041).

2.5. Tamoxifen Treatment

Prepare a 1 mg/mL tamoxifen solution by suspending 10 mg tamoxifen (Sigma; cat. no. T-5648) in 1 mL ethanol, followed by the addition of 9 mL sunflower oil (grocery store). Vortex for 2 min and sonicate for 30 min. Store as aliquots at –20°C for several months.

2.6. Cre Immunohistochemistry

1. Cryomatrix (Frozen Embedding Resin; Shandon Inc, Pittsburg, PA; cat. no. 6769006).
2. Dulbecco's phosphate-buffered saline (PBS; Sigma; cat. no. D5652).
3. CY3-conjugated streptavidine (Jackson ImmunoResearch, West Grove, PA; cat. no. 016-160-084).
4. Diamidino Phenylindole (Roche DIAGNOSTICS, Mannheim, Germany; cat. no. 236276).
5. Normal goat serum (Vector, Burlingame, CA; cat. no. S-1000).
6. Vectashield Mounting Medium (Vector; cat. no. H-1000).

2.7. LacZ Staining

1. Fixation solution (freshly made at 4°C): 2.5 mL formaldehyde (40%); 0.4 mL glutaraldehyde (25%); and 47.1 mL PBS (1X).
2. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Roche Diagnostics; cat. no. 745740). Stored at 40 mg/mL in dimethylformamide at –20°C (protected from light).

3. β-Galactosidase staining solution (prepared just before use): 43.65 mL PBS (1X), 2.5 mL K-ferricyanide (0.1 *M*), 2.5 mL K-ferrocyanide (0.1 *M*), 0.1 mL $MgCl_2$ (1 *M*), and 1.25 mL X-gal stock solution (40 mg/mL) filtered with a 0.22-µm stericup (Millipore) and protected from light.
4. Safranin (Merck, Darmstadt, Germany; cat. no. 1.15948; 0.05% in H_2O).
5. Histosol plus (Shandon Inc; cat. no. histol + I).

2.8. Instrumentation

1. Thermocycler (Gene Amp PCR system 9700; Perkin-Elmer Corporation, Wellesley, MA).
2. Cryostat (Leica, Wetzlar, Germany; CM 3050S).
3. Fluorescent microscope (Leica DM LB Type 307-072.057).

3. Methods

An example of cell-specific temporally controlled somatic mutagenesis, targeting floxed genes in basal epidermal keratinocytes, is detailed.

3.1. Establishment of Transgenic Mice Expressing Cre-ER^{T2} in Basal Epidermal Keratinocytes

3.1.1. Production of K14-Cre-ER^{T2} Transgenic Mice

To express Cre-ER^{T2} in epidermal basal keratinocytes, transgenic mice harboring Cre-ER^{T2} under the control of the Keratin K14 (K14) regulatory sequences are established. To this end, a transgene encompassing the 2-kb human K14 promoter/enhancer that is active in the dividing basal layer keratinocytes of the epidermis, the outer root sheath of hair follicles, and some other stratified squamous epithelia (e.g., oral and tongue epithelia; **ref. *15***), located upstream of the Cre-ER^{T2} encoding sequence **(Fig. 1A)**, was injected into C57BL/6 x SJL zygotes ***(16)***. The detailed description of the methods used to construct the transgene and establish transgenic founder mice (F0) is not within the scope of this chapter (*see* **refs. *17*** and ***18***).

3.1.2. Identification of K14-Cre-ER^{T2} Transgenic Founder Animals

1. Take 0.5-cm tail biopsies from 2-wk-old F0 mice.
2. Extract genomic DNA: Add 250 µL proteinase K digestion buffer and 7.5 µL proteinase K stock solution (10 mg/mL) to tail biopsies and incubate at 55°C overnight. Add an equal volume of phenol/chloroform (1 : 1), mix well and microfuge at 15,000*g* for 5 min at room temperature. Precipitate the supernatant with 500 µL ethanol (95%), microfuge at 15,000*g* for 5 min at room temperature. Wash the deoxyribonucleic acid (DNA) pellet with 70% ethanol, air-dry, and dissolve in 100 µL H_2O. DNA can be stored at 4°C for weeks.
3. PCR genotyping. One PCR contains the following in a 0.2-mL thermocycler tube: 3 µL 10X PCR buffer; 0.6 µL dNTP (10 m*M* each); 0.06 µL 5′-primer (1 µg/µL) (TK 139); 0.06 µL 3′-primer (1 µg/µL) (TK 141); 0.2 µL *Taq* polymerase; 1 µL genomic DNA; and 20 µL H_2O for a total volume of 25 µL. Place the tubes in a thermocycler, and run the following procedure: 94°C, 5 min; 94°C, 20 s/55°C 30 s/72°C 5 s for 28 cycles; 72°C, 5–10 min.

Analyze the PCR products on an ethidium bromide-stained 2.0% agarose gel ***(19)***. A 301-bp product is indicative for the presence of the transgene (**Fig. 1A**, and data not shown), and thus allows the identification of K14-Cre-ER^{T2} founder mice.

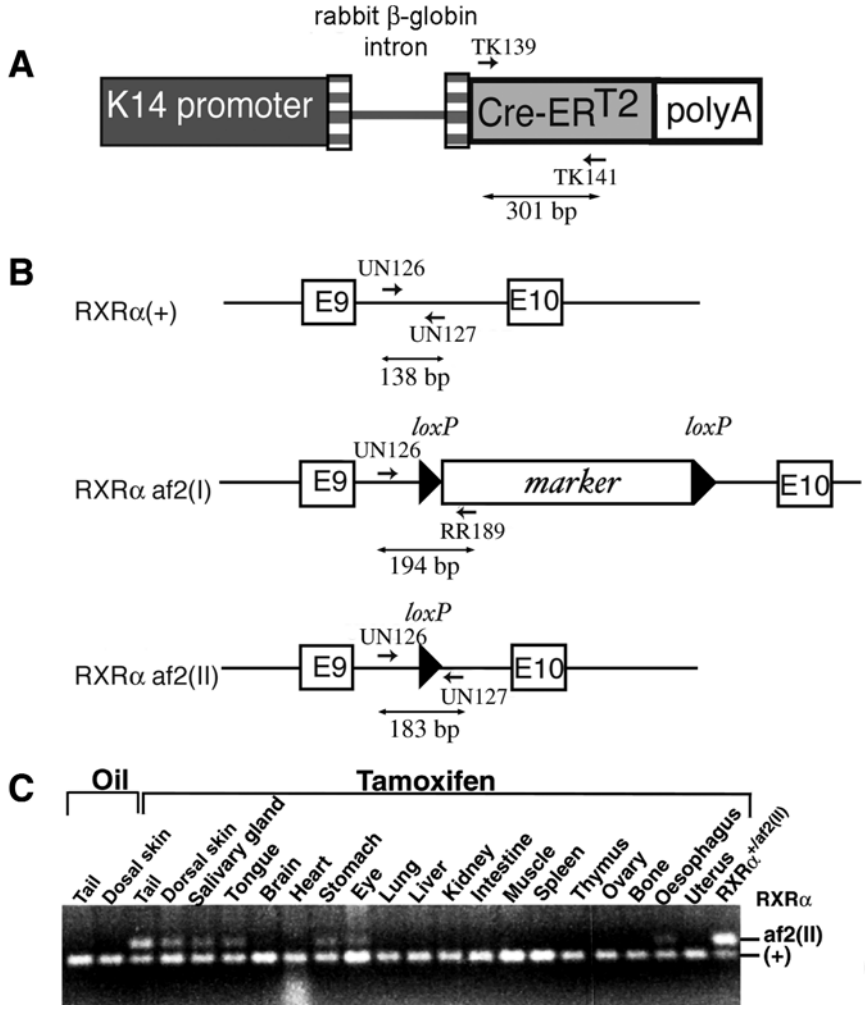


Fig. 1. Characterization of the K14-Cre-ER^{T2} transgenic line. **(A),** Structure of the K14-Cre-ER^{T2} transgene. The human K14 promoter, the Cre-ER^{T2} coding sequence and the simian virus 40 polyadenylation signal (polyA) are represented by black, grey, and open boxes, respectively. The rabbit β-globin intron and splice donor and acceptor sites are depicted by a line and hached boxes, respectively. **(B),** Genomic structure of the RXRα WT, the RXRα af2(I) target allele and the recombined RXRα af2(II) allele, and PCR strategies to identify these RXRα alleles. **(C),** PCR detection of Tam-induced Cre-mediated DNA excision in mice. PCR was performed on DNA isolated from the indicated organs 1 d after oil- (vehicle) or Tam-treated K14-Cre-ER$^{T2\ (tg/0)}$/RXRα$^{+/af2(I)}$ mice, as well as from tail of RXRα$^{+/af2(II)}$ mice (last lane on the right), as indicated. The position of the PCR products amplified from the WT RXRα allele (+) and the RXRα af2(II) allele are shown.

3.1.3. Identification of Founder Mice Transmitting the Transgene Through the Germ Line

Transgenic founder mice are bred with C57BL/6 mice. 2-wk-old transgenic F1 offsprings are identified by genotyping (*see* **Subheading 3.1.2.**).

3.1.4. Identification of K14-Cre-ERT2 Transgenic Lines Exhibiting Tamoxifen-Dependent Cre Recombinase Activity in Basal Keratinocytes

3.1.4.1. Semiquantitative Analysis of Cre-ER^{T2}-Mediated LoxP-Flanked (floxed) DNA Excision in Various Tissues

To analyze the recombinase activity of Cre-ER^{T2} transgenic mice in various organs, F1 Cre-ER^{T2} mice are bred with mice bearing a floxed DNA segment within one of the two alleles of an autosomal gene, thus allowing for the identification of the WT and recombined alleles by PCR using a single primer pair. For that purpose we use here the RXR$\alpha^{+/af2(I)}$ mice (**ref. *12***; *see* **Fig. 1B**).

1. Breed F1 K14-Cre-ER^{T2} and RXR$\alpha^{+/af2(I)}$ mice.
2. Genotype offsprings as described in **Subheadings 3.1.2.** The PCR primers to identify the RXR$\alpha^{af2(I)}$ allele are UN126 and RR189 (PCR product, 194 bp; **Fig. 1B**).
3. Tamoxifen (Tam) treatment: Inject 8-wk-old K14-Cre-ER$^{T2(tg/0)}$/RXR$\alpha^{+/af2(I)}$ mice intraperitoneally with 100 µL Tam solution (0.1 mg) or oil (vehicle) daily for 5 d with a 1-mL syringe equipped with a 25-G needle.
4. Sacrifice Tam- and oil-treated mice by cervical dislocation 1 d after the last Tam injection and take organs/tissues (e.g., tail, brain, oesophagus, lung, heart, liver, tongue, salivary gland, intestine, kidney, thymus, eye, spleen, muscle, stomach, testis/ovary, and uterus). Genomic DNA is isolated from the various samples and analyzed by PCR as in **Subheading 3.1.2.**, except that UN126 and UN127 are used as PCR primers to amplify the 183 bp recombined RXR$\alpha^{af2(I)}$ allele [RXR$\alpha^{af2(II)}$] and the 138 bp RXRα WT allele (*see* **Fig. 1B**,**C**).
5. K14-Cre-ER^{T2} lines exhibiting, after Tam treatment, efficient conversion of the RXR$\alpha^{af2(I)}$ allele into the RXR$\alpha^{af2(II)}$ allele in the skin and tail and no conversion in vehicle-treated (oil) control animals (**Fig. 1C**, and data not shown) are selected for further analysis (*see* also **Note 1**).

3.1.4.2. Characterization of the Expression Pattern of the Chimeric Cre Recombinase in the Skin of the Selected K14-Cre-ER^{T2} Lines by Immunohistochemistry

1. Inject Tam to K14-Cre-ER^{T2} mice as in **Subheading 3.1.4.1.**, to induce Cre-ER^{T2} nuclear translocation ***(20)***, thus facilitating Cre-ER^{T2} detection. One day after the last Tam injection, take a tail biopsy from which bones are removed (or take shaved dorsal skin). Place the samples into molds filled with Cryomatrix and freeze on dry ice. Store tissue blocks at –20°C until sectioning at 10 µm with a cryostat.
2. Cre Immunohistochemistry
   a. Fix cryosections in 2% paraformaldehyde for 5 min at room temperature.
   b. Wash with PBS/Triton X-100 0.1% (3 × 5 min) and with PBS (5 min).
   c. Incubate for 30 min at room temperature in PBS/5% normal goat serum/Triton X-100 0.1%.
   d. Incubate for 2 h at room temperature or overnight at 4°C with the Biotinylated mouse monoclonal 2CRE 2D8-1-2 (1 : 1000 in PBS/5% normal goat serum/Triton X-100 0.1%).
   e. Wash slides with PBS/Triton-X 100 0.1% (3 × 5 min) and with PBS (5 min).
   f. Incubate with CY3-conjugated streptavidine (1 : 400 in PBS) for 1 h at room temperature.
   g. Wash with PBS/Triton X-100 0.1% (3 × 5 min) and with PBS (5 min).
   h. Mount the slides with Vectashield containing DAPI (10 µg/mL).
   i. Examine by fluorescent microscopy (excitation, 545 nm; emission 610 nm).

3.1.4.3. Characterization of the Recombinase Activity at the Cellular Level in K14-Cre-ER^{T2} Mice

Transgenic mice exhibiting Cre-ER^{T2} expression in most, if not all basal keratinocytes are bred with transgenic lines that express a reporter gene such as *lacZ*, alkaline phosphatase or green fluorescent protein after Cre-mediated floxed DNA excision. Note that the currently established Cre reporter lines (ACZL, RosaR26R, Z/AP, Z/EG etc.) do not allow reporter gene expression in all cells/tissues of adult mice (**refs. *13*,*20–25*** and unpublished results). Thus it is essential to breed the transgenic Cre-ER^{T2} lines to be analysed with appropriate reporter mice. For example, in epidermal keratinocytes, the ACZL ***(25)*** is a good reporter line in suprabasal keratinocytes but not in basal keratinocytes ***(22)***, whereas the RosaR26R (**ref. *13***; hereafter called Rosa$^{fl/+}$) expresses *lacZ* in both basal and suprabasal cells after Cre-mediated recombination ***(14)***.

1. Breed K14-Cre-ER$^{T2(tg/0)}$ with Rosa$^{fl/+}$ mice and select K14-Cre-ER$^{T2(tg/0)}$/Rosa$^{fl/+}$ double transgenic offsprings by PCR genotyping as described in **Subheading 3.1.2.** The primers used for genotyping the ROSA fl allele are VD23 and VD24.
2. Inject 8- to 10-wk-old K14-Cre-ER$^{T2(tg/0)}$/Rosa$^{fl/+}$ mice for 5 d with 0.1 mg Tam or oil as in **Subheading 3.1.4.1., step 3**.
3. Take tail biopsies at d 5, 30, and 60 after Tam treatment and prepare 10-µm cryosections as in **Subheading 3.1.4.2., step 1**.
4. β-gal staining:
   a. Incubate the slides with fixation solution at 4°C for 15 min.
   b. Wash 3 × 5 min with PBS at room temperature.
   c. Incubate in β-galactosidase staining solution overnight at 37°C (protect from light).
   d. Wash 3 × 5 min with PBS at room temperature.
   e. Rinse 2 × 30 s in distilled H_2O (*see* **Note 2**).
   f. Dehydrate the slides in 90% ethanol for 30 s, 100% ethanol for 30 s, 100% ethanol for 2 min, and in Histosol plus 2 × 3 min.
   g. Mount the slides.
   h. Examine by light microscopy.

The absence of X-gal staining in skin of untreated or vehicle treated K14-Cre-ER$^{T2(tg/0)}$/Rosa$^{fl/+}$ mice demonstrates that the recombinase activity of K14-Cre-ER$^{T2(tg/0)}$ mice is tightly controlled ***(8)***.

Suprabasal cells are renewed after 5–10 d in mouse tail epidermis. As keratinocytes of the basal and suprabasal layers of the epidermis and of the outer root sheath of hair follicle are X-gal stained 5 d after the beginning of Tam injection, recombination is rapidly induced by Tam. Furthermore, the X-gal staining observed in most if not all keratinocytes of the epidermis and ORS of the hair follicle 30 and 60 d after Tam treatment shows that recombination is efficiently induced in epidermal stem cells of this K14-Cre-ER^{T2} transgenic line (*see* **ref. *8***).

3.2. RXRα Ablation as an Example of Conditional Somatic Mutagenesis of Target Genes in Epidermal Keratinocytes of Adult Mice

To determine the function of RXRα in epidermal keratinocytes of adult mice, RXRα is conditionally disrupted in basal keratinocytes of 8- to 10-wk-old mice. The following steps are performed:

3.2.1. Establishment of K14-Cre-ER$^{T2(tg/0)}$/RXRα$^{L2/L2}$ and Control Mice

Breed floxed RXRα mice (RXRα$^{L2/L2}$) with K14-Cre-ER$^{T2(tg/0)}$ mice and identify K14-Cre-ER$^{T2(tg/0)}$/RXRα$^{L2/+}$ offsprings by genotyping as described in **Subheading 3.1.2.** ZO243 and ZO244 primers are used to identify RXRα L2 and (+) alleles **(Fig. 2A)**; the PCR program is: 94°C, 5 min; 94°C 10 s/56°C 30 s/72°C 1 min for 32 cycles; 72°C 5–10 min. Breed K14-Cre-ER$^{T2(tg/0)}$/RXRα$^{L2/+}$ mice with RXRα$^{L2/L2}$ and identify K14-Cre-ER$^{T2(tg/0)}$/RXRα$^{L2/L2}$ offspring (pro-mutant mice), as well as K14-Cre-ER$^{T2(tg/0)}$/RXRα$^{L2/+}$ and K14-Cre-ER$^{T2(0/0)}$/RXRα$^{L2/L2}$ littermates (control mice) by genotyping.

3.2.2. Temporally Controlled RXRα Ablation in Epidermal Basal Keratinocytes

Treat 8- to 10-wk-old sex- and age-matched K14-Cre-ER$^{T2(tg/0)}$/RXRα$^{L2/L2}$ and control littermates with oil or Tamoxifen as under **Subheading 3.1.4.1., step 3**. Analyze the excision efficiency of the target gene in the tail epidermis 2 and 8 wk after Tam/oil treatment. To isolate dermis and epidermis from tail biopsies, samples are incubated in Dispase (4 mg/mL in PBS) overnight at 4°C (or for 2 h at 37°C) after bone removal. The dermis is separated from the epidermis with forceps. Genomic DNA is extracted and analyzed by PCR as in **Subheading 3.2.1.** Primers ZO243 and UD196 are used to amplify the RXRαL^{-} allele **(Fig. 2A)**.

The example shown in **Fig. 2B** demonstrates that RXRα L2 alleles were fully converted into RXRα L^{-} alleles in the epidermis, but not in the dermis of K14-Cre-ER^{T2} expressing mice, 2 wk after Tam treatment, demonstrating the selectivity and efficiency of the K14-Cre-ER^{T2} transgene for mediating somatic ablation of RXRα in epidermal keratinocytes after Tam-treatment (RXRα$^{ep-/-}$ mice). The absence of L2 to L^{-} allele conversion in control mice expressing the K14-Cre-ER^{T2} transgene without Tam treatment shows that RXRα ablation is strictly dependent on Tam **(Fig. 2B)**. Moreover, the presence of only RXRα L^{-} alleles in K14-Cre-ER$^{T2(tg/0)}$/RXRα$^{L2/L2}$ mouse epidermis 8 wk after Tam-treatment indicates that RXRα was disrupted in most, if not all epidermal stem cells (data not shown).

Efficient RXRα protein ablation in the interfollicular keratinocytes and outer root sheath of the hair follicle in Tam-treated K14-Cre-ER$^{T2(tg/0)}$/RXRα$^{L2/L2}$ mice can be verified in skin biopsies by immunohistochemistry using an antibody directed against RXRα, according to standard procedures (*see* **ref. *8***).

The phenotypic analysis of mice in which a target gene is ablated in adult mouse skin can be investigated by macroscopic examination and histological analysis. For example, weekly examination of mutant and control mice revealed hair loss in the ventral region of K14-Cre-ER$^{T2(tg/0)}$/RXRα$^{L2/L2}$ mice 6–7 wk after Tam treatment but not in oil-treated mice of same genotype or in Tam-treated K14-Cre-ER$^{T2(tg/0)}$/RXRα$^{L2/+}$ mice. Twelve to sixteen wk after Tam treatment, large regions of ventral skin and smaller regions of dorsal skin of mutant animals were hairless. Cysts became visible under the skin surface and these enlarged and spread all over the body with time. With increasing age (>20 wk), minor focal lesions appeared on hairless dorsal skin, on chins and behind the ears. Histology of hairless skin showed disappearance of hair follicles and presence of utriculi and dermal cysts. Abnormal keratinocyte proliferation and differentiation was also observed, as well as inflammatory reactions ***(8,16)***.

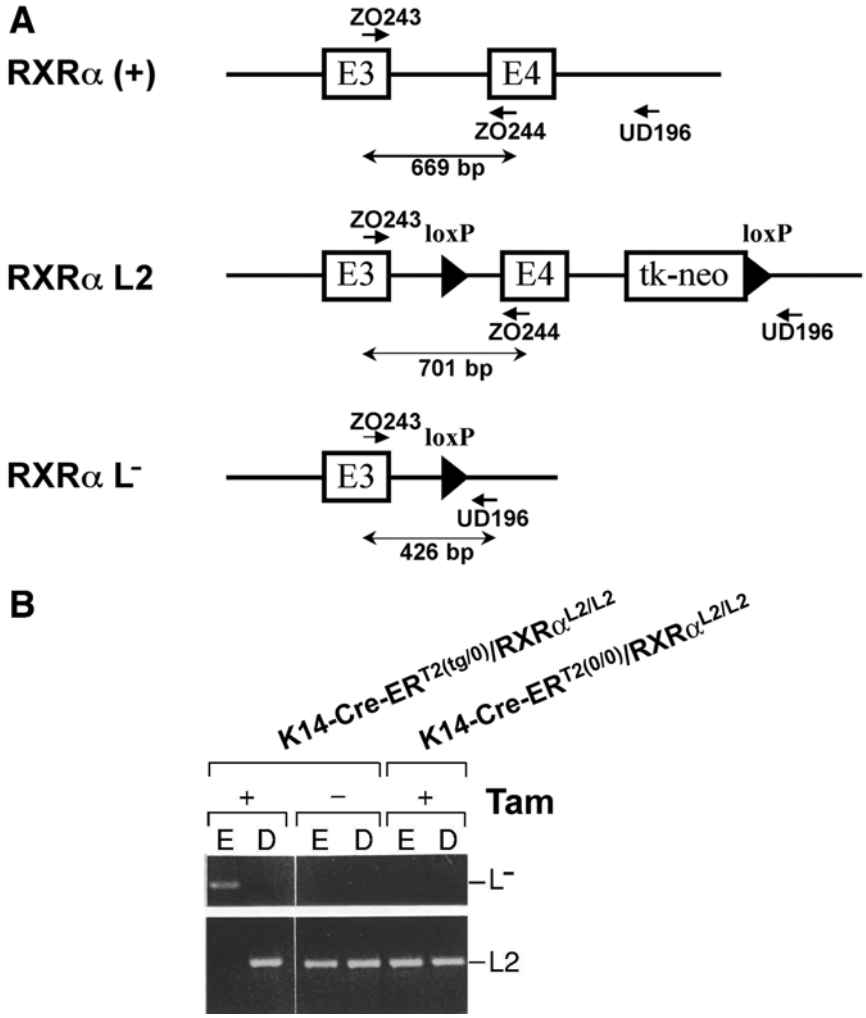


Fig. 2. Temporally controlled RXRα ablation in epidermal keratinocytes. Schematic diagram of the RXRα WT (+) genomic locus, the floxed RXRα L2 allele, and the RXRα L⁻ allele obtained after Tam-induced Cre-mediated excision of exon 4. PCR primers to identify the RXRα +, L2, and L⁻ alleles are indicated. Arrowheads represent LoxP sites. (B) Efficiency of K14-Cre-ER^{T2}-mediated RXRα recombination in adult skin. L2 and L⁻ RXRα alleles were identified by PCR analysis of genomic DNA extracted from epidermis "E" or dermis "D" isolated from tail two weeks after administration of either Tam (+) or oil (vehicle; –) to K14-Cre-ER$^{T2(tg/0)}$/RXRα$^{L2/L2}$ and K14-Cre-ER$^{T2(0/0)}$/RXRα$^{L2/L2}$ mice, as indicated. The PCR fragments corresponding to the RXRα L2 and L⁻ alleles are displayed.

3.3. Conclusion

The K14-Cre-ER^{T2} mice established and characterized according to the described procedure very efficiently induce Cre-mediated floxed DNA recombination in basal keratinocytes after a Tam treatment at low concentration (0.1 mg for 5 d). Importantly, no background recombination was detected in the absence of Tam administration, thus allowing a tight temporal control of the appearance of the genetic modification.

Induction of the recombinase activity of Cre-ER^T proteins is not only effective in basal keratinocytes but in all cell types tested up to now. Over the last years we have established a number of mouse lines allowing temporally controlled somatic mutagenesis in various cell types and tissues (e.g. adipocytes, hepatocytes, nervous system, male germ cells; **refs.** ***21,26–28)***. One major drawback is the availability of promoter-containing segments that allow the expression of transgenes selectively in all cells of a given cell type. Indeed, using small promoter regions, no or mosaic transgenic expression is often observed. Flanking the transgene with insulator sequences or using large genomic promoter-containing segments, present in Bacs or Pacs, known to reduce gene silencing, might facilitate the establishment of potent Cre-ER^{T2} transgenic mice ***(29–31)***.

Temporally controlled somatic mutagenesis in other skin cell types might be achieved by establishing transgenic mice expressing Cre-ER^{T2} under the control of previously characterised promoters, for instance, K1 or K10 for suprabasal keratinocytes ***(32,33)***; Versican for dermal papilla ***(34)***; tyrosinase for melanocytes ***(35)***.

4. Notes

1. Recombination in the tongue, salivary gland, eye, stomach, and oesophagus is in agreement with the previously described promoter activity of the human K14 promoter ***(15)***.
2. Optional: counterstain with safranin (0.05%) for 1 min and wash 3 × 30 s in distilled H_2O.

Acknowledgments

We are grateful to P. Soriano for the generous gift of the RosaR26R mice. We thank present and past members of the laboratory who have contributed to developing the foregoing protocols, as well as E. Metzger and A. Van Es and the animal facility staff for animal care, the secretariat staff for typing, and the illustration staff for preparing the figures. This work was supported by funds from the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale, the Collège de France, the Hôpital Universitaire de Strasbourg, the Association pour la Recherche sur le Cancer, the Fondation pour la Recherche Médicale, the Human Frontier Science Program, and the Ministère de l'Éducation Nationale de la Recherche et de la Technologie.

References

1 Metzger, D., and Chambon, P. (2001) Site- and time-specific gene targeting in the mouse. *Methods* **24,** 71–80.
2 Jonkers, J. and Berns, A. (2002) Conditional mouse models of sporadic cancer. *Nat. Rev. Cancer* **2,** 251–265.
3 Rajewsky, K., Gu, H., Kuhn, R., Betz, U. A., Muller, W., Roes, J., et al. (1996) Conditional gene targeting. *J. Clin. Invest.* **98,** 600–603.
4 Lewandoski, M. (2001) Conditional control of gene expression in the mouse. *Nat. Rev. Genet.* **2,** 743–755.
5 Zhu, Z., Zheng, T., Lee, C. G., Homer, R. J., and Elias, J. A. (2002) Tetracycline-controlled transcriptional regulation systems: advances and application in transgenic animal modeling. *Semin Cell Dev. Biol.* **13,** 121–128.
6 Metzger, D. and Feil, R. (1999) Engineering the mouse genome by site-specific recombination. *Curr. Opin. Biotechnol.* **10,** 470–476.
7 Garcia, E. L. and Mills, A. A. (2002) Getting around lethality with inducible Cre-mediated excision. *Semin. Cell Dev. Biol.* **13,** 151–158.

8 Metzger, D., Indra, A. K., Li, M., Chapellier, B., Calleja, C., Ghyselinck, N., et al. (2003) Targeted conditional somatic mutagenesis in the mouse: temporally-controlled knock out of retinoid receptors in epidermal keratinocytes. *Meth. Enzymol.* **364,** 379–408.
9 Fuchs, E. (1997) Keith R. Porter Lecture, 1996. Of mice and men: genetic disorders of the cytoskeleton. *Mol. Biol. Cell* **8,** 189–203.
10 Hardy, M. H. (1992) The secret life of the hair follicle. *Trends Genet.* **8,** 55–61.
11 Paus, R. and Cotsarelis, G. (1999) The biology of hair follicles. *N. Engl. J. Med.* **341,** 491–497.
12 Mascrez, B., Mark, M., Dierich, A., Ghyselinck, N. B., Kastner, P., and Chambon, P. (1998) The RXRalpha ligand-dependent activation function 2 (AF-2) is important for mouse development. *Development* **125,** 4691–4707.
13 Soriano, P. (1999) Generalized LacZ expression with the ROSA26 Cre reporter strain. *Nat. Genet.* **21,** 70–71.
14 Li, M., Chiba, H., Warot, X., Messaddeq, N., Gérard, C., Chambon, P., et al. (2001) RXR-alpha ablation in skin keratinocytes results in alopecia and epidermal alterations. *Development* **128,** 675–688.
15 Vassar, R., Rosenberg, M., Ross, S., Tyner, A., and Fuchs, E. (1989) Tissue-specific and differentiation-specific expression of a human K14 keratin gene in transgenic mice. *Proc. Natl. Acad. Sci. USA* **86,** 1563–1567.
16 Li, M., Indra, A. K., Warot, X., Brocard, J., Messaddeq, N., Kato, S., et al. (2000) Skin abnormalities generated by temporally controlled RXRalpha mutations in mouse epidermis. *Nature* **407,** 633–636.
17. Sambrook, J. and Russel, D. W. (2001) *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
18. Nagy, A., Gertsenstein, M., Vintersten, K., and Behringer, R. (2003) *Manipulating the Mouse Embryo. A Laboratory Manual*, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
19 Feil, R., Brocard, J., Mascrez, B., LeMeur, M., Metzger, D., and Chambon, P. (1996) Ligand-activated site-specific recombination in mice. *Proc. Natl. Acad. Sci. USA* **93,** 10,887–10,890.
20 Brocard, J., Warot, X., Wendling, O., Messaddeq, N., Vonesch, J. L., Chambon, P., et al. (1997) Spatio-temporally controlled site-specific somatic mutagenesis in the mouse. *Proc. Natl. Acad. Sci. USA* **94,** 14,559–14,563.
21 Weber, P., Metzger, D., and Chambon, P. (2001). Temporally controlled targeted somatic mutagenesis in the mouse brain. *Eur. J. Neurosci.* **14,** 1777–1783.
22 Indra, A. K., Warot, X., Brocard, J., Bornert, J. M., Xiao, J. H., Chambon, P., et al. (1999) Temporally-controlled site-specific mutagenesis in the basal layer of the epidermis: comparison of the recombinase activity of the tamoxifen-inducible Cre-ER(T) and Cre-ER(T2) recombinases. *Nucleic Acids Res.* **27,** 4324–4327.
23 Lobe, C. G., Koop, K. E., Kreppner, W., Lomeli, H., Gertsenstein, M., and Nagy, A. (1999). Z/AP, a double reporter for cre-mediated recombination. *Dev. Biol.* **208,** 281–292.
24 Novak, A., Guo, C., Yang, W., Nagy, A., and Lobe, C. G. (2000) Z/EG, a double reporter mouse line that expresses enhanced green fluorescent protein upon Cre-mediated excision. *Genesis* **28,** 147–155.
25 Akagi, K., Sandig, V., Vooijs, M., Van der Valk, M., Giovannini, M., Strauss, M., et al. (1997) Cre-mediated somatic site-specific recombination in mice. *Nucleic Acids Res.* **25,** 1766–1773.
26 Weber, P., Schuler, M., Gérard, C., Mark, M., Metzger, D., and Chambon, P. (2003) Temporally controlled site-specific mutagenesis in the germ cell lineage of the mouse testis. *Biol. Reprod.* **68,** 553–559.

27 Imai, T., Jiang, M., Chambon, P., and Metzger, D. (2001) Impaired adipogenesis and lipolysis in the mouse upon selective ablation of the retinoid X receptor alpha mediated by a tamoxifen-inducible chimeric Cre recombinase (Cre-ERT2) in adipocytes. *Proc. Natl. Acad. Sci. USA* **98,** 224–228.

28 Imai, T., Jiang, M., Kastner, P., Chambon, P., and Metzger, D. (2001). Selective ablation of retinoid X receptor alpha in hepatocytes impairs their lifespan and regenerative capacity. *Proc. Natl. Acad. Sci. USA* **98,** 4581–4586.

29 Yang, X. W., Model, P., and Heintz, N. (1997) Homologous recombination based modification in Escherichia coli and germline transmission in trangenic mice of a bacterial artificial chromosome. *Nat. Biotechnol.* **15,** 859–865.

30 Bell, A. C., West, A. G., and Felsenfeld, G. (2001) Insulators and boundaries: versatile regulatory elements in the eukaryotic genome. *Science* **291,** 447–450.

31 Giraldo, P., and Montoliu, L. (2001) Size matters: use of YACs, BACs and PACs in transgenic animals. *Transgenic Res.* **10,** 83–103.

32 Bailleul, B., Surani, M. A., White, S., Barton, S. C., Brown, K., Blessing, M., et al. (1990) Skin hyperkeratosis and papilloma formation in transgenic mice expressing a ras oncogene from a suprabasal keratin promoter. *Cell* **62,** 697–708.

33 Greenhalgh, D. A., Rothnagel, J. A., Quintanilla, M. I., Orengo, C. C., Gagne, T. A., Bundman, D. S., et al. (1993) Induction of epidermal hyperplasia, hyperkeratosis, and papillomas in transgenic mice by a targeted v-Ha-ras oncogene. *Mol. Carcinog.* **7,** 99–110.

34 Kishimoto, J., Ehama, R., Wu, L., Jiang, S., Jiang, N., and Burgeson, R. E. (1999) Selective activation of the versican promoter by epithelial-mesenchymal interactions during hair follicle develoment. *Proc. Natl. Acad. Sci. USA* **96,** 7336–7341.

35. Porter, S. D., and Meyer, C. J. (1994) A distal tyrosinase upstream element stimulates gene expression in neural-crest-derived melanocytes of transgenic mice: position-independent and mosaic expression. *Development* **120,** 2103–2111.

34

Methods to Study Protein–Protein Interactions

Jin-Jun Meng, Meghan Rojas, Willis Bacon, John T. Stickney, and Wallace Ip

Summary

Protein–protein interactions are the underpinnings of a vast number of cellular processes. In recent years, the convergence of biochemistry, cellular, and molecular biology has made available a number of powerful techniques for studying such interactions. These techniques vary in their sensitivity, efficiency, and rapidity, but judicial deployment of a combination of them has proved to be effective and reliable. Here, we highlight a version of the yeast two-hybrid assay originally pioneered by Fields and Song (1989) and subsequently enhancements by other investigators. We also briefly describe a number of new fluorescent imaging-based biophysical techniques for studying protein–protein interactions—FRET, FCS, and BiFC. Together, these constitute an impressive collection of tools for studying interactions among proteins.

Key Words:

Two-hybrid assays; protein interactions; fluorescence; imaging.

1. Introduction

The skin is a dynamic organ, and the epidermis is arguably the most dynamic of the three dermal compartments. Epidermal cells divide, differentiate, and die, all in an exquisitely orchestrated series of events that culminates in the formation of a protective layer for the body that effectively safeguards against moisture, infection, and mechanical trauma. The vast majority of these events are mediated by interactions among proteins, and understanding these interactions is key to a full appreciation of the development and function of the skin.

The protein–protein interactions in the epidermis are extremely complex. An example may be found in one of the final steps in the terminal differentiation of an epidermal cell—the formation of an insoluble proteinaceous layer subjacent to the plasma membrane called the cell envelope. This process involves more than 20 proteins and follows a tightly regulated sequence of events ***(1)***. Moreover, the interactions are not limited to binary interactions, but often involve large, multiprotein complexes in which some of the associations are permanent whereas others are transient. The experimental approaches that one must use to study these interactions are accordingly varied and diverse. In the following pages, we discuss two broad types of approaches—two-hybrid assays and microscope-based biophysical assays—and discuss their relative merits and pitfalls. Most of these approaches are ones that are commonly used to study proteins in general,

From: *Methods in Molecular Biology, vol. 289: Epidermal Cells: Methods and Protocols*
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

although when applied to cytoskeletal proteins certain limitations must be kept in mind. These will be identified and discussed as they arise. A theme that we wish to emphasize is that the combination of approaches, rather than any single method, is the most successful and reliable means to learn about how proteins interact with one another. In our experience, it is no longer the case that a laboratory has to acquire all the expertise and resources before embarking on a new experimental approach. Most research institutions now have core facilities to assist in or perform outright many specialized techniques such as protein expression or live cell imaging. Collaborating with experienced colleagues is also an excellent (and scholarly) way to solve one's scientific problems using an approach unfamiliar to one's own laboratory.

1.1. Biochemical Approaches

Biochemical approaches are by far the most extensively used to study protein–protein interactions. The most common biochemical methods are co-immunoprecipitation, glutathione-*S*-transferase (GST) pulldown, chemical crosslinking, and blot overlay. In coimmunoprecipitation, a protein is bound to its cognate antibody, and the antigen–antibody complex, together with any other protein binding partners, are precipitated by treatment with Protein A- or Protein G-coupled agarose beads and analyzed by denaturing gel electrophoresis and western blotting. In a GST pulldown assay, the probe protein is expressed as a fusion protein with GST in bacteria and purified; it is then incubated with a cell lysate containing putative binding partners. The protein complex is then precipitated by treatment with glutathione-coupled beads. Although technically different, both assays rely on the assumption that the proteins involved exist in a complex that is sufficiently stable to survive the cell or tissue fractionation and the assay procedure itself. For example, the identification of a protein complex by co-immunoprecipitation would be successful only if the protein complex remains intact during the antibody treatment, the extensive washing that follows, the binding to Protein A- or Protein G-conjugated beads and the subsequent centrifugation. Chemical crosslinking, however, involves the addition of a bifunctional reagent to covalently crosslink the probe protein to its binding partners and subsequent analysis by denaturing gel electrophoresis and Western blotting. When two proteins are found to have been crosslinked, this is taken as evidence that they interact with one another. A large number of crosslinking reagents are available commercially. Some are zero-length crosslinkers, whereas others specify known distances of separation over which the reagent is effective. Most reagents form crosslinks between e-amine groups of lysines or sulfhydryl groups of cysteine residues. As with the two-hybrid assays discussed below, the assumption that one makes when doing a crosslinking experiment is that when two proteins are within a certain distance of one another, they are interacting. This is most often, but formally not always, the case, and should be borne in mind. In blot overlay experiments, proteins separated on a denaturing polyacrylamide gel electrophoresis gel are transferred to a nitrocellulose or similar membrane and purified, native potential interacting proteins are overlaid on top of the membrane. Bands on the blot that interact with the overlaid protein are then visualized by incubating the blot with antibodies to the overlaid protein. A limitation of this technique is that the proteins separated on the gel have been denatured and may or may not fully renature. The lack of interaction is therefore inconclusive, and any putative interactions detected must be further verified by independent means.

Detailed descriptions of these protocols are widely available and will not be discussed here. The reader is referred to authoritative manuals such as those by Harlow and Lane ***(2)*** and Golemis **(3)**.

1.2. Two-Hybrid Approaches

In recent years, assays based on the two-hybrid principle have become essential tools for studying protein–protein interactions. In general, the yeast two-hybrid approach uses the putative protein–protein interaction (binding) to reconstitute a functionally active molecule, most often a transcription activator in yeast. Reconstitution of this transcription activator stimulates expression of a reporter gene, the product of which can be measured readily. Appearance of the reporter gene product is then taken as indication that the putative protein–protein interaction has occurred. Two prime examples of the two-hybrid assay are the GAL4 system ***(4)*** and the lexA system ***(5)***. In the GAL4 system, the DNA binding (DB) domain and the transcriptional activation (TA) domain of the transcription activator, GAL4, are separately encoded in two vectors. The complimentary deoxyribonucleic acid (cDNA) encoding the proteins whose interaction is to be studied are subcloned into the two vectors such that they are expressed as fusion proteins with either the DB or TA domain. The two vectors are then introduced by transformation into a strain of *Saccharomyces cerevisiae* that has a copy of the *lacZ* gene (which encodes the enzyme, β-galactosidase) driven by the GAL1 promoter. Interaction between the two putative proteins recombines the two domains of GAL4 as a functional transcription factor and drives expression of the *lacZ* reporter gene. A simple colorimetric or fluorescence assay is then used to measure expression of β-galactosidase. Since the original description of the GAL4 two-hybrid assay ***(4)***, numerous others have been constructed, and many of them have been marketed commercially **(Table 1)**. Today, to implement a yeast two-hybrid assay is a simple matter of purchasing the vectors and reagents from a vendor, and performing the subcloning and transformation according to the vendor's instructions.

2. Materials

Materials and recipes that we use in our laboratory are listed in Tables 1–3. We purchase reagents and chemicals from Sigma (St. Louis, MO).

3. Methods

3.1. Yeast Two-Hybrid Methods

Numerous articles have been written on the use of yeast two-hybrid assays, either to study the interaction between two known proteins or to "mine" novel interactions involving a given protein of interest. Two excellent discussions of the method and its variations may be found in Bolger ***(6)*** and a more recent chapter by Serebriiskii and Joung ***(7)*** in Golemis's 2002 manual on protein–protein interactions ***(3)***. Both articles provide detailed descriptions of the vectors, yeast strains, and methods, the latter focusing primarily on the lexA system. We presented a brief description of a GAL4-based two-hybrid method for studying interaction among two or three known proteins ***(8)***, using vectors that originated from Nathans' laboratory ***(9)***. Updated versions of these vectors are now marketed by Invitrogen as part of their ProQuest Two-Hybrid System (http://www.invitrogen.com/content/sfs/manuals/10835023.pdf). Although we do not

Table 1
Partial Listing of Commercially Available Yeast Two-Hybrid Vectors

Name of product	Vector	Bait or target vector	Vendor and Web site	Reference
HybriZAP 2.1	pAD-GAL4-2.1	Target	Strategene http://www.stratagene.com/display Product.asp?productId=256	*4*
HybriZAP 2.1	pBD-GAL4-Cam	Bait	Strategene http://www.stratagene.com/display Product.asp?productId=256	*4*
Hybrid Hunter	pYESTrp2	Target	Invitrogen http://www.invitrogen.com/content/ sfs/manuals/hybrid_man.pdf	*5*
Hybrid Hunter	pHybLex/Zeo	Bait	Invitrogen http://www.invitrogen.com/content/ sfs/manuals/hybrid_man.pdf	*5*
ProQuest	pPC86	Target	Invitrogen http://www.invitrogen.com/content/ sfs/manuals/10835023.pdf	*9*
ProQuest	pDBLeu	Bait	Invitrogen http://www.invitrogen.com/content/ sfs/manuals/10835023.pdf	*9*
MatchMaker 3	pGADT7	Target	Clontech http://www.clontech.com/products/ literature/pdf/brochures/matchmaker.pdf	*4*
MatchMaker 3	pGBKT7	Bait	Clontech http://www.clontech.com/products/ literature/pdf/brochures/matchmaker.pdf	*4*

use the kit in our laboratory, new users of the assay may find the commercial kit more convenient and user-friendly. Any one of the articles mentioned above can be relied upon for detailed, step-by-step instructions to carry out a two-hybrid assay. Here we share our experience with the Nathans system and discuss several methods of measuring β-galactosidase activity. With the exception of vector construction and yeast strains for transformation, most of the methods described below are applicable to two-hybrid assays in general. Recipes for reagents are detailed in **Tables 2** and **3**.

3.1.1. Construction of Vectors, Preparation, and Transformation of Competent Cells

Standard methods are used to clone cDNAs of interest into two-hybrid vectors. We routinely sequence our constructs to ensure that the cDNA insert is in-frame with the DB or TA domain such that a fusion protein is expressed. It is important to use caution when mixing plasmids from different two-hybrid systems. Although many combinations of DB and TA domains will activate transcription, one must ensure that the two desired plasmids possess different selection markers.

3.1.1.1. Preparation of Competent Yeast Cells

We use the lithium acetate method to prepare competent yeast cells, in the following steps.

1. From a freshly spread yeast plate, pick a single colony to inoculate 25 mL of YEPD medium and culture at 28°C overnight in a shaking incubator at 200 rpm until the cells reach mid-log phase (A_{600} = 0.8–1.0, or 0.5–1.0 × 10^7 cells/mL).
2. Pellet the cells at low speed-typically 3000 rpm in a bench-top centrifuge-for 2 min and discard the supernatant.
3. Resuspend the pellet in 10 mL LiOAc solution and pellet the cells again. Repeat two more times.
4. Add 2 mL LiOAc–glycerol solution to the final pellet and gently shake by hand to resuspend the cells.
5. Aliquot the competent cells into 1.5-mL Eppendorf microcentrifuge tubes (50 µL/tube). The competent cells can be either used immediately for transformation or stored frozen at –70°C or lower for future use.
6. It is important to freeze competent cells slowly. A convenient way to do this is to wrap the microcentrifuge tubes containing cells in 8–10 layers of paper towel before placing in the –80° freezer.

3.1.1.2. Yeast Transformation

Either freshly prepared or previously frozen competent cells (thawed at room temperature) can be used for transformation ***(10,11)***. Use the stepwise procedure below:

1. Add 1–2 µL of each vector (0.1–0.5 µg/µL DNA) to 50 µL of competent cells with gently mixing and incubate at room temperature for 5 min. Transforming 50–100 µL of competent cells with 1–3 µg of each DNA usually results in 20–100 well-separated colonies on a MinGal plate.
2. Add 140 µL PEG1500 solution and mix the content thoroughly by inverting the tube four to five times.
3. Place the cells in a 30°C incubator for at least 45 min, then mix the contents by flicking and heat shock the cells in a water bath at 42°C for 5 min.
4. Pellet the cells in a bench-top centrifuge at 3000 rpm for 30 s.
5. Wash the cells twice with 1-mL sterile distilled water, using a pipet to resuspend the cells, and discard the supernatant.
6. Resuspend the cells in 100-µL sterile distilled water.
7. Spread the transformed cells onto a plate containing selective medium, invert the plate and incubate at 28°C for 3–4 d to allow growth of transformants.
8. When the yeast colonies reach 1–2 mm in diameter, use a single colony to inoculate 2 mL of MinGal medium supplemented with the appropriate amino acid drop-out mix.
9. Incubate at 28–30°C with shaking for 48–72 h. This culture can now be used to assay for β-galactosidase activity.

3.1.2. β-Galactosidase Assay

The most common reporter gene used in two-hybrid studies is β-galactosidase. There are many available assays for β-galactosidase activity; the ones described below are used routinely in our laboratory and each has its own advantages and disadvantages. A comparison of the three assays is provided in **Note 1**.

This is a rapid but qualitative assay for β-galactosidase (12). Two disadvantages of this method are that it is not possible to obtain numerical data and that when the blue

Table 2
Media, Reagents, and Buffers

Name	Composition
YEPD medium for liquid culture (500 mL)	5.0 g yeast extract 10 g peptone 300 mL deionized distilled H_2O Autoclave at 110°C for 20 min Cool to 55°C Add 50 mL of 20% glucose Add H_2O to a final volume of 500 mL
YEPD medium for plates (500 mL)	Same as for liquid YEPD medium except add 7.5 g granulated agar before autoclaving.
MinGal medium for liquid culture (per 500 mL)	3.5 g yeast nitrogen base without amino acids 15 mL glycerol 300 mL deionized distilled H_2O Autoclave at 110°C for 20 min and cool to 55°C Add 50 mL amino acid drop-out mix solution Add 50 mL of 20% galactose Add H_2O to a final volume of 500 mL
MinGal medium for plates (per 500 mL)	3.5 g yeast nitrogen base without amino acids 7.5 g granulated agar 15 mL glycerol 300 mL deionized distilled H_2O Autoclave at 110°C for 20 min and cool to 55°C Add 50 mL amino acid drop-out mix solution Add 50 mL 20% galactose Add 2.5 mL 20% glucose Add H_2O to a final volume of 500 mL
MinGlu medium for broth (per 500 mL)	3.5 g yeast nitrogen base without amino acids 15 mL glycerol 300 mL deionized distilled H_2O Autoclave at 110°C for 20 min and cool to 55°C Add 50 mL amino acid drop-out mix solution Add 50 mL 20% glucose Add H_2O to a final volume of 500 mL
MinGlu medium for plates (per 500 mL)	Same as for liquid MinGlu medium except add 7.5 g granulated agar before autoclaving
X-gal stock solution	20 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (GIBCO-BRL) dissolved in *N*,*N*-dimethylformamide. Store at –20°C in the dark.
ONPG test solution	4 mg/mL *o*-nitrophenyl β-D-galactopyranoside (ONPG) (Sigma) in 0.1 *M* potassium phosphate, pH 7.0
4-MUG stock solution	4-methylumbelliferyl-galactopyranoside, 1 m*M* in 10 m*M* phosphate buffer, pH 7, 1 m*M* $MgCl_2$. 4-MUG should be made fresh. It dissolves very slowly and we usually place the solution in a light-proof container and allow it to stir overnight, or at least 3–4 h before using.

(continued)

Table 2 *(continued)*

Name	Composition
50% Polyethylene glycol, m.w. 1500 (PEG1500)	4 mL/vial, sterile, fusion tested (Boehringer Mannheim, Germany). Add 1 mL of LiOAc solution before using. Stable for 2 wk at 4°C. Glass beads, acid-washed, 425–600 microns (Sigma). 15% glycerol in LiOAc solution, 0.2 µm filter-sterilized
LiOAc-glycerol solution	
Yeast lysis buffer	20 m*M* Tris-HCl, pH 7.5, 50 m*M* KCl, 5 m*M* $MgCl_2$, 0.1 m*M* EDTA, 0.5 m*M* dithiothreitol (DTT), 10% (v/v) glycerol, 1 m*M* phenylmethylsulfonyl fluoride (PMSF), 2 µ*M* pepstatin A, and 0.6 µ*M* leupeptin

Table 3
Stock Solutions for Amino Acid Drop-Out Mix

Amino Acid	Stock solution (g/100 mL)	Milliliters of Stock Solution per Liter of Medium
Adenine sulfate	0.4	5
L-glutamic acid	1.0	20
L-arginine	1.0	2
L-aspartic acid	1.0	20
L-histidine	1.0	2
L-leucine	1.0	3
L-isoleucine	1.0	3
L-lysine HCl	1.0	3
L-methionine	1.0	2
L-phenylalanine	1.0	5
L-serine	8.0	4
L-threonine	4.0	4
L-tryptophan	1.0	2
L-tyrosine	0.2	15
Uracil	0.2	10
L-valine	3.0	5

To make a drop-out mix solution, combine the appropriate volumes of all amino acid stock solutions except the one or more to be selected for. Substitute an equivalent volume of water to achieve the final volume.

coloration is faint the decision as to whether a yeast colony is positive often becomes subjective.

1. Grow transformed yeast on MinGal plates supplement with the appropriate amino acid drop-out mix at 28°C for 2–3 d.
2. When colonies reach 1–2 mm in diameter, carefully lay an autoclaved nitrocellulose membrane on top of the plate.
3. Leave the membrane on the plate in a 30°C incubator for 30 min.

4. Using blunt forceps, carefully peel the membrane away form the agar surface. Immerse the membrane, colony side up, in an ice bucket containing liquid nitrogen for 5 s to permeabilize the cells.
5. Thaw the nitrocellulose membrane at room temperature and place the colony side against a disk of Whatman 3MM filter paper soaked with 2 ml Z buffer containing 1 mg/mL X-gal in a 100-mm Petri dish.
6. Cover the dish and incubate at 37°C for 10–60 min to develop the blue color.

3.1.2.2. ONPG Assay

This colorimetric method is quantitative and uses *o*-nitrophenyl β-D-galactopyranoside (ONPG) as substrate ***(13)***. Enzymatic cleavage of the substrate releases a bright yellow reaction product that can be read at 420 nm in a standard spectrophotometer.

1. The assay should be performed in triplicate. Use a single colony of yeast transformants grown on a MinGal plate supplement with the appropriate amino acid drop-out mix to inoculate 2 mL of liquid medium.
2. Incubate at 28°C with shaking for 2 to 3 d until the cells reach mid-log phase (A_{600} = 0.8–1.0).
3. Record A_{600}, then pipet 0.1 mL yeast culture in a 1.5-mL microcentrifuge tube, add 0.8 mL Z buffer, 0.1 mL chloroform and 50 µL of 0.1% sodium dodecyl sulfate, and vortex for 30 s.
4. Add 0.15 mL of ONPG test solution and vortex well.
5. Incubate at 30°C for 60 min or until the yellow color is developed.
6. Add 0.3 mL of 1 *M* Na_2CO_3 to stop the reaction.
7. Centrifuge at 12,000 rpm in a microcentrifuge to clear cell debris, and carefully remove the supernatant to a fresh tube.
8. Read the absorbance at 420 nm in a spectrophotometer.
9. Calculate the β-galactosidase activity using the following equation:

$$\text{Activity (U/min•mL)} = [A_{420}/t \times V \times A_{600}] \times 1000$$

where A_{420} = absorbance at 420 nm, A_{600} = absorbance of yeast culture at 600 nm, t = time of incubation with ONPG in minutes, V = volume (in milliliters) of yeast culture used in assay.

3.1.2.3. Fluorescence Assay

This assay uses the fluorescent substrate, 4-methylumbelliferyl β-D-galactopyranoside (4-MUG; **ref. *14***). Enzymatic cleavage produces a reaction product that emits at 450 nm when excited at 360 nm. In our experience this is the most sensitive method of detecting β-galactosidase activity and thus lends itself most readily to discriminating between weak interactions and noninteractions in two-hybrid transformations.

1. The assay should be performed in triplicate. Use a single colony of yeast transformants grown on a MinGal plate supplement with the appropriate amino acid drop-out mix to inoculate 2 mL of liquid medium.
2. Incubate at 28°C with shaking for 2 to 3 d until the cells reach mid-log phase (A_{600} = 0.8–1.0).
3. Record A_{600} of the culture.
4. Shake the yeast culture well and pipet 0.1 mL into a 1.5-mL microcentrifuge tube.
5. Pellet the cells at 12,000 rpm for 30 s and carefully remove and discard the supernatant.
6. Permeabilize the yeast cells by freezing in liquid nitrogen for 2 min, then thawing at room temperature, or by placing them in a –20°C or –70°C freezer for several hours, then adding 3/4 vol of acid-washed glass beads (425–600 microns) and vortex rigorously.

7. Add 350 µL 4-MUG reaction buffer (10 m*M* phosphate, pH 7.5; 1 m*M* $MgCl_2$, 0.1% [w/v] bovine serum; and 0.1% NaN_3) and 50 µL 1 m*M* 4-MUG in 10 m*M* phosphate buffer, pH 7.0, 1 m*M* $MgCl_2$.
8. Vortex and incubate at 37°C for 60 min.
9. Add 400 µL 4-MUG stop solution (0.1 *M* glycine, pH 10.3) to terminate the reaction.
10. Centrifuge the reaction tube at top speed in a microcentrifuge for 1 min.
11. Carefully remove the supernatant to a fresh tube for fluorescence reading.
12. In a fluorescence spectrophotometer set for excitation at 360 nm, record the emission of each sample at 450 nm.
13. Fluorescence is readily detectable after 5 min of incubation with 4-MUG and increases steadily with time. After termination of the reaction by adding stop solution, the fluorescence remains stable for at least 4 h. The total change in fluorescence after 16 h of storage is less than 10%.
14. Calculate the β-galactosidase activity using the following equation:

$$\text{Activity (U•mL)} = 1.62 \times [F \times M/V \times A_{600}]$$

where F = fluorescence emission at 450 nm, in arbitrary units; M = dilution factor of the sample before reading; V = the volume (in milliliters) of yeast sample used in assay; and A_{600} = absorbance of the yeast culture at 600 nm.

3.1.3. Controls

It is of great importance to include all necessary controls in two-hybrid assays. These assays are sometimes technically challenging and there are a number of pitfalls (*see* **Notes 2–4**). At a minimum, controls should include the following:

1. *A positive control.* Commercially available two-hybrid kits often include control plasmids that encode a pair of proteins known to interact with one another. Positive reporter gene activity resulting from this transformation will ensure that all the reagents are in working order.
2. *Transformation with the cDNA insert omitted from one of the two-hybrid plasmids.* No reporter gene activity should be detectable. If a significant signal is detected in a transformation with one insert-containing and one blank vector, the cDNA insert likely encodes a polypeptide that activates transcription, or is nonspecifically sticky. In this case, trying a different set of two-hybrid vectors may be the most expedient solution. A more laborious but more systematic solution is to replace the full-length protein with increasingly truncated versions, in the hope that the truncation eventually removes the offending region of the protein.
3. *Transformation with the cDNA inserted into the other two-hybrid vector.* Swapping cDNA inserts and vectors should not alter the reporter gene activity significantly. We have found that, in general, a 10–15% variation is tolerable. In cases where the variation exceeds this practical limit, the result may still be valid, but caution should be exercised and confirmation by other methods should be sought.

A convenient and effective way of confirming a positive interaction is to ascertain that the two fusion proteins do indeed bind one another. This can be accomplished by chemical cross-linking of a protein extract from transformed yeast and demonstrating by Western blotting that a complex of the proper molecular size (the sum of the two proteins plus the DB and TA domains) does exist. Coimmunoprecipitation is also an excellent confirmation of a protein–protein interaction. If available, antibodies to the

proteins of interest should be used. If not, antibodies to the DB and TA domains of GAL4, LexA and others are available commercially and may be used for western blotting.

3.2. Bacterial and Mammalian Two-Hybrid Systems

A brief mention should be made of the bacterial version of the two-hybrid assay, introduced by Dove et al. ***(18)***. A kit for this assay called BacterioMatch is now marketed by Stratagene (http://www.stratagene.com/displayProduct.asp?productId=52). The strategy of the BacterioMatch system is similar to that of a yeast two-hybrid system in that the protein–protein interaction reconstitutes a functional transcriptional activator. In this case, the two proteins of interest are fused to either the bacteriophage lambda repressor protein, λc1, which has a DNA binding domain, or the α-subunit of RNA polymerase. *Escherichia coli* is transformed with the constructs. Upon interaction of the two proteins, λc1 binds to the λ operator and recruits RNA polymerase to the promoter and activates transcription of two reporter genes, an ampicillin resistance gene, Amp^r, and *lacZ*, which encodes β-galactosidase. The readout for interaction is then growth of the *E. coli* in the presence of carbenicillin and expression of β-galactosidase.

A key advantage of the bacterial system is that *E. coli* grows much faster than yeast, so that the entire process takes far less time than using a yeast two-hybrid system. One readout of this assay—the growth of *E. coli* in the presence of carbenicillin, is qualitative, but the other—β-galactosidase activity—can be measured quantitatively.

It should come as no surprise to the reader that a mammalian two-hybrid system is also available. The system (http://www.stratagene.com/displayProduct.asp?productId=509) is marketed by Stratagene and used the CMV promoter to drive expression of the two vectors. The bait vector is a fusion between one protein of interest and the DNA binding domain of GAL4, whereas the target vector encodes a fusion between the other protein of interest and the NF-κB activating domain ***(19)***. The reporter gene consists the luciferase gene preceded by five copies of the GAL4 binding site and a TATA sequence. All three plasmids are co-transformed into a mammalian cell line—COS, HeLa, Chinese hamster ovary, and 293 cells are all suitable. Interaction between the two proteins brings the NF-κB-activating domain into close proximity to the luciferase gene, thereby activating it. The activity of luciferase, which oxidizes luciferin to light-emitting oxyluciferin, is then assayed with a kit available from many vendors (e.g., http://www.Promega.com), using a luminometer.

The mammalian two-hybrid system is particularly useful in confirming putative interactions identified by yeast or bacterial two-hybrid screens, because the interaction takes place within a mammalian cell, thus ruling out artifacts that potentially could arise from working in the nucleus of yeast or in bacteria. Interactions that require posttranslational modification of the proteins of interest are also more likely to occur.

3.3. Imaging-Based Approaches for Studying Protein–Protein Interactions

In this section, we briefly discuss several techniques that we have explored recently and found to be excellent complements to two-hybrid analysis for studying protein–protein interactions. These are techniques based on the use of either a standard wide-field fluorescence microscope or a laser scanning confocal microscope. Key advantages of imaging-based assays are that they are typically carried out in the context of living cells, and that some assays allow one to "visualize" the interaction within the living cell. We

have found that the latter very often provides welcomed spatial information that is lacking in a result derived from standard yeast two-hybrid assays, and we highly recommend this dual approach to understanding protein–protein interactions in depth. Many imaging-based assays take advantage of recent advances in green fluorescent protein (GFP) technology ***(21,22)***. Because of space limitations, only a very cursory introduction is provided here. The World Wide Web links provided with each technique contains either detailed information about the technique or the companies that provides reagents and protocols for them.

3.3.1. Fluorescence Resonance Energy Transfer (FRET)

FRET was recognized more than 25 yr ago as a biophysical technique for measuring distances between macromolecules ***(22)***. It is an interaction between two fluorescent dye molecules in which excitation is transferred from the donor to the acceptor without emission of a photon. Two requirements for this to occur are 1) that the emission spectrum of the donor must overlap the excitation spectrum of the acceptor, and 2) the two molecules must be in very close proximity. The latter is necessary because the efficiency of FRET is dependent on the inverse sixth power of the intermolecular separation ***(22)***. For commonly used donor-acceptor pairs, the typical distance within which FRET occurs is on the order of 5–10 nm. Beyond this range, FRET drops off very rapidly, making it an exquisitely sensitive tool for measuring interactions over distances comparable with the dimensions of biological macromolecules.

Until the advent of GFP technology, FRET was primarily used in a biochemical context with proteins chemically labeled with fluorescent probes. Measurements were made in solution using a fluorescence spectrophotometer ***(23)***. The availability of GFP and its derivatives stimulated development in imaging techniques that led to the current popularity of FRET microscopy. It is now routine to construct expression vectors that encode fusion proteins of cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), whose emission and excitation spectra overlap. Alternatively, the GFP moiety in a GFP-fusion protein can be used as a donor for the fluorescent dye, Cy3, which can be chemically conjugated to a putative protein interacting partner. The former is typically used to assay for interaction between two proteins—the two plasmids encoding CFP and YFP fusions are cotransfected into cultured cells and the presence of FRET is taken to indicate positive interaction. The latter donor-acceptor pair, GFP and Cy3, is suitable for assaying posttranslational modification of a given protein ***(24)***. Typically, the protein of interest is fused to GFP and transfected into cultured cells. An antibody specific for the modified state of the protein, for example, an antibody that recognizes only the tyrosine-phosphorylated state of a given signaling protein, is labeled with Cy3 and either microinjected into living cells, or used to label fixed cells, and FRET is then assayed.

FRET results in the augmentation of acceptor emission and diminution of donor emission. Either parameter can be measured, but each has its own drawback ***(25)***. Direct measurement of increase in acceptor emission is complicated by the fact that, owing to the unavoidable spectral overlap between donor and acceptor excitation, the acceptor is excited not only by the donor emission but also to a small degree by the donor excitation directly. This small component must be subtracted from the total increase in acceptor emission and this requires recording of additional images and background correction. The measurement of the quenching of donor emission resulting from FRET is also done

indirectly, because both the donor and acceptor are in the same cell, making it impossible to establish a pre-FRET level of donor emission. To measure donor quenching, the acceptor dye molecules within an area of a cell are destroyed by photobleaching. This makes that area of the cell incapable of accepting donor emission. As the donor is excited, this area of the cell is expected to have a higher level of emission than the surrounding, non-photobleached areas because of the lack of functional acceptor. This elevation of donor emission in the bleached area upon donor excitation is taken as positive FRET.

FRET can be measured on a standard, wide field fluorescence microscope or a confocal microscope. These instruments are widely available at research institutions. A typical wide field setup may consist of a high-quality research microscope with epi-fluorescence illumination, a high quantum efficiency CCD camera, such as the Hamamatsu Orca ER, imaging software such as Universal Imaging's Metamorph, and appropriate filter sets. An electronic shutter is also helpful. The advantage of a wide field setup for FRET is that it is usually simpler to use than a confocal microscope, but it suffers from two drawbacks. The first is emission signals that originate from above and below the plane of focus, which tends to degrade the final image. The second is that, unlike the case of a confocal microscope, it is not possible to photobleach a small region of a cell using the region of interest feature. In a wide field instrument, one can only photobleach the entire field. FRET using a confocal microscope alleviates these problems, but in turn may be somewhat restricted in the choice of donor/acceptor pairs because the wavelengths of light available for excitation of the donor are limited to the laser lines available in the instrument.

The above is by necessity a very brief overview of FRET. Many excellent reviews and books ***(24–27)*** have been written about FRET, and the reader is encouraged to consult them for theoretical treatment of the subject as well as detailed protocols. The publication by Periasamy ***(25)***, in particular, is an outstanding resource and an essential book for any laboratory interested in applying imaging techniques to study protein–protein interactions.

3.3.2. Bimolecular Fluorescence Complementation (BiFC)

Brief mention should also be made of BiFC, a simple-yet-powerful new method to detect protein–protein interaction that is based on the two-hybrid principle but conducted in the context of a fluorescence microscope ***(28)***. This method takes advantage of the observation that a derivative of green fluorescent protein (GFP), known as yellow fluorescent protein (YFP), can be split into two nonfluorescent fragments that, when recombined, reconstitute a fluorescent molecule.

Like the two-hybrid assays described previously, BiFC calls for the creation of two constructs. One construct encodes a fusion between residues 1-154 of YFP (YN) and one of the proteins whose interaction is to be studied. The other construct encodes a fusion between residues 155-238 of YFP (YC) and the other protein of interest. The two fusion constructs are then transfected into cells. When interaction between the two proteins occurs, YFP is functionally reconstituted and becomes fluorescent. Negative controls consist of the omission of cDNA insert in one or both vectors. The binding of the bZIP domains of the immediate early genes, Fos and Jun, fused to the two YFP fragments, was used as a positive control in the original report.

A recent report by the same authors ***(29)*** suggests that the power of this approach may be even greater than originally conceived. This report documents that GFP itself and two other GFP variants—CFP and BFP—also can be split in a similar fashion to produce nonfluorescent fragments that become fluorescent when recombined. Furthermore, when fragments from different fluorescent proteins are recombined, the resultant hybrid molecule fluoresces with different spectral properties from their parent fluorescent proteins. Thus, by fusing proteins with different fluorescent protein fragments, and cotransfecting them into the same cell, multiple interactions can be visualized simultaneously and their selectivity by the cell can be assessed by the color of the resultant protein complex.

The vectors encoding the two YFP fragments, pBiFC-YN155 and pBiFC-YC155, as well as the vectors for positive control, may be obtained via a material transfer agreement from the author of the original report, Dr. Changdeng Hu, at cdhu@umich.edu. In our hands, BiFC is simple and straightforward to implement, and it allows the interaction of interest to be studied in cultured mammalian cells as long as they are amenable to transfection. The equipment requirement for this technique is extremely simple—any research quality fluorescence microscope with the appropriate filter set will suffice. Interpretation of data is also considerably simpler compared to FRET. YFP emission can be detected readily using a standard filter set for FITC, but filter sets constructed specially for YFP are also available from Omega Optical (http://www.omegafilters.com) and Chroma Technology Corp. (http://www.chroma.com).

3.3.3. Fluorescence Correlation Spectroscopy (FCS)

A very powerful, though not universally accessible, microscope-based technique for studying protein–protein interaction is FCS ***(30,31)***. For those who are fortunate enough to have access to an FCS-capable instrument, measuring protein–protein interaction is fast (on the order of a few minutes), of extremely high in spatial resolution (typically approx 0.25 femtoliter), and can be done in living cells. As its name suggests, FCS is a spectroscopic technique; although it is performed on a confocal microscope specially fitted for this purpose (the Zeiss ConfoCor2), the readout is not an image, but a correlation curve. An excellent overview of this powerful technique may be found at http://www.zeiss.de/C12567BE0045ACF1/allBySubject/6736A9B858525267C12569B4002FD671.

FCS measures the random diffusion of fluorescently labeled molecules within a defined volume element irradiated by a focused laser beam (the confocal volume), and reports a fluorescence fluctuation correlation function. This provides information about the diffusion rates of a particle, which, in turn, depends on the mass of the particle. When two particles interact, their mass is additive and this is reflected in a change in the correlation function. The diffusion times for the molecular aggregate can be derived from the correlation function and its molecular size can then be calculated. Furthermore, if the two particles are labeled with two different fluorescent dyes, their correlation functions can be recorded in two separate channels and a cross-correlation can be established.

In practice, cultured cells can be transfected with separate vectors encoding fusions of proteins of interest and two different fluorescent proteins, for example, GFP and BFP. The cells can then be analyzed directly using the ConfoCor2. Alternatively, lysates from the cells can be analyzed. At this time, the FCS approach is only available at a few sites around the country. However, this is likely to change. The power and versatility of the

approach make it well worthwhile, even at this point in time, to contact investigators with the instruments for collaborative investigations.

3.4. Concluding Remarks

Although much of this chapter is not specifically focused on problems related to epidermal biology, it can be appreciated that the approaches discussed are easily adapted to specific problems without significant modifications. For the epidermal biologist interested in protein–protein interactions, this is an exciting time. Molecular biology, genomics, proteomics, bioinformatics and imaging technology have contributed a wide array of techniques and approaches for identifying and studying protein interactions and interaction networks. Our limited experience with this impressive tool chest has already taught us that the diverse approaches complement one another extremely well, and that the power of this convergence is immense. We urge our colleagues to avail themselves to these opportunities.

4. Notes

1. A comparison of the three assays. The X-gal (replica plating) assay for β-galactosidase activity is at best semi-quantitative and involves an element of subjectivity. The ONPG colorimetric assay is quantitative with a detection limit of approx 1 ng or 1×10^9 molecules. The fluorometric assay using 4-MUG as substrate is 1000 times more sensitive, with a detection limit of 1 fg or 1×10^6 molecules. Although results from X-gal assays do not always correspond strictly with results from the fluorescent assay, we have found that for the vast majority of the time the following correlations are valid:

Coloration in X-gal Assay	Fluorescence (arbitrary Units)
White	<500
Very light blue	500
Light blue	1000–3000
Blue	3000–7000
Dark blue	>7000

   How useful is the two-hybrid system for comparing strengths of protein–protein interactions? Estojak et al. ***(15)*** assayed interactions of known rate constants with the yeast two-hybrid system and compared the linearity of response of several reporters. They concluded that the strength of interaction as judged by quantitative reporter gene assays generally correlates with that determined in vitro, so that comparing *relative* strengths is acceptable. However, there was no single reporter for which the amount of gene expression linearly reflected affinity measured in vitro, so that absolute values of interaction strength are not very meaningful. Our own experience echoes these conclusions. We have found that we can quite accurately compare relative strength of interaction between, say, protein X and a series of deletion mutants of protein Y, but extending the comparison to the interaction between protein X and a third, unrelated protein, Z, is not always meaningful.
2. The GAL4 two-hybrid system does have several limitations. First, because it relies on transcriptional activation by GAL4, it cannot be used if one of the proteins of interest is itself an activator of transcription. (This limitation also applies to the LexA system.) One solution to this problem is to make constructs that encode progressively larger truncations of the bait protein and repeat the assay until self-activation does not occur. This is often

feasible, but quite time- and labor-intensive. An example that comes to mind is the adhesion protein, plakoglobin. Another solution is to use a two-hybrid system that does not rely on transcriptional activation. Stratagene markets one such system, called the Cytotrap (http://www.stratagene.com/displayProduct.asp?productId=255). In this system, subcloning one cDNA into pMyr fuses a myristylation signal to the expressed protein so that it is targeted to the inner surface of the plasma membrane. The other vector, pSos, encodes a fusion protein consisting of the other protein of interest and Sos, a GDP-GTP exchange factor. Interaction between the proteins of interest brings Sos to the membrane where it activates the Ras pathway and promotes cell growth ***(16)***. The yeast host is a strain that carries a temperature-sensitive mutation at the cdc25 (the yeast homolog of Sos) locus; it grows at 25°C but not 37°C. Rescue of the Ras pathway by Sos restores its ability to grow at 37°C and serves as a qualitative readout for protein–protein interaction.

3. Another commonly perceived limitation of the yeast two-hybrid system is that the protein–protein interaction occurs in the nucleus. The question is often raised as to whether the nuclear milieu can be taken as equivalent to a cytoplasmic environment for protein–protein interactions. The Cytotrap provides a means to explore this question. We have compared the GAL4 system with the Cytotrap using dimerization of the ezrin–radixin–moesin family of cytoskeleton-membrane linker proteins as a model system, and have found the two systems to corroborate one another closely ***(17)***, with the exception of one case in which both isoforms of merlin were found to interact with ezrin by coimmunoprecipitation and the Cytotrap assay, but only isoform II of merlin interacted with ezrin in the GAL4 assay. We are not aware of specific examples of spurious interactions detected by the yeast two-hybrid system specifically because the interaction took place in the nucleus. However, it is prudent to be cautious and seek corroborating evidence.
4. A third limitation of the yeast two-hybrid assay is that protein–protein interactions that require post-translational modification, for example, phosphorylation, may not take place because those particular kinds of modifications may not be present or may be regulated differently in yeast (this limitation also applies to bacterial two-hybrid systems). Using a two-hybrid system such as the Cytotrap, in which the interaction occurs in the cytoplasm, increases the probability, but does not assure, that the protein modification will occur. In these cases, corroboration from another method is the only prudent alternative. Either coimmunoprecipitation or the mammalian two-hybrid system is a good choice for such corroboration.

References

1 Steinert, P. M. (2000) The complexity and redundancy of epithelial barrier function. *J. Cell Biol.* **151,** F5–F7.

2. Harlow, E. and Lane, D. (1988) *Antibodies: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
3. Golemis, E. (2002) *Protein–Protein Interactions. A Molecular Cloning Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

4 Fields, S. and Song, O. (1989) A novel genetic system to detect protein–protein interactions. *Nature* **340,** 245–246.

5 Gyuris, J., Golemis, E. A., Chertkov, H., and Brent, R. (1993) Cdi1, a human G1 and S phase protein phosphatase that associates with Cdk2. *Cell* **75,** 791–803.

6. Bolger, G. B. (1998) Molecular genetic approaches I: Two-hybrid systems, in *Methods in Molecular Biology* (Clegg, R. A., ed.), Humana Press, Totowa, NJ, pp. 101–131.
7. Serebriiskii, I. and Joung, J. K. (2002) Yeast and bacterial two-hybrid selection systems for studying protein–protein interactions, in *Protein–Protein Interactions. A Molecular Cloning Manual* (Golemis, E., ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

8. Meng, J. J. and Ip, W. (2001) A yeast two-hybrid approach for probing cytoskeletal protein interactions, in *Methods in Molecular Biology, Vol. 161: Cytoskeleton Methods and Protocols* (Gavin, R. H., ed.), Humana Press Inc., Totowa, NJ.
9 Chevray, P. M. and Nathans, D. (1992) Protein interaction cloning in yeast: identification of mammalian proteins that react with the leucine zipper of Jun. *Proc. Natl. Acad. Sci. USA* **89,** 5789–5793.
10 Hill, J., Donald, K. A., and Griffiths, D. E. (1991) DMSO-enhanced whole cell yeast transformation. *Nucleic Acids Res.* **19,** 5791.
11 Gietz, R. D. and Schiestl, R. H. (1991) Applications of high efficiency lithium acetate transformation of intact yeast cells using single-stranded nucleic acids as carrier. *Yeast* **7,** 253–263.
12 Breeden, L. and Nasmyth, K. (1985) Regulation of the yeast HO gene. Cold Spring Harbor *Symp.* Quant. Biol. ***50,*** *643–650.*
13 Yocum, R. R., Hansley, S., West, R., and Ptashne, M. (1984) Use of LacZ fusions to delimit regulatory domains of the inducible divergent GAL1-GAL10 promoter in Saccharomyces cerevisiae. *Mol. Cell Biol.* **4,** 1985–1998.
14 Meng, J. J., Khan, S. A., and Ip, W. (1996) Intermediate filament protein domain interactions as revealed by two-hybrid screens. *J. Biol. Chem.* **271,** 1599–1604.
15 Estojak, J., Brent, R., and Golemis, E. A. (1995) Correlation of two-hybrid affinity data with in vitro measurements. *Mol. Cell Biol.* **15,** 5820–5829.
16 Aronheim, A., Engelberg, D., Li, N., Al-Alawi, N., Schlessinger, J., and Karin, M. (1994) Membrane targeting of the nucleotide exchange factor Sos is sufficient for activating the Ras signaling pathway. *Cell* **78,** 949–961.
17 Meng, J. J., Lowrie, D. J., Jr., Sun, H., Dorsey, E., Pelton, P. D., Bashour, A. M., et al. (2000) Interactions between two isoforms of the NF2 tumor suppressor protein, merlin, and between merlin and ezrin, suggests modulation of ERM proteins by merlin. *J. Neurosci. Res.* **62,** 491–502.
18 Dove, S. L., Joung, J. K., and Hochschild, A. (1997) Activation of prokaryotic transcription through arbitrary protein–protein contacts. *Nature* **386,** 627–630.
19 Dang, C. V., Barrett, J., Villa-Garcia, M., Resar, L. M., Kato, G. J., and Fearon, E. R. (1991) Intracellular leucine zipper interactions suggest c-Myc hetero-oligomerization. *Mol. Cell Biol.* **11,** 954–962.
20 Heim, R. and Tsien, R. Y. (1996) Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer. *Curr. Biol.* **6,** 178–182.
21 Hanson, M. R. and Kohler, R. H. (2001) GFP imaging: methodology and application to investigate cellular compartmentation in plants. *J. Exp. Bot.* **52,** 529–539.
22 Stryer, L. (1978) Fluorescence energy transfer as a spectroscopic ruler. *Annu. Rev. Biochem.* **47,** 819–846.
23 dos Remedios, C. G., Miki, M., and Barden, J. A. (1987) Fluorescence resonance energy transfer measurements of distances in actin and myosin: a critical evaluation. *J. Muscle Res. Cell Motil.* **8,** 97–117.
24. Verveer, P. J., Harpur, A. G., and Bastiaens, P. I. H. (2002) Imaging protein interactions by FRET microscopy, in Protein–Protein Interactions. A Molecular Cloning Manual. *(Golemis, E., ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.*
25. Periasamy, A. (2001) *Methods in Cellular Imaging.* Oxford Univ. Press, New York.
26 Day, R. N. (1998) Visualization of Pit-1 transcription factor interactions in the living cell nucleus by fluorescence resonance energy transfer microscopy. *Mol. Endocrinol.* **12,** 1410–1419.
27 Kenworthy, A. K., Petranova, N., and Edidin, M. (2000) High-resolution FRET microscopy of cholera toxin B-subunit and GPI-anchored proteins in cell plasma membranes. *Mol. Biol. Cell* **11,** 1645–1655.

28 Hu, C. D., Chinenov, Y., and Kerppola, T. K. (2002) Visualization of interactions among bZIP and Rel family proteins in living cells using bimolecular fluorescence complementation. *Mol. Cell* **9,** 789–798.

29 Hu, C. D. and Kerppola, T. K. (2003) Simultaneous visualization of multiple protein interactions in living cells using multicolor fluorescence complementation analysis. *Nat. Biotech.* **21,** 539–545.

30 Elson, E. L. (2001) Fluorescence correlation spectroscopy measures molecular transport in cells. *Traffic* **2,** 789–796.

31. Lippincott-Schwartz, J., Snapp, E., and Kenworthy, A. (2001) Studying protein dynamics in living cells. *Nat. Rev. Mol. Cell Biol.* **2,** 444–466.

35

Isolation of Recombinant Phage-Displayed Antibodies Recognizing Skin Keratinocytes

Kim Bak Jensen and Peter Kristensen

Summary

Advances in our understanding of biology at the molecular level are very much driven by improvements in the scientist's tool box. Such improvements may not only be an introduction of new techniques like polymerase chain reaction, but as much an increment of for example the sensitivity of existing methods. The in vitro generation of antibodies using phage display is one such technique, which continuously has been developed since its introduction more than 10 yr ago. As a result, selection of phage-displayed antibodies is emerging as a proteomic tool for the identification of differentially expressed cell surface antigens. Here, a method is described that enables the rapid isolation of a panel of recombinant antibodies recognizing epidermal skin keratinocytes. The method exploits the properties of a protease sensitive helper phage and facilitates the isolation of affinity-binders after a single round of selection. This assures a high diversity of binders owing to the reduction of experimental noise.

Key Words:

Epidermal keratinocyte; scFv; phage display; helper phage; recombinant antibody; proteomics.

1. Introduction

In recent years tremendous strides have been achieved concerning the knowledge of the human genome at the level of deoxyribonulceic acid (DNA) and ribonucleic acid (RNA). Still facing us is the challenge of converting this information to its functional parts, the proteins. To access information relating to posttranscriptional modifications and expression levels of proteins, such analysis needs to be performed directly. In this respect antibodies are very important tools both for novel findings and for verification and further analysis of data established at the DNA and RNA level. The pioneering works of, among others, the group of Dr. Greg Winter, have led to the establishment of an in vitro alternative, known as phage display, to the traditional generation of antibodies by immunization of animals (reviewed in **refs. *1*** and ***2***). The method is based on the filamentous class FF bacteriophage, a family of single-stranded bacterial DNA vira. The phage gene III, which encodes the protein responsible for infection of *Escherichia coli*, can be fused to foreign genes, thereby facilitating the display of the fusion on the surface of the phage particle. Moreover, the gene fusion product, part of the phage particle, is physically linked to the corresponding gene encapsulated in the particle. Such linkage facilitates the selection of phage displayed gene products with desired properties such

From: *Methods in Molecular Biology, vol. 289:*
Epidermal Cells: Methods and Protocols
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

as affinity for particular targets. The corresponding genes can subsequently be analyzed by PCR after infection of *E. coli* with the purified phage. Accordingly, antibody repertoires of recombined antibody variable heavy and light chains displayed on the surface of the phage constitute an in vitro alternative to the mammalian immune system for isolation of target specific antibodies. Compared with the traditional generation of antibodies, phage display facilitates a more rapid and cost-effective isolation of monoclonal antibodies. Moreover, the amount of target antigen needed can be reduced tremendously. The protocol described here is based on recent improvements in phage display antibody technology, which reduce the experimental noise originating from background binding of phage particles. Consequently, affinity binders can be isolated after a single round of selection, which assures a high diversity of binders ***(3)***. Because of these improvements phage display can now be applied as a high-throughput method in studies of the functional genome.

2. Materials

2.1. Selection of Antibodies

1. Griffin repertoire (available from MRC, Cambridge, UK; *see* **Note 1**); Tomlinson repertoires (available from MRC, Cambridge, UK; *see* **Note 2**; **ref. *4***); ETH2 repertoire (available from ETH, Zürich, Switzerland; *see* **Note 3**; **ref. *5***). Prepare these antibody repertoires as described in the protocols provided with the repertoires.
2. The protease sensitive helper phage, KM13 (supplied with repertoires from MRC, Cambridge; **ref. *6***).
3. The amber stop codon suppressing bacteria strain TG-1 (K12 supE hsdD5 Δ(lac-proAB)thi F′ {traD36 pro AB^+ $lacI^q$ *lacZ*Δ}) for propagation of phage particles (supplied with repertoires from MRC, Cambridge).
4. Epidermal skin keratinocytes obtained from skin biopsies and cultured using traditional culturing methods ***(7)***.
5. Keratinocyte medium supplemented with EGF and pituitary extract according to manufacturer (Gibco; cat. no. 17005-042) as well as gentamicin (Sigma Aldrich; cat. no. G 8648, 5 µg/mL).
6. Bacterial culture medium (2xTY): 16 g/L casein peptone (Merck; cat. no. 1.07213), 10g/L yeast extract (Merck; cat. no. 1.03753) and 5 g/L NaCl.
7. Bacterial TYE agar plates: 8 g/L casein peptone, 5 g/L yeast extract, 5 g/L NaCl, and 15 g/L agar (Life Technologies; cat. no. 80814943-2) supplemented after autoclaving with 100 µg/mL ampicillin, and 1% glucose.
8. Bacterial minimal agar plate: 15 g/L agar autoclaved separately in 800 mL dH_2O and supplemented with 200 mL/L 5 × M9 medium (30 g/L Na_2HPO_4, 15 g/L KH_2PO_4, 5 g/L NH_4Cl, and 2.5 g/L NaCl), 1 mL 1 *M* $MgSO_4 \cdot 7H_2O$, 10 mL 20% glucose as well as 0.1 mL 0.5% vitamin B1 (Thiamine hydrochloride; Sigma Aldrich; cat. no. T4625).
9. 20% weight/vol. glucose in dH_2O sterilized by autoclaving.
10. 100 mg/mL Ampicillin: (1 : 1000 stock solutions) in dH_2O sterilized by filtration using a 0.45-µ*M* disposable filter.
11. 25 mg/mL Kanamycin: 25 mg/mL (1 : 1000 stock solutions) in dH_2O sterilized by filtration using a 0.45-µ*M* disposable filter.
12. 50% glycerol in dH_2O sterilized by autoclaving.
13. 10 L Phosphate-buffered saline (PBS): 58.4 g of NaCl, 44.5 g $Na_2HPO_4 \cdot 2H_2O$, 35.1 g of $NaH_2PO_4 \cdot H_2O$, pH 6.8; 100 m*M* NaCl, 50 m*M* NaH_2PO_4/Na_2HPO_4.
14. PBS supplemented with low-fat milk powder (MPBS).

15. Trypsin buffer: 50 m*M* Tris-HCl, 1 m*M* $CaCl_2$ pH 7.4 supplemented with 10 mg/mL trypsin (Sigma Aldrich; cat. no. T 1426).
16. Six-well tissue culture plates.

2.2. Screening of Output

1. 96-well tissue culture plates.
2. 96-well bacteria culture microplates.
3. HRP-conjugated mouse anti-M13 antibody (Amersham-Pharmacia; cat. no. 27-9421-01).
4. 50 m*M* $NaHCO_3$, pH 9.6.
5. Murine anti-Myc tag antibody: a monoclonal antibody clone, 9E10, can be obtained by the European Tissue and culture collection. Alternatively, purified antibody from 9E10 can be purchased from Sigma Aldrich (cat. no. M4439).
6. HRP-conjugated rabbit anti-mouse antibody (DAKO; Denmark; cat. no. P0447).
7. *Ortho*-Phenylenediamine dihydrochloride (OPD) 2-mg tablets, for enzyme-linked immunosorbent assay (ELISA) (DAKO, Denmark; cat. no. S2045).
8. 1 *M* H_2SO_4.
9. 35% H_2O_2.
10. *Taq* DNA polymerase (Invitrogen; cat. no. 10342-012).
11. dNTP (Promega).
12. Primers: Rev: 5′-AAACAGCTATGACCATG-3′; M13back: 5′-CAAAACCTCATACA-GAAAATTCA-3′

2.3. Downstream Application of Antibodies

1. Restriction enzymes *Bst*NI, *Nco*I, and *Not*I (New England Biolabs).
2. T4-DNA ligase (New England Biolabs).
3. The expression plasmid pKBJ3 (available from Peter Kristensen, Department of Molecular Biology, University of Aarhus).
4. Ni-NTA beads (Qiagen™; cat. no. 30410).
5. Eight well glass slides (Nunc, Roskilde, Denmark; cat. no. 154941).
6. PVDF Western blotting membrane (Millipore; cat. no. IPVH 000 10).
7. Streptavidin coated M-280 Dynabeads® (Dynal®, Norway; cat. no. 112.05).
8. Dynal MPC®-S magnet for retrieval of Dynabeads® (Dynal®, Norway; cat. no. 120.20).
9. ECL protein biotinylation module (Amersham Pharmacia Biotech; cat. no. RPN2202).
10. [S^{35}] Redivue cell labeling mix (Amersham Pharmacia Biotech; cat. no. AGQ0080).
11. Phospor imager or films for autoradiograms.
12. Fluorescein isothiocyanate-conjugated Extravidin™ for indirect immunofluorescence studies (Sigma Aldrich; cat. no. E2761).

3. Methods

3.1. Selection of Phage-Displayed Antibody Repertoires (see Fig. 1)

This section describes the initial isolation of phage-displayed antibodies binding to a complex mixture of antigens such as the variety of antigens displayed on the plasma membrane and antigens secreted for integration in the extracellular matrix. Moreover, the procedure can in a slightly modified version be used to generate antibodies recognizing specified antigens, when these are applied as purified targets (*see* **Note 4**). The method described requires that cultured cells are available (e.g., epidermal keratinocytes).

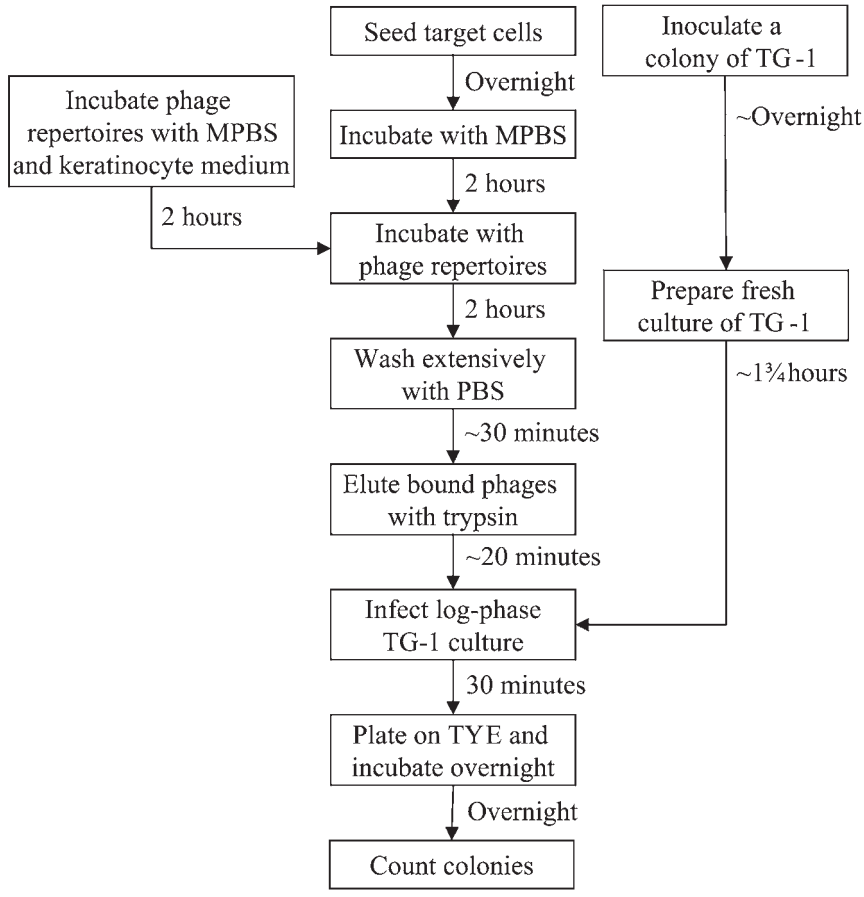


Fig. 1. Flow chart showing the steps in selecting phage displayed antibodies against epidermal keratinocytes as outlined in the text.

3.1.1. The Selection Process

1. One day prior to selection, inoculate a colony of the bacterial strain TG-1 from a minimal plate into 20 mL of 2xTY and incubate overnight at 37°C with agitation. After overnight incubation store the culture at 4°C.
2. One day prior to selection, keratinocytes are seeded in keratinocyte medium at a density of approx 2×10^5 cells per well in a six-well tissue culture plate and incubated overnight.
3. On the day of selection rinse keratinocytes from **step 2** twice in PBS and incubate for 2 h at room temperature with freshly made 2% weight volume MPBS added to the brim of the well.
4. Simultaneously, incubate 0.5–1.0 mL (approx 10^{13} colony-forming units) of the phage displayed antibody repertoire for 2 h at room temperature with 1 : 1 vol of keratinocyte medium and PBS supplemented with fat-free milk powder to a final concentration of 2% in a final volume of 3 mL.
5. Rinse the keratinocytes from **step 3** in PBS.
6. Add the preblocked phage-displayed antibody repertoire from **step 4** into the preblocked well from **step 5** and incubate for 2 h at room temperature.

7. Inoculate 500 µL of the freshly prepared overnight culture of TG-1 in 50 mL of 2xTY and place at 37°C for approx 1 h and 45 min with agitation until OD_{600nm} approx 0.5. Phage infection of *E. coli* requires the presence of a F-pilus, which is expressed by log phase *E. coli* when grown at 37°C.
8. Empty the well from **step 6** and wash six times for 5 min with PBS.
9. Prepare a trypsin solution (10 mg/mL in 50 m*M* Tris-HCl, pH 7.4; 1 m*M* $CaCl_2$) and make a 10× dilution in trypsin buffer for elution of bound phage displayed antibodies.
10. Incubate the washed well from **step 8** with 500 µL of the diluted trypsin for 20 min at room temperature. Trypsin cleaves the linker between the coat protein used for display and the antibody, thereby eluting specifically bound phage (*see* **Note 5**).
11. Infect 1.75 mL of the fresh culture of TG-1 at OD_{600nm} approx 0.5 with 250 µL of the eluted phage from **step 10** for 30 min at 37°C. Store the remaining 250 µL of the eluted phage at –20°C as backup.
12. Make four 10× dilutions of the infected bacteria and transfer to dry TYE plates (13.5 cm in diameter) containing 100 µg/mL ampicillin and 1% glucose. The dilutions are made by transferring 200 µL of the initial sample to 1.8 mL 2xTY and repeating this three times (*see* **Note 6**).
13. After the plates have dried, they are placed overnight at 30°C.
14. Determine the approximate number of infecting phage retrieved in the selection by counting the number of colonies in the dilution series. If the number of retrieved phage is below 100 or exceeds 10^6, the selection should be repeated; as such numbers of retrieved phage indicate that an error has occurred.

3.2. Screening of the Retrieved Phage-Displayed Antibodies (see Fig. 2)

To isolate monoclonal antibodies, which have affinity for the target, phage-displayed antibodies need to be expressed as individual clones. These are tested for their reactivity both towards keratinocytes, the low-fat milk powder present during selections, plastic as well as keratinocyte medium because all of these constituents are targets in the selection. To get the largest diversity of antibodies, screening is performed after the first round of selection. In order to be certain that positive antibodies recognise epitopes produced by the target, the procedure is repeated a second time for antibody clones that score positive in the first screening (*see* **Note 7**).

3.2.1. Production of Monoclonal Phage

1. After having counted the colonies, make "master plates" by inoculating single colonies with sterile toothpicks into 150 µL 2xTY supplemented with 100 µg/mL ampicillin and 1% glucose in 96-well culture microplates.
2. Incubate the 96-well culture microplates overnight at 37°C with agitation in a humidity chamber, for example, a plastic box with humidified paper tissues (*see* **Note 8**).
3. Replicate the master plate using a 96-pin microplate replicator (*see* **Note 9**) into a new 96-well culture microplate containing 150 µL 2xTY supplemented with 1% glucose and 100 µg/mL ampicillin.
4. Add 50 µL sterile 50% v/v glycerol to each well in the master plate and store at –80°C as backup.
5. Incubate the new microplates with agitation for 5 h at 37°C in a humidity box.
6. Add helper phage, KM13, in a multiplicity of infection of 20 (add 10^9 plaque-forming units of helper phage in 25 µL 2xTY to each well; *see* **Note 10**) and incubate at 37°C for 1 h without shaking. Before the addition of helper phage the bacteria contain a phagemid, which encodes antibody fused to the coat protein as well as ampicillin resistance. The remaining

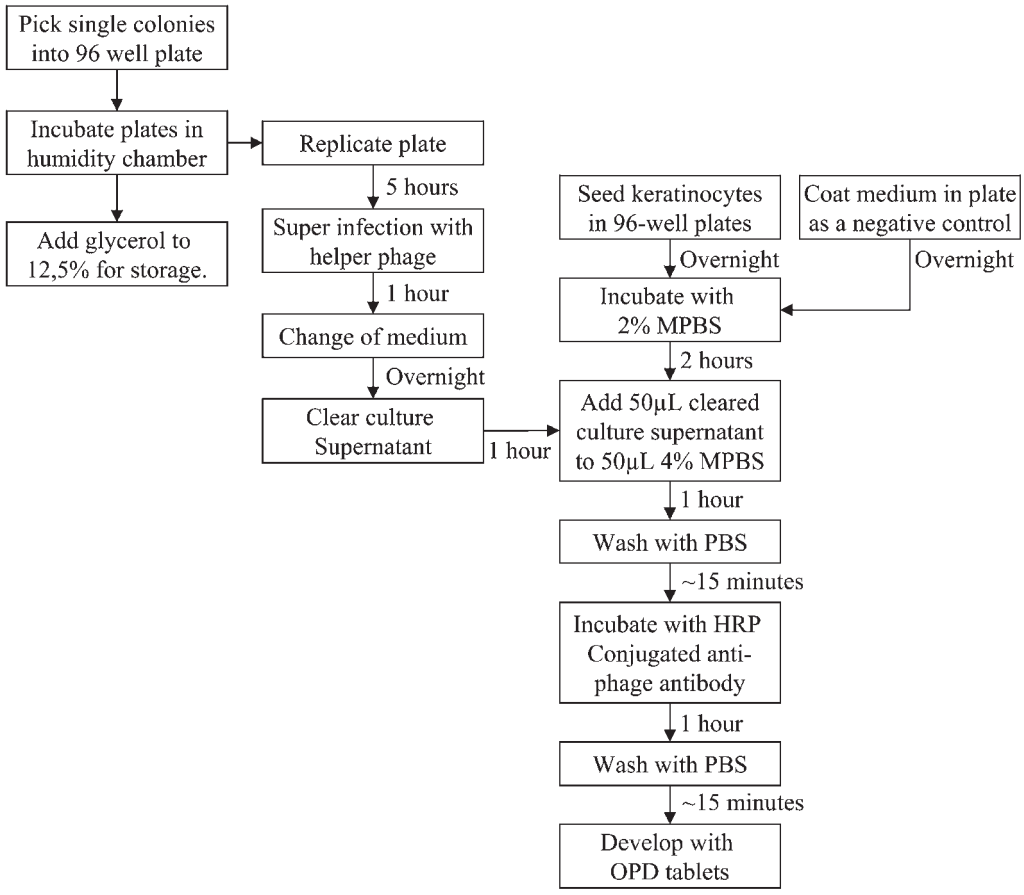


Fig. 2. Flow chart outlining the steps in the identifications of monoclonal antibodies selected against the target cell type, as described in the text.

phage proteins needed for the production of viable phage particles are added by infecting with a helper phage, which in addition confers kanamycin resistance.

7. Centrifuge the 96-well culture microplate at 900*g* for 20 min at 4°C.
8. Remove the supernatant carefully to avoid cross contamination
9. Resuspend pellets in 150 µL 2xTY supplemented with 100 µg/mL ampicillin and 25 µg/mL kanamycin.
10. Incubate plates in a humidity chamber overnight at 30°C with agitation (*see* **Note 11**).
11. Centrifuge plates at 900*g* for 30 min at 4°C and transfer the supernatants containing the phage containing supernatants to new microplates and store at 4°C until ELISA screening (*see* **Note 12**).

3.2.2. ELISA Screening

1. For screening, keratinocytes are seeded in keratinocyte medium at a density of approx 5000 cells per well in a 96-well tissue culture plates and incubate overnight.
2. Simultaneously, add 50 µL keratinocyte medium to separate wells in tissue culture plates in 50 µL 50 m*M* $NaHCO_3$, pH 9.6, and incubate overnight at 4°C.

3. Remove the medium from **steps 1** and **2**.
4. Add 4% MPBS to the brim of each well (~300–330 µL) and incubate for 2 h.
5. Empty plates and add 50 µL 4% MPBS as well as 50 µL of the monoclonal phage antibody containing supernatant from **Subheading 3.2.1., step 11** to each well (*see* **Note 13**) and incubate for 1 h with agitation.
6. Empty plates and wash six times in PBS (*see* **Note 14**).
7. Incubate for 1 h with 100 µL of a 1 : 5000 dilution of the HRP-conjugated monoclonal anti-phage antibody in 2% MPBS.
8. Empty and wash as in **step 6**.
9. Develop the ELISA by incubating with 100 µL OPD solution. Prepare buffer by dissolving 4 OPD tablets per 12 mL of water and supplement with H_2O_2 to 0.0015% (5 µL of a 35% H_2O_2 in 12 mL dH_2O).
10. When a general background color starts to develop (*see* **Note 15**) quench the reaction by addition of 50 µL 1 *M* H_2SO_4.
11. Read the ELISA plates at 490 nm with 655 nm as background reference.
12. Analyze the obtained ELISA signals and select clones for further characterization, which give a signal above the general background and are only positive in the assay on seeded keratinocytes. Between 10% and 40% of the screened antibodies are traditionally tested positive after one round of selection, however, only between 1% and 10% recognize the target specifically, whereas the remaining antibodies are isolated as binders to the medium, milk powder protein and plastic.

3.2.3. Re-Screening of Positive Clones

1. Make new master plates from the former master plates (**Subheading 3.2.1., step 4**) by picking positive clones from the first master plate into new wells as described in **Subheading 3.2.1.**
2. Repeat **Subheading 3.2.1.** through **3.2.2.** for the positive clones.
3. Truly positive clones can now be analyzed in more detail.

3.3. Investigation of Antibody Diversity

Because the same antibody can be isolated several times from the applied repertoires, the diversity of positive antibodies needs to be determined to minimize the characterization work. Two different methods can be used; however, the repertoire type dictates the method applied. Single framework repertoires such as the Tomlinson I and J as well as the ETH2 repertoire require sequencing, whereas the diversity of a semisynthetic repertoire, such as the Griffin-1 repertoire, initially can be determined by restriction fingerprinting.

3.3.1. Fingerprinting

1. Run PCR using standard conditions for *Taq* DNA–polymerase and primers Rev and M13back.
2. Purify PCR products using, for instance, PCR purification kit from Qiagen.
3. Set up a restriction digestion with *Bst*NI.
4. Digest for 2 h at 60°C and analyze on a 2% DNA agarose gel. Based on the observed pattern, it is possible to establish the diversity of the retrieved antibody clones, although clones with similar fingerprints can contain different sequences.

3.3.2. Sequencing and Database Searching

1. Use either phagemids purified from minipreps or purified PCR products from, for example, Qiagen DNA purification kits, when sequencing with primers Rev and M13back. For

phagemid purifications supplement medium with glucose as well as antibiotics in order to repress the expression of fusion proteins, which is toxic to *E. coli*.
2. Align the retrieved sequences from the Griffin repertoire with that of the expressed human antibody genes using DNA plot sampling of the V-base (*see* **Note 16**) to gain information of the variable gene family.
3. Analyze the sequences according to the model outlined by Kabat et al. ***(8)*** to obtain the sequences for the complementarity determining regions of the heavy and light chains of the recovered antibodies.

3.4. Further Analysis and Application of the Generated Phage Display Antibodies

As for traditional monoclonal antibodies, the phage display-derived antibodies can be used for a variety of down stream applications, such as Western blotting, indirect immunofluorescence, and immunoprecipitation. Before applying isolated antibodies in such experiments, it is often advantageous to express them as soluble proteins instead of as phage displayed antibodies, because phage displayed antibodies give rise to a low signal to noise ratio compared to soluble antibodies in certain experiments. Several strategies have been evolved for the expression of soluble phage derived antibodies, such as the recently published FuncFAb system, which exploits fusion to the N-terminal domain of filamentous phage protein III ***(9)***. Fusion proteins can subsequently be purified via their C-terminal His-6-tag and detected in immunoassays via a c-myc tag.

3.4.1. Subcloning of Antibodies Into the FuncFAb System

1. Purify phagemids using, for example, basic Qiagen protocols supplementing 1% glucose to the medium to repress the expression of antibody fusion protein.
2. Digest with the appropriate restriction enzymes, in the case of the FuncFAb vector pKBJ3 use *Nco*I and *Not*I.
3. Gel purify the approx 750-bp antibody gene.
4. Ligate the gene into a *Nco*I- and *Not*I-digested and gel-purified pKBJ3 vector, then electroporate the ligated vector into a bacterial expression strain, for example, TG-1.

3.4.2. Expression and Testing of Antibodies in the FuncFAb System

1. Inoculate a single colony of the bacteria containing the pKBJ3 subcloned antibody gene in 2xTY supplemented with 100 µg/mL ampicillin and 1% glucose overnight at 37°C.
2. Inoculate 10 mL of the overnight culture in 1 L of 2xTY supplemented with 100 µg/mL ampicillin and 0.1% glucose for 4 h at 37°C with agitation.
3. Induce expression of the antibody through the addition of isoproylthiogalactoside to 1 m*M* and grow overnight at room temperature shaking.
4. Pellet bacteria by centrifugation at 8000*g* for 10 min and resuspend the pellet in 30 mL of PBS.
5. Disrupt the bacterial cell membranes using either a Sonicator or a French press.
6. Clear the suspension by centrifugation at 25,000*g* for 30 min.
7. Add the cleared supernatant to Ni-NTA beads and purify antibodies according to the protocol provided by Qiagen™.
8. Analyze the purified antibodies by ELISA for activity as described in **Subheading 3.2.2.** using the following modifications: 1) Use 1 µg and 5 µg of the purified antibodies as primary antibody; 2) detect antibodies expressed in the FuncFAb system with 9E10, a murine anti-myc tag antibody; 3) An HRP conjugated anti mouse antibody is used as tertiary antibody.

9. Between each of these incubations wash six times with PBS and develop as described in **Subheading 3.2.2.**

3.4.3. Western Blotting Using Either Soluble or Phage-Displayed Antibodies

1. Separate cell extracts by sodium dodecyl sulfate polyacrylamide gel electrophoresis according to Laemmli ***(10)***. Instead of boiling the sample in sample buffer, incubate at 37°C for 30 min to preserve some structural motifs.
2. Transfer proteins to a polyvinylidene difluoride membrane (Millipore) according to Sambrook et al. ***(11)***. 1) Before transferring the protein, activate the membrane by embedding it in 96% ethanol for 30–45 s. 2) After transfer deactivate the membrane by immersing it in 96% ethanol for 30–45 s (*see* **Note 17**).
3. Incubate the membrane overnight in 10% MPBS supplemented with 10% glycerol.
4. Develop the Western blot as described for ELISA with phage displayed antibodies followed by an HRP anti-M13 antibody or with soluble antibodies, 9E10 and HRP conjugated anti-mouse antibody. In order to reduce the noise wash six times for 5 min between incubations.
5. Develop with chemiluminescence.
6. Determine the identity of the cognate antigen by comparing the observed spot pattern to those of 2D PAGE databases. Various databases exist for the proteome expressed by different cell types, for example, http://proteomics.cancer.dk.

3.4.4. Indirect Immunofluorescence

1. Biotinylate purified antibodies using a biotinylation module (Amersham Pharmacia; *see* **Note 18**).
2. Seed keratinocytes on eight-well glass slides and culture overnight in a cell incubator.
3. Fix and permeabilize the cells in 1% formaldehyde 0.1% Tween-20 in PBS for 5 min.
4. Wash three times in PBS.
5. Block residual binding to the glass surfaces by incubating for 2 h at room temperature with 2% MPBS.
6. Rinse the chambers briefly in PBS.
7. Incubate with varying concentrations of biotinylated antibody from **step 1** in 2% MPBS for 1 h at room temperature.
8. Wash four times with PBS supplemented with 0.05% Tween-20 and subsequently four times with PBS.
9. Incubate with fluoresceine isothiocyanate-conjugated Extravidin™ in 2% MPBS for 30 min at room temperature.
10. Wash as in **step 8**.
11. Mount slides with mounting medium and analyze slides by fluorescence microscopy.

3.4.5. Immunoprecipitation and Determination of Cognate Antigen Identities

1. Biotinylate a purified antibody.
2. Prepare cell extracts by seeding 10^6 keratinocytes in 13.5-cm tissue culture dishes and culture overnight in medium supplemented with 1 : 1000 dilution of [^{35}S]-Redivue cell labeling mix.
3. Incubate with PBS supplemented with a detergent, for example, 1-5 m*M* Tx-100 (*see* **Note 19**) as well as a protease inhibitor such as 100 µ*M* phenylmethyl sulfoxyl fluoride for 1 h on ice.
4. Scrape the remaining cell material of the plastic with a rubber police man.
5. Incubate on ice for 1 h.
6. Clear the suspension for material not extracted by the detergent by centrifugation at maximum speed in a microfuge for 15 min at 4°C.

7. Incubate biotinylated antibodies with the cell extracts for 1 h at 4°C with end-over-end rotation.
8. Add streptavidin-coated magnetic beads (Dyna™, Norway) and incubate for 15 min.
9. Isolate the beads via a magnet.
10. Wash four times with PBS supplemented with the extraction detergent and three times with PBS.
11. Analyze the samples by sodium dodecyl sulfide polyacrylamide gel electrophoresis.
12. Dry the gel and use, for example, a phosphor imager to visualise precipitated protein.
13. After initial positive identification of extracted bands, additional precipitations are performed in larger scale.
14. Use either Coomassie staining or silver staining to visualise precipitated protein, cut out the precipitated bands, in-gel-digest the protein, analyze the peptide fragments by mass spectrometry and peptide database searching to determine the protein identity ***(3)***.

4. Notes

1. Link to the phage display section of the MRC, Cambridge homepage, where phage displayed antibody repertoires can be ordered http://www.mrc-cpe.cam.ac.uk/winter-hp.php?menu=1808.
2. Link to information on the Tomlinson repertoires http://www.hgmp.mrc.ac.uk/geneservice/reagents/products/descriptions/scFv_tomlinsonIJ.shtml
3. Link to information on the ETH2 repertoire http://www.pharma.ethz.ch/bmm/protocols/eth.html
4. a. It is important to use disposable plasticware and have devoted pipets when working with filamentous bacteriophage, as phage contaminations can accumulate in glassware as well as in pipets.
   b. If a single purified antigen is the target for the selection it can either be immobilized on an immunotube (Nunc, Roskilde, Denmark) or via a tag. Phage repertoires are subsequently incubated with the immobilised target.
5. Traditionally, selections with phage displayed repertoires have suffered from a low signal-to-noise ratio because of retrieval of a large fraction of nonspecific phage particles. The introduction of a protease sensitive helper phage, KM13, has overcome this problem. This particular helper phage contains an engineered version of the coat protein responsible for infection of *E. coli*, protein III, with a protease sensitive linker inserted between the two domains responsible for infection and the domain anchoring the coat protein in the phage particle. Accordingly, the linker between the antibody and coat protein III is not the only substrate cleaved by the trypsin treatment, since trypsin also removes the two domains of helper phage-encoded protein III responsible for infection ***(6)***. Thus, the treatment with trypsin renders phage not carrying a fusion to the coat protein III nonviable. However, phage, which consist of both helper phage encoded proteins and fusion coat proteins, still remains infective upon trypsin elution. A tremendous increase in the signal to noise ration is hereby established, which facilitates screening of the retrieved output upon only one round of affinity selection ***(3)***.
6. The serial dilutions have two purposes:
   a. enable determination of the number of retrieved phage particles.
   b. facilitate easy picking of individual colonies for the subsequent screening if colonies are too confluent on the first dilution.
7. Fluctuations occur in ELISAs with phage displayed antibodies as primary antibodies, especially when phage infected *E. coli* are grown in 96-well culture plates. Therefore, an additional screening is required.

8. Be aware of cross-contamination between the different wells. One way of circumventing this problem is to seal the top of the microtiter plate with parafilm.
9. Microplate replicators can be purchased from Boekel Scientific.
10. A bacteria culture OD_{600nm} of approx 0.5 contains approx 5×10^8 bacteria per mL.
11. To produce the highest amounts of phage particle overnight, rescue of phage displayed antibodies is performed for at least 16 h.
12. Phage containing supernatants should be stored no more than 2 d at 4°C. It is preferred to use fresh supernatants for screenings.
13. It is at this point very important to use new tips for each and every well in order not to get cross contaminations. Fifty microliters of a supernatant corresponds to approx 5×10^{10} phage.
14. If an ELISA washer is not available a very efficient method for washing is to fill a box with PBS and embed the plate into the buffer. The buffer is subsequently removed by inverting the plate over a sink.
15. The speed varies by which color starts to develop.
16. Alignment of antibodies to the V-base can be conducted at the MRC, Cambridge homepage: http://www.mrc-cpe.cam.ac.uk/DNAPLOT.php?menu=901.
17. These incubations in ethanol increases the signal to noise ratio in particular when phage displayed antibodies are used for the detection of antigen.
18. Biotinylation increases the sensitivity tremendously when antibodies are applied for staining as well as precipitation purposes.
19. No known single detergent is capable of extracting all membrane proteins. Consequently, it is advantageous to apply a panel of detergents in initial experiments to evaluate which detergent extracts the particular antigen, for example, by ELISA, and which detergent does it in a way that preserves the antigen antibody interaction.

Acknowledgments

This work was supported by The Danish Natural Science Research Counsil, The Carlsberg Foundation, The Danish Technical Research Counsil, The Novo Nordic Foundation, The Danish Centre for Molecular Gerontology, and Nemod Immunoterapie AG. We acknowledge Professor Brian F. C. Clark for encouragement and support.

References

1 Winter, G., Griffiths, A. D., Hawkins, R. E., and Hoogenboom, H. R. (1994) Making antibodies by phage display technology. *Annu. Rev. Immunol.* **12,** 433–455.
2 Kristensen, P., Ravn, P., Jensen, K. B., and Jensen, K. H. (2000) Applying phage display technology in aging research. *Biogerontology* **1,** 67–78.
3 Jensen, K. B., Jensen, O. N., Ravn, P., Clark, B. F. C., and Kristensen, P. (2003) Identification of keratinocyte specific markers using phage display and mass spectrometry. *Mol. Cell. Proteomics* **2,** 61–69.
4 de Wildt, R. M., Mundy, C. R., Gorick, B. D., and Tomlinson, I. M. (2000) Antibody arrays for high-throughput screening of antibody-antigen interactions. *Nat. Biotechnol.* **18,** 989–994.
5 Pini, A., Viti, F., Santucci, A., Carnemolla, B., Zardi, L., Neri, P., and Neri, D. (1998) Design and use of a phage display library. Human antibodies with subnanomolar affinity against a marker of angiogenesis eluted from a two-dimensional gel. *J. Biol. Chem.* **273,** 21,769–21,776.
6 Kristensen, P. and Winter, G. (1998) Proteolytic selection for protein folding using filamentous bacteriophages. *Fold. Des.* **3,** 321–328.

7. Norsgaard, H., Glenting, M., Kristensen, P., Clark, B. F. C., and Rattan, S. I. S. (1994) Ageing, differentiation and apoptosis in human epidermal keratinocytes in culture. *Gerontol. Geriatr. Suppl. Arch.* **4,** 185–196.
8. Kabat, E. A., Wu, T. T., Perry, H. M., Gottesman, K. S., and Foeller, C. (eds.) (1991) *Sequences of Proteins of Immunological Interest*, U.S. Health Services, NIH, Washington DC.
9 Jensen, K. B., Larsen, M., Pedersen, J. S., Christensen, P. A., Alvarez-Vallina, L., Goletz, S., et al. (2002) Functional improvement of antibody fragments using a novel phage coat protein III fusion system. *Biochem. Biophys. Res. Commun.* **298,** 566–573.
10 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.
11. Sambrook, J., Fritsch, E. F., and Miniatis, T. (eds.) (1989) *Molecular Cloning*, Cold Spring Harbor Laboratory Press, New York.

36

Analysis of Tissue-Specific DNA Methylation During Development

Jun Ohgane, Naka Hattori, and Kunio Shiota

Summary

The establishment of a cell identification method more accurate than the conventional morphological and cell-type-specific marker analyses has been desired. DNA methylation is related to the gene activity including gene-silencing and is a key mechanism of epigenetics underling cellular differentiaion and development in mammals. Recent studies indicated that there exist unique genomic DNA methylation profiles specific to the cell type. DNA methylation profiles a mechanism for memorizing the set of genes inherent in individual type of cells. In this chapter, we present the methods to analyze DNA methylation status for identifying cells or tissues.

Key Words:

DNA methylation; CpG island; cell-type-specific methylation pattern; epigenetics.

1. Introduction

Cells have been identified using morphological characteristics and molecular markers. For therapeutic use, however, these traditional procedures do not provide enough information for the production of cells by new in vitro technologies, including stem cell cultures, nuclear transfer, and so on. Furthermore, there is a possibility that produced cells might not exhibit expected functions when transplanted or a possibility that the growth of the produced cells might become uncontrollable after transplantation. Thus, the establishment of a cell identification method more accurate than the conventional and traditional ones has been desired.

In mammals including humans, cells, once differentiated during the course of development, inherit the same properties as those of the parental cells. Such cell characteristics are retained throughout the lives of individuals. Each cell possesses basically the same set of genomic information regardless of the cell type in an individual, but the set of genes expressed in one type of cell is different to that of the other types of cells. We have found that there exist unique genomic deoxyribonucleic acid (DNA) methylation patterns depending on the cell type ***(1–5)***, since the methylation of genomic DNA is related to the gene activity including gene-silencing ***(6)***. DNA methylation patterns specific to cells are considered to function as a mechanism for memorizing the set of genes inherent in individual type of cells.

From: *Methods in Molecular Biology, vol. 289: Epidermal Cells: Methods and Protocols*
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

This chapter provides the methods to analyze DNA methylation status for identifying cells or tissues as follows: 1) DNA methylation sensitive Southern blotting; 2) DNA methylation-sensitive polymerase chain reaction (PCR); and 3) Genome-wide analysis of DNA methylation by restriction landmark genomic scanning (RLGS; **refs. *7*** and ***8***). RLGS was originally developed by Hayashizaki et al. ***(7)*** and a fine manual with the trouble shootings and illustrations were published ***(9)***. We describe the RLGS protocol according to this manual with modifications.

2. Materials

2.1. Equipment

1. Dialysis tube (Sanko Jun-yaku, Tokyo, Japan, molecular cut off 14,000, 100 ft; cat. no. UC27-32-100): Cut a piece of dialysis tube to approx 15 cm in length, boil or autoclave the tubes in 2% sodium bicarbonate twice, rinse the tubes in sterilized distilled water, and autoclave in distilled water (store at 4°C up to several months until use).
2. Scotch 3M tape (483 Labo-sealing tape, 25.4 mm × 32.9 m; cat. no. JT-1400-0802-3).
3. Teflon tubing for first-dimensional electrophoresis of RLGS (2.4-mm inner diameter 3.0-mm outer diameter, Sanplatec Corp., Osaka, Japan; cat. no. 5265E).
4. Teflon tubing for in-gel digestion of RLGS (3.0-mm inner diameter, 3.7-mm outer diameter, Sanplatec Corp., Osaka, Japan; cat. no. 5267E).
5. The following RLGS electrophoretic apparatuses listed are available at Bio Craft, Tokyo, Japan (Phone no. +81-3-3964-6561, Fax no.+81-3-3964-6443): first-dimensional apparatus; glass tube for first-dimensional electrophoresis (the top of the glass tube is beveled and narrowed for fixation of teflon tubing); silicone tubing; second dimensional apparatus; glass plate with slant at the top for second dimensional electrophoresis; and spacer for second-dimensional gel plates (1-mm thick).
6. Gel-holder of disc gel for first-dimensional electrophoresis of RLGS:
   a. Cut teflon tubing (2.4 mm inner diameter) in 70-cm length (the top is cut sharply).
   b. Pass the teflon tubing through a glass tube from the broader end, and pull up the top about 1 cm with pliers.
   c. Cut off the top of the teflon tubing leaving about 2 mm from the top of the glass tube.
   d. Push the top of the teflon tubing onto the heated top of flanging tool, and push the heated top of the tubing onto a cool and flat surface.
   e. Cut off the bottom of the teflon tubing leaving 2 cm below the glass tube.
   f. Seal the gap between the teflon tubing and glass holder at the bottom with teflon tape.
7. Electroeluter (Bio Craft, Tokyo, Japan; cat. no. BE-883).

2.2. Purification of High-Molecular-Weight Genomic DNA From Mammalian Tissues and Cells

1. Proteinase K (Merck, Darmmstadt, Germany; cat. no. 70663-4; store up to several months at –20°C at 10 mg/mL in 1-mL aliquot in sterilized-distilled water).
2. RNase (DNase-free; Roche, Basel, Switzerland; cat. no. 1 119 915).
3. Lysis buffer: 10 m*M* Tris-HCl, pH 8.0, 150 m*M* ethylene diamine tetraacetic acid (EDTA), 1% sodium dodecyl sulfate (SDS). Make sure that the final pH is approx 8.0 using pH indicator paper.
4. 2-Mercaptoethanol (Nacalai Tesque, Kyoto, Japan; cat. no. 214-17).
5. Phenol : chloroform : isoamylalcohol 50 : 49 : 1 (PCI). Melt 500 g of crystal phenol (Nacalai Tesque, Kyoto, Japan; cat. no. 26728-45) at 65°C and add 8-hydroxyquinoline (Wako Pure Chemicals, Osaka, Japan; cat. no. 085-01212) to a final concentration of 0.1%. Saturate

the phenol with 500 mL of 0.5 *M* Tris-HCl, pH 8.0, once followed by 500 mL of 0.1 *M* Tris-HCl, pH 8.0, three times. Add 490 mL of chloroform (Wako Pure Chemicals, Osaka, Japan; cat. no. 038-02606) and 10 mL of isoamylalcohol (Wako Pure Chemicals, Osaka, Japan; cat. no. 135-12015) to the saturated phenol. Store at 4°C in a light-tight bottle up to 2 mo.

6. Ethanol (stored at –20°C).
7. TE: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, pH 8.0.

2.3. Southern Blotting to Evaluate Methylation Status

1. Methylation-sensitive restriction enzyme (*see* **Note 1**).
2. pBluescript II SK$^-$ (Stratagene, San Diego, CA; cat. no. 212206).
3. SeaKem GTG agarose (FMC Inc., Philadelphia, PA; cat. no. 50070).
4. 1X TAE buffer: 40 m*M* Tris-acetate, 1 m*M* EDTA.
5. Gel loading dye: 0.25% BPB, 0.25% XC, 30% glycerol in water.
6. 0.25 *N* HCl.
7. Denaturation buffer: 0.5 *N* NaOH, 1.5 *M* NaCl.
8. Neutralization buffer: 0.5 *M* Tris-HCl, pH 7.6, 1.5 *M* NaCl.
9. 20X standard saline citrate (SSC): 3 *M* NaCl, 0.3 *M* sodium citrate.
10. Hybridization buffer: 0.4 *M* phosphate buffer, pH 7.2, 7% SDS, 1 m*M* EDTA, 5X Denhalt's solution.
11. 50X Denhalt's solution: 1% each of Ficoll, polyvinylpyrrolidone and bovine serum albumin (BSA) in water (stored at –20°C up to 6 mo).
12. 10 mg/mL salmon sperm DNA: dissolve appropriate amount of salmon sperm DNA in TE buffer and sonicate for several mins the DNA to reduce average molecular weight. Extract the DNA with PCI and precipitate with ethanol. Dissolve the DNA in TE and adjust the concentration to 10 mg/mL with TE.
13. Probe: probes should be located at either side of methylation-sensitive enzyme recognition site (not containing the enzyme site within the probe). This allows for the detection of one methylated band and one unmethylated band in the autoradiogram with the intensities of both bands proportional to copy number. Thus, the methylation degree of the enzyme recognition site can be calculated by the intensities of methylated and unmethylated bands.
14. DIG DNA labeling kit (cat. no. 1 175 033), antidigoxygenin-AP Fab fragment (cat. no. 1 093 274), and CDP-star (cat. no. 1 685 627; Roche, Basel, Switzerland).
15. Maleate buffer: 0.1 *M* maleic acid, 0.15 *M* NaCl (adjust pH to 7.5 with NaOH and store at room temperature up to 2 mo).
16. 10% blocking buffer: Add 10 g blocking reagent (Roche, Basel, Switzerland; cat. no. 1 096 176) to 100 mL maleate buffer, autoclave to dissolve the reagent and store at 4°C up to 2 mo. For working solution (1%), dilute 10% blocking buffer to 1% with maleate buffer just before use.
17. TBS-T (Tris-buffered saline + 0.1% Tween-20): dilute 10X TBS (0.2 *M* Tris-HCl, pH 7.6, 1.4 *M* NaCl) and 20% Tween-20 to final concentrations of 1X TBS and 0.1% Tween-20, respectively, with distilled water.
18. Buffer 3: 0.1 *M* Tris-HCl, pH 9.5, 0.1 *M* NaCl.

2.4. PCR to Evaluate Methylation Status

Primers: Design a primer pair (primer MS: 25-mer each for forward and reverse primers) flanking the methylation-sensitive restriction enzyme site (*see* **Note 1**). For

internal control, design another primer pair (primer CNTL) that does not contain the methylation-sensitive enzyme site and that amplifies a different length from primer MS pair. PCR is performed in a single tube for both primer MS and primer CTRL pairs. Methylated or unmethylated enzyme recognition site of the interest results in amplification or no amplification, respectively, by PCR with the primer MS pair following the methylation-sensitive enzyme digestion.

2.5. RLGS

1. Klenow fragment (Takara, Kyoto, Japan; cat. no. 2140A).
2. Sequenase Ver. 2 (USB, Cleveland, OH; cat. no. E70775Y).
3. *Not*I, *Pvu*II, and *Pst*I (Nippongene, Toyama, Japan; cat. no. 310-01454, 311-00281 and 318-01771).
4. dCTPαS and dGTPαS (NEN, Boston, MA; cat. no. NLP-011 and NLP-012).
5. ddATP, ddTTP, ddCTP, and ddGTP (Takara, Kyoto, Japan; cat. no. 4031, 4034, 4033, and 4032).
6. [α-^{32}P]dCTP (6,000 Ci/mmol) and [α-^{32}P]dGTP (6000 Ci/mmol; NEN, Boston, MA; cat. no. NEN513Z and NEN514Z).
7. *Sca*I (Takara, Kyoto, Japan; cat. no. 1084A).
8. pBluescript II SK$^-$ (Stratagene, San Diego, CA; cat. no. 212206).
9. 0.1% BSA and 0.1% Triton X-100 (Takara, Kyoto, Japan).
10. 10X H buffer: 500 m*M* Tris-HCl, pH 7.5; 100 m*M* $MgCl_2$, 1 *M* NaCl; 10 m*M* dithiothreitol. Store at room temperature up to several months.
11. Masking buffer: 4X H buffer, 40 m*M* dithiothreitol, 1.6 μ*M* dGTPαS, 0.8 μ*M* dCTPαS, 1.6 μ*M* ddATP, 1.6 μ*M* ddTTP, stored at –20°C up to 6 mo.
12. 2.5X SHB: 375 m*M* NaCl, 0.025% BSA, 0.025% Triton X-100, stored at –20°C up to 1 yr.
13. Second digestion buffer: 132 μ*M* ddGTP, 132 μ*M* ddCTP, 15.8 m*M* $MgCl_2$, stored at –20°C up to 6 mo.
14. 10X first-dimension buffer: dissolve 242 g Tris, 109 g sodium acetate trihydrate, 42 g NaCl, 23.4 g EDTA–2Na in 1.8 L distilled water. Adjust the pH to 8.15 with acetic acid. Fill up the volume to 2 L with distilled water and autoclave. Store at room temperature up to several months.
15. SeaKem GTG agarose (FMC Inc., Philadelphia, PA; cat. no. 50070).
16. 6X dye solution for first-dimension electrophoresis: 0.1% Bromphenol blue (BPB), 0.1% xylene cyanol (XC), 30% glycerol, 150 mM EDTA, pH 8.0.
17. 5X TBE: dissolve 270 g Tris, 138 g boric acid, 23.3 g EDTA–2Na in 4 L distilled water and adjust the volume to 5 L.
18. Acrylamide and methylenebisacrylamide (Wako Pure Chemicals, Osaka, Japan; cat. no. 019-110025 and 130-08172).
19. Ammonium Persulfate and *N*′-tetramethyl ethylene diamine (TEMED) (Amersham Pharmacia Biotech, Uppsala, Sweden; cat. no. 17-1311-01 and 17-1312-9).
20. Connecting gel: 0.8% GTG agarose and 5% sucrose in 1X TBE buffer.
21. Second-dimensional dye solution: 0.25% BPB, 0.25% XC in TE buffer (without glycerol).
22. T4 DNA ligase (Promega, Madison, WI; cat. no. M1801).
23. LA-Taq DNA polymerase with GC-rich buffer (Takara, Kyoto, Japan; cat. no. RR02BG).
24. MicroSpin™ S-400 column (Amersham Pharmacia Biotech; cat. no. 27-5140-01).
25. *Not*I adaptor (5′-ACGCCAGGGTTTTCCCAGTCACGACGC-3′ and 5′-pGGCCGCG TCGTGACTGGGAAAACCCTGGCGT-3′): Incubate the two oligomers (2 nmol each) in 200 μL TE for 10 min at 70°C and cool to room temperature (*see* **Note 2**). The annealed adaptor generates 5′-protruding end of *Not*I (final adaptor concentration is 10 pmol/μL).

26. *Pst*I adaptor (5′-pGTGTACTGCACCAGCAAATCC-3′ and 5′-GGATTTGCTGGT GCAGTACACTGCA-3′): Annealing reaction is performed under the same conditions as *Not*I adaptor (*see* **Subheading 2.2.4., step 25**). The annealed *Pst*I adaptor generates 3′-protruding end of *Pst*I (10 pmol/μL).
27. Primers: Ad-NI (5′-AGGGTTTTCCCAGTCACGACGCGG-3′) and Ad-PI (5′-TTGCTG GTGCAGTACACTGCAG-3′).

3. Methods

3.1. Purification of High-Molecular-Weight Genomic DNA From Mammalian Tissues, Cultured Cells, and Sperm

Pretreat samples before PCI extraction as follows:

3.1.1. Tissue Samples

1. Freeze tissue with 2 mL lysis buffer that is broken into small pieces with a hammer.
2. Prechill the mortar, pestle and 50-mL tubes in liquid nitrogen.
3. Grind down 0.1–0.5 g tissue into powder using the mortar and pestle.
4. Transfer the tissue powder into a prechilled 50-mL tube and add 10–25 mL lysis buffer containing 150 μL of 10 mg/mL proteinase K.
5. Mix gently with a spatula and incubate for 20 min at 55°C.

3.1.2. Cultured Cells

1. Collect 1–5 × 10^7 cells by centrifugation at 500*g* for 5 min at 4°C and discard the supernatant.
2. Add 5 mL lysis buffer (without SDS) and suspend the pellet.
3. Add 500 μL of 10% SDS to a final concentration of 1% and mix well gently up and down in the tube.
4. Add 24 μL of 10 mg/mL proteinase K, mix gently up and down in the tube, and incubate for 20 min at 55°C.

3.1.3. Sperm

1. Collect sperm from cauda epididymis in a culture dish with phosphate-buffered saline and transfer to a 50-mL tube with phosphate-buffered saline (sperm from 10–20 mice or 3–5 rats is put together for DNA extraction).
2. Centrifuge at 3000*g* for 10 min at 4°C and discard the supernatant.
3. Suspend sperm in 5-mL lysis buffer without SDS by pipetting.
4. Add 500 μL of 10% SDS and 50 μL of 2-mercaptoethanol (14.4 *M*) and mix gently by pipetting (final concentrations of SDS and 2-melcaptoethanol are 1% and 0.144 *M*, respectively).
5. Incubate for 30 min at 37°C. (2-mercaptoethanol reduces S-S bonds of protamines, this step is crucial for complete digestion by proteinase K.)
6. Add 200 μL of 10 mg/mL proteinase K and incubate at 55°C for 30 min.

Next, add an equal volume of PCI, mix by rotating (25 rpm) for 30 min at room temperature and centrifuge at 3000*g* for 30 min at room temperature. Transfer the aqueous layer to a new 50-mL tube with a wide-pored cutoff 1000-μL or 5000-μL tip. Repeat the PCI treatment and centrifugation and transfer the aqueous layer to a dialysis tube preequilibrated with 10 m*M* Tris-HCl pH 8.0. Dialyze three times (2 h, 2 h, and overnight) against 1 L of 10 m*M* Tris-HCl pH 8.0 per four dialysis tubes; transfer the dialysate to a 50-mL tube; and add 1 : 1000 volume of 1 mg/mL RNaseA and incubate

for 2 h at 37°C. Next, divide the sample into several 50-mL tubes (less than 10 mL of sample in each tube) and slowly add 40 mL ice-cold ethanol retaining the interface between DNA solution and ethanol, and rotate slowly to mix the DNA solution and ethanol at 15 rpm for 30 min. Pick up the DNA pellet with a 200-µL yellow tip and transfer to a 1.5-mL tube and carefully remove any ethanol in the tube with a 200-µL yellow tip and dry the pellet briefly for several mins (do not dry too much). Finally, dissolve in TE buffer for several days at 4°C before methylation analyses (*see* **Note 3**).

3.2. Southern Blotting With Methylation-Sensitive Restriction Enzyme

This section describes the procedure for Southern blotting to evaluate the methylation status of genomic DNA by methylation-sensitive restriction enzymes using *Not*I as an example. To simplify the results, genomic DNA is first fragmentized with *Pst*I that does not contain methylatable CpGs within its recognition sequence.

3.2.1. Restriction Enzyme Digestion and Electrophoresis (see **Note 4**)

1. Digest genomic DNA with *Pst*I as follows: 20 µg genomic DNA; 5 µL 10X H buffer; and 3 µL *Pst*I (10 U/µL). Top up to 50 µL with sterilized distilled water and mix well.
2. Transfer 2 µL of the mixture to a 1.5-mL tube containing *Sca*I-linearized pBluescript II SK⁻ vector (0.1 µg in 8 µL of 1X H buffer) to ensure the complete digestion of the genomic DNA.
3. Incubate both tubes overnight at 37°C.
4. Run a sample of the plasmid on a 1.5% agarose gel. The completely digested plasmid results in 1.9-kb and 1.1-kb bands in addition to the genomic band. If a 3-kb undigested plasmid band can still be seen, the sample should be further digested with *Pst*I until the complete digestion is confirmed (*see* **Note 5**).
5. Precipitate *Pst*I-digested DNA with ethanol and dissolve in 30 µL TE.
6. Digest half of the *Pst*I-digested genomic DNA as follows: 15 µL *Pst*I-digested DNA; 3 µL 10X H buffer; 3 µL 0.1% BSA; 3 µL 0.1% Triton X-100; and 2 µL *Not*I (10 U/µL). Top up to 30 µL with sterilized-distilled water and mix well.
7. Transfer 2 µL of the mixture to a 1.5-mL tube containing *Sca*I-linearized pBluescript II SK⁻ vector (0.1 µg in 8 µL of 1X H buffer) for digestion confirmation.
8. Incubate both tubes overnight at 37°C.
9. Ensure the complete digestion as for **step 4**.
10. Precipitate the double-digested DNA with ethanol and dissolve in 16 µL TE.
11. Measure the DNA concentration and adjust to 5 µg/15 µL by TE.
12. Apply 15 µL of the DNA (5 µg) with gel-loading buffer and run on a 1.4% GTG agarose gel in 1X TAE buffer.
13. Stain the DNA in 0.5 µg/mL ethidium bromide solution.
14. Photograph the gel under UV light placing a transparent ruler alongside the gel.

3.2.2. Capillary Transfer of DNA to Nylon Membrane

1. Gently rock the gel in distilled water to remove excess ethidium bromide.
2. Soak upper one-third of the gel in 0.25 *N* HCl by slanting the plastic tray containing the gel and gently rock the gel for 10 min for partial degradation of high molecular-weight DNA.
3. Denature the DNA by soaking the gel for 2X 15 min in denaturation buffer with gentle rocking.

4. Neutralize the gel by soaking the gel for 2X 15 min in neutralization buffer with gentle rocking.
5. While the gel is soaking in neutralization buffer, cut a nylon membrane (BIODYNE A: PALL, Port Washington, NY) to the same size as the gel and equilibrate the membrane with distilled water followed by 20X SSC (before equilibration, record information such as date and sample names with a pencil to identify the membrane).
6. Capillary-transfer the neutralized DNA from the gel to the membrane with 20X SSC (remove air bubbles between the gel and membrane completely).
7. Mark the well positions with a ballpoint pen and peel the gel.
8. Air-dry the membrane and UV-crosslink at 120,000 μJ, three times (the membrane is sealed in a heat-sealable plastic bag and can be stored at room temperature until use).

3.2.3. Probe Labeling

1. Prepare DNA fragment of the interested region of genomic DNA.
2. Denature 300 ng of purified DNA by boiling for 10 min followed by chilling on ice for 5 min.
3. Assemble the following (DIG DNA labeling kit, Roche): 15 μL heat-denatured DNA; 2 μL hexanucleotide mixture; 2 μL dNTP mixture; and 1 μL Klenow fragment for a total of 20 μL. Incubate for overnight at 37°C.
4. Stop the reaction by adding 1 μL of 0.5 *M* EDTA and precipitate the labeled DNA with ethanol in the presence of co-precipitant.
5. Dissolve the DNA in 50 μL TE. Using 300 ng template DNA, 500 ng labeled probe is generated by an overnight reaction. Thus, the final probe concentration is about 10 ng/μL.
6. Store the labeled probe at –20°C up to 6 mo.

3.2.4. Hybridization and Detection

1. Boil 150 μL of 10 mg/mL salmon sperm DNA for 10 min followed by chilling on ice for 3 min.
2. Add the denatured salmon sperm DNA to the membrane with 15 mL of hybridization buffer in a plastic tray.
3. Incubate for 1 h at 60°C for prehybridization.
4. Boil 160 ng of DIG-labeled probe in 100 μL TE for 10 min followed by chilling on ice for 3 min.
5. Add the denatured probe to the membrane with hybridization buffer (8 mL for a 6-cm × 11-cm membrane) in a plastic bag and mix well before sealing (final probe concentration is 20 ng/mL).
6. Remove air bubbles and seal the plastic bag.
7. Incubate overnight at 60°C for hybridization.
8. Open the bag and discard the probe-containing hybridization buffer.
9. Wash the membrane three times in a plastic tray containing 2X SSC/0.1% SDS for 5 min each at 68°C.
10. Wash the membrane three times in 0.1X SSC/0.1% SDS for 5 min at 68°C.
11. Rinse the membrane in TBS-T to remove SDS for 3 min with gentle rocking.
12. Briefly rinse the membrane with maleate buffer followed by 1% blocking buffer for 1 min each.
13. Place the membrane in 15 mL of 1% blocking buffer and rock the membrane gently for 1.5 h at room temperature for blocking.
14. Add 1.5 μL of anti-digoxigenin-AP Fab fragment to the membrane in blocking buffer and incubate for 1 h with gentle rocking (antibody is diluted to 1 : 10,000).
15. Wash the membrane three times in a plastic tray containing TBS-T for 10 min at room temperature.

16. Equilibrate the membrane in buffer 3 for 10 min at room temperature.
17. Dilute 4 µL of CDP-star to 400 µL with buffer 3 and spot on a plastic bag as small drops.
18. Place the membrane on the plastic bag with DNA side touching to CDP-star and seal the plastic bag after removal of air bubbles.
19. Expose to an X-ray film for 15 min and develop the film.
20. Re-expose and develop X-ray films depending on the hybridization signal intensity.

3.3. PCR to Evaluate Methylation Status

This section describes a simple PCR method to roughly evaluate the methylation status of methylation-sensitive restriction enzyme sites using *Not*I as an example.

1. Digest 5 µg of genomic DNA with an appropriate restriction enzyme that does not have CpGs in its recognition site. Make sure that the target sequence of PCR amplification does not have the recognition site (this step is only to simplify handling of genomic DNA and does not require complete digestion of genomic DNA).
2. Extract the digested DNA with PCI, precipitate with ethanol and dissolve in 30 µL TE.
3. Digest half of the DNA with *Not*I as follows: 15 µL size-fragmented genomic DNA; 3 µL 10X H buffer; 3 µL 0.1% BSA; 3 µL 0.1% Triton X-100; and 2 µL *Not*I (10 U/µL). Top up to 30 µL with sterilized distilled water.
4. Transfer 2 µL of the mixture to a 1.5-mL tube containing *Sca*I-linearized pBluescript II SK^- vector (0.1 µg in 8 µL of 1X H buffer) for digestion confirmation.
5. Incubate both tubes for overnight at 37°C.
6. Check the plasmid digestion by electrophoresis to confirm complete digestion of genomic DNA (*see* **Note 5**).
7. Precipitate the *Not*I-digested DNA with ethanol and dissolve in 16 µL TE.
8. Measure the DNA concentration and adjust to 100 ng/µL by TE.
9. Assemble the following for PCR and mix gently by pipetting: 3 µL enzyme-digested DNA (100 ng/µL); 2 µL 10X PCR buffer; 2 µL dNTP mixture (2 m*M* each); 0.5 µL Primer MS-F (10 nmol/µL); 0.5 µL Primer MS-R (10 nmol/µL); 0.5 µL Primer CNTL-F (10 nmol/µL); 0.5 µL Primer CNTL-R (10 nmol/µL); 1.2 µL $MgCl_2$ (25 m*M*); 0.2 µL *Taq* DNA polymerase; 9.6 µL sterilized-distilled water for a total of 20 µL.
10. Amplify the enzyme-digested DNA for 30 cycles of 94°C for 1 min, 50–65°C (depending on primer sequences) for 30 sec, and 72°C for 1 min.
11. Run PCR products on an agarose gel and stain with ethidium bromide.

3.4. RLGS Procedure

This section describes the protocol to study genome-wide methylation status of gene loci (CpG islands) by restriction landmark genomic scanning (RLGS) based on two-dimensional electrophoresis of genomic DNA.

3.4.1. Genomic DNA Treatment Before Electrophoresis

1. Mask nicks in genomic DNA to avoid nonspecific labeling before digestion by restriction enzymes as follows: 7 µL sample genomic DNA (0.5–1.0 µg/µL); 2.5 µL masking buffer; and 0.5 µL Klenow fragment for a total of 10 µL.
2. Mix well by gentle pipetting, incubate for 20 min at 37°C and inactivate Klenow fragment for 30 min at 65°C.
3. Perform *Not*I digestion: 10 µL masked sample; 8 µL 2.5X SHB; and 2 µL *Not*I (10 U/µL) for a total of 20 µL.
4. Transfer 2 µL of the mixture to a 1.5-mL tube containing *Sca*I-linearized pBluescript II SK^- vector (0.1 µg in 8 µL of 1X H buffer) for digestion confirmation.

5. Incubate both tubes for 4 h at 37°C.
6. Confirm complete digestion of genomic DNA with electrophoresis (*see* **Note 5**).
7. Labeling: 18 µL *Not*I-digested sample; 1 µL [α-^{32}P] dCTP; 1 µL [α-^{32}P] dGTP; 0.3 µL 1 *M* DTT; and 0.3 µL Sequenase Ver. 2 (13 U/µL) for a total of 20.6 µL.
8. Incubate for 1 h at 37°C.
9. *Pvu*II digestion: 20.6 µL labeled sample; 7.6 µL second-digestion buffer; 2 µL of *Pvu*II (10 U/µL) for a total of 30.2 µL.
10. Incubate overnight at 37°C (the *Pvu*II-digested samples can be stored at 4°C for a few days before electrophoresis).

3.4.2. First-Dimensional Gel Electrophoresis and In-Gel DNA Size Fragmentation With the Third Restriction Enzyme (PstI)

1. Prepare first-dimensional gel with the following: 10 mL 10X first-dimension buffer; 0.9 g GTG agarose (final 0.9%); and 5 g sucrose (final 5 %). Top up to 90 mL with distilled water, and melt agarose in a microwave oven. Filter through 0.22-µm filter, adjust to 100 mL with prewarmed distilled water, and warm the gel solution in a 55–60°C water bath.
2. Connect the top of the gel holder with 2- to 3-cm silicone tubing at the bottom of a three-way stopcock and connect a 5-mL plastic syringe at the top of the three-way stopcock.
3. Hold the gel holder connected with the syringe on a stand. Suck up the first-dimensional gel gradually from the bottom to the height 1 cm below the top of gel holder and close the stopcock.
4. After the gel solidifies, open the stopcock and remove the silicone tubing with stopcock.
5. Set the gel holders to the first-dimensional electrophoresis tank and fill 350 mL of 1X first-dimension buffer to the bottom tank and 250 mL of first-dimension buffer to upper tank, respectively.
6. Remove air inside the gel holder using a 5-mL syringe with a 19-gauge needle (check the current of electricity before applying hot samples: 100V with four gels is usually approx 2–3 mA).
7. Apply 12.5 µL of sample mixed with 2.5 µL of 6X dye (*see* **Note 6**).
8. Run the electrophoresis at 100 V for 1 h and at 230 V overnight until the BPB reaches 50 cm from the gel top.
9. Remove the buffer from the top tank with an aspirator and bring out the gel holders (note that the buffer after electrophoresis is contaminated with ^{32}P).
10. Push out the gel noodle slowly with the in-gel digestion buffer (1X H buffer) using a cutoff 200-µL tip on a 1-mL syringe.
11. Cut the gel noodle at the point about 5-cm lower molecular size from BPB (about 500 bp) and discard the gel less than the 500-bp position.
12. Put the gel noodle into a 50-mL tube containing 40 mL of 1X H buffer and rock the tube gently for 10 min to equilibrate the gel.
13. Change the buffer and rock the tube once more for 10 min.
14. Put the equilibrated gel noodle into a white plastic tray laid a black celluloid sheet (about the half size of the tray) on the bottom of the tray (white color helps to distinguish the BPB and XC dyes and black color visualizes the gel shape).
15. Pour the 1X H buffer into the tray.
16. Gently suck up the gel noodle from the BPB side into the teflon tubing (inner diameter of 3.0 mm, 42 cm long) connected to a 5-mL syringe with a 1.5-cm silicone tubing.
17. Remove the buffer inside the teflon tubing into the syringe by pulling slowly the piston.
18. Slowly suck up 1200 µL of 1X H buffer containing 1200 U of *Pst*I and 0.01% BSA into the tubing (*see* **Note 7**).

19. Remove the syringe and connect one end of the tube to the other with a 1.5-cm silicone tubing to loop the teflon tubing.
20. Incubate for 2 h at 37°C.

3.4.3. Second-Dimensional Electrophoresis

1. Wipe the surface of glass plates for second-dimensional apparatus with 70% ethanol and siliconize one side of glass plates (the side without slope).
2. Assemble the second-dimensional apparatus and seal the side holes near the bottom of the apparatus with Scotch 3M tape.
3. Prepare 2.5 L of 5% acrylamide (acrylamide 120.8 g and methylenebisacrylamide 4.2 g, acrylamide : bis 29 : 1) gel solution in 1X TBE and add 1.7 g of ammonium persulfate.
4. Add 677 µL of TEMED and pour the acrylamide gel solution into second-dimensional electrophoretic tank.
5. Cover the gel surface with water-saturated 2-butanol and wait for about 2 hr until the gel is solidified.
6. Remove the tape around the side holes.
7. Wash out 2-butanol and fill the top of the gels with 1X TBE buffer until electrophoresis (check that the buffer does not leak). **Steps 1–7** must be completed before the in-gel digestion is finished.
8. Rinse the top of the second acrylamide gel with 1X TBE and gently wipe the gel surface with Whatman 3MM filter paper.
9. Expel the *Pst*I-treated gel noodle from the teflon tubing into a 50-mL tube containing 40 mL of 1X TBE.
10. Rock the tube gently for 10 min to equilibrate the gel.
11. Discard the buffer and put the equilibrated gel onto a black celluloid sheet.
12. Wipe the surface of the gel with Kim wipe.
13. Transfer the gel noodle onto the top of second acrylamide gel.
14. Connect the gel noodle and second acrylamide gel with approx 2 mL of connecting agarose gel using a 5-mL syringe with 19-gauge needle.
15. Cover the connected gel surface with 6X dye solution without glycerol.
16. Run electrophoresis at 100 V for 1 h and at 150 V for about 20 h until BPB reaches the side holes at the bottom.

3.4.4. Autoradiography

1. Carefully remove the glass plate over the gel.
2. Place a Whatman 3MM filter paper (34.5 cm × 42.5 cm: 5 mm each smaller than 35 cm × 43 cm cassette size) at the center of acrylamide gel and cut the gel along the filter paper with a razor blade.
3. Pick up the excised gel with the filter paper and cover the gel surface with Saran Wrap.
4. Dry the gel for about 20 min at 80°C.
5. Check the radioactivity of the gel at the center and the most radioactive point of the top side of second dimension with a survey meter.
6. Autoradiograph at –80°C for 2–4 wk depending radioactivity (*see* **Note 8**).

3.4.5. Spot DNA Cloning

3.4.5.1 Two-Dimensional Electrophoresis for Cloning

1. Label the genomic DNA sample that can visualize spots of interest by the same method as in the **Subheading 3.4.1.** of RLGS.

2. Digest the same genomic DNA sample with *Not*I and *Pvu*II (without labeling).
3. Mix the labeled and unlabeled DNA samples in the following order (*see* **Note 9**): 30.2 µL labeled DNA; 25 µL 6X dye solution for first-dimensional electrophoresis (containing 150 m*M* EDTA); 39.6 µL TE; and 30.2 µL unlabeled DNA for a total of 125 µL.
4. Apply 12.5 µL each of mixed sample (a total of 10 gels) and run RLGS.
5. Dry the second-dimensional gel at 65°C and staple the dried gel with X-ray film at multiple sites (more than 10 per gel) to identify the gel and X-ray film position.
6. Autoradiograph for 1 mo, develop, and re-staple the gel and autoradiogram.

3.4.5.2. Adaptor Ligation and PCR Amplification

1. Punch out spots of interest and peel Saran Wrap from dried spot gel.
2. Electroelute the DNA for 20 min at 200 V in 1X TBE buffer.
3. Extract the DNA with equal volume of PCI and centrifuge at 15,000*g* for 1 min.
4. Precipitate with ethanol in the presence of coprecipitant and dissolve in 3 µL TE.
5. Assemble the following and mix well by pipetting: 3 µL eluted spot DNA; 0.5 µL *Not*I adaptor (10 pmol/µL); 0.5 µL *Pst*I adaptor (10 pmol/µL); 5 µL 2X ligation buffer; and 1 µL T4 DNA ligase for a total of 10 µL.
6. Incubate for overnight at 4°C.
7. Add 30 µL TE and pass through MicroSpin S-400 column at 700*g* for 1 min to remove excess adaptors.
8. Mix the following reagents to perform PCR: 14.5 µL adaptor-ligated spot DNA; 25 µL 2X GC-rich buffer II; 8 µL dNTP (3.2 m*M* each); 1 µL Ad-NI primer (10 pmol/µL); 1 µL Ad-PI primer (10 pmol/µL); and 0.5 µL LA-Taq (Takara) for a total of 50 µL.
9. Amplify the linker-ligated DNA for 30 cycles of 94°C for 1 min, 65°C for 30 s, and 72°C for 1 min.
10. Electrophoresis 5 µL of the PCR product in agarose gel. If resulting band is faint or is not seen, reamplify the spot DNA using 1 µL of first PCR solution as a template.
11. Double-digest the PCR product with *Not*I and *Pst*I.
12. Ligate into pBluescript II SK$^-$ digested with *Not*I and *Pst*I for 2 h at 16°C.
13. Transform into *Escherichia coli*-competent cells (XL-1Blue MRF′) and select positive colonies containing plasmid with *Not*I site.

3.4.6. Further Analyses

It has been reported that approx 90% of *Not*I sites are in CpG islands, which are the dense CpG regions. Genomic regions including *Not*I sites as restriction landmarks, which are revealed to be differentially methylated among cells, can be further analyzed by other methods, including DNA methylation-sensitive Southern blotting and PCR (described in this chapter) and sodium bisulfite sequencing (described elsewhere; **ref. *10***).

4. Notes

1. The REBASE homepage (http://rebase.neb.com/rebase/rebase.html) has an excellent list for hundreds of methylation-sensitive restriction enzymes as well as methylases. This list also contains information on enzyme suppliers and detailed position effects of methylated bases on digest ability. Thus, you can refer to the information to decide which enzyme is appropriate to evaluate the methylation status of the genes of interest.
2. The 1.5-mL annealing reaction tube is floated on water at 70°C in a 500-mL beaker. Leave the tube in the warm water until it becomes room temperature.
3. Pellet is dissolved in 100–300 µL for tissue samples and 50–100 µL for sperm and cultured cell samples. To obtain fine profiles of RLGS, purification of high quality genomic

DNA is most important. Accordingly, degradation by endogenous DNases and mechanical shearing must be avoided.

4. Recognition sites of methylation-sensitive enzymes containing methylatable CpG dinucleotides exist one quarter to one fifth less frequently in the mammalian genome than expected. Thus, to separate methylated and unmethylated bands clearly on ordinary agarose gels, genomic DNA must be first fragmented with one or two appropriate restriction enzyme(s) whose recognition sites do not contain methylatable CpGs.
5. Restriction enzymes can be simply added to the reaction mixture unless the total enzyme volume does not exceed to 10% of reaction mixture. Purifying genomic DNA by PCI extraction and ethanol precipitation may help to digest DNA in the case of certain contaminants inhibiting the enzyme reaction.
6. Labeled genomic DNA applied to first-dimensional electrophoresis must be less than 1 µg. Applying large amount of DNA results in unclear spot pattern in high molecular weight region and high background. Usually, 1 : 1–1 : 5 dilution of labeled DNA with TE gives appropriate DNA concentration for first-dimensional electrophoresis.
7. High glycerol hangover from in-gel digestion can cause high background in autoradiograms. Thus, *Pst*I enzyme of high concentration should be purchased and used for in-gel digestion to minimize glycerol content.
8. To check RLGS spot pattern with an image analyzer is simple and quick. However, it is better to use X-ray films for obtaining fine profiles and for comparison of spot pattern.
9. Labeled DNA gives spots in autoradiograms, and unlabeled DNA at the same position with the spots can be cloned. Since addition of 150 m*M* EDTA in 6X dye ensures the complete inactivation of Sequenase, unlabeled DNA must not be added before the 6X dye is added to the labeled DNA.

References

1 Shiota, K., Kogo, K., Ohgane, J., Imamura, T., Urano, A., Nishino, K., et al. (2002) Epigenetic marks by DNA methylation specific to stem, germ and somatic cells in mice. *Genes Cells* **7,** 961–969.

2 Ohgane, J., Wakayama, T., Kogo, Y., Senda, S., Hattori, N., Tanaka, S., et al. (2001) DNA methylation variation in cloned mice. *Genesis* **30,** 45–50.

3 Imamura, T., Ohgane, J., Ito, S., Ogawa, T., Hattori, N., Tanaka, S., et al. (2001) CpG island of rat sphingosine kinase-1 gene: tissue-dependent DNA methylation status and multiple alternative first exons. *Genomics* **76,** 117–125.

4 Cho, J.-H., Kimura, H., Minami, T., Ohgane, J., Hattori, N., Tanaka, S., et al. (2001) DNA methylation regulates placental lactogen-I gene expression. *Endocrinology* **142,** 3389–3396.

5 Shiota, K. and Yanagimachi, R. (2002) Epigenetics by DNA methylation for development of normal and cloned animals. *Differentiation* **69,** 162–166.

6 Bird, A. P. (2002) DNA methylation patterns and epigenetic memory. *Genes Dev.* **16,** 6–21.

7 Hayashizaki, Y., Hirotsune, S., Okazaki, Y., Hatada, I., Shibata, H., Kawai, J., et al. (1993) Restriction landmark genomic scanning method and its various applications. *Electrophoresis* **14,** 251–258.

8 Ohgane, J., Aikawa, J., Ogura, A., Hattori, N., Ogawa, T., and Shiota, K. (1998) Analysis of CpG islands of trophoblast giant cells by restriction landmark genomic scanning. *Dev. Genet.* **22,** 132–140.

9. Hayashizaki, Y. and Watanabe, S. (eds.) (1997) *Restriction Landmark Genomic Scanning (RLGS).* Springer-Verlag, Tokyo.

10. Frommer, M., McDonald, L. E., Millar, D. S., Collis, C. M., Watt, F., Grigg, G. W., et al. (1992) A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc. Natl. Acad. Sci. USA* **89,** 1827–1831.

37

Serial Analysis of Gene Expression in Human Keratinocytes and Epidermis

Bastiaan J. H. Jansen, Gys de Jongh, Joost Schalkwijk, and Fred van Ruissen

Summary

The demand for large-scale gene expression analysis tools is on the rise now that several genomes have been sequenced. One of these tools, serial analysis of gene expression (SAGE), allows the qualitative as well as quantitative analysis of a large number of genes in a defined tissue or culture model. SAGE has already been successfully used to identify differentially expressed genes in normal physiological processes and pathological conditions. This chapter focuses on the SAGE protocol and its application to cultured human keratinocytes, and on MicroSAGE, an adapted protocol that allows the use of small amounts of mRNA from isolated epidermis or a skin biopsy.

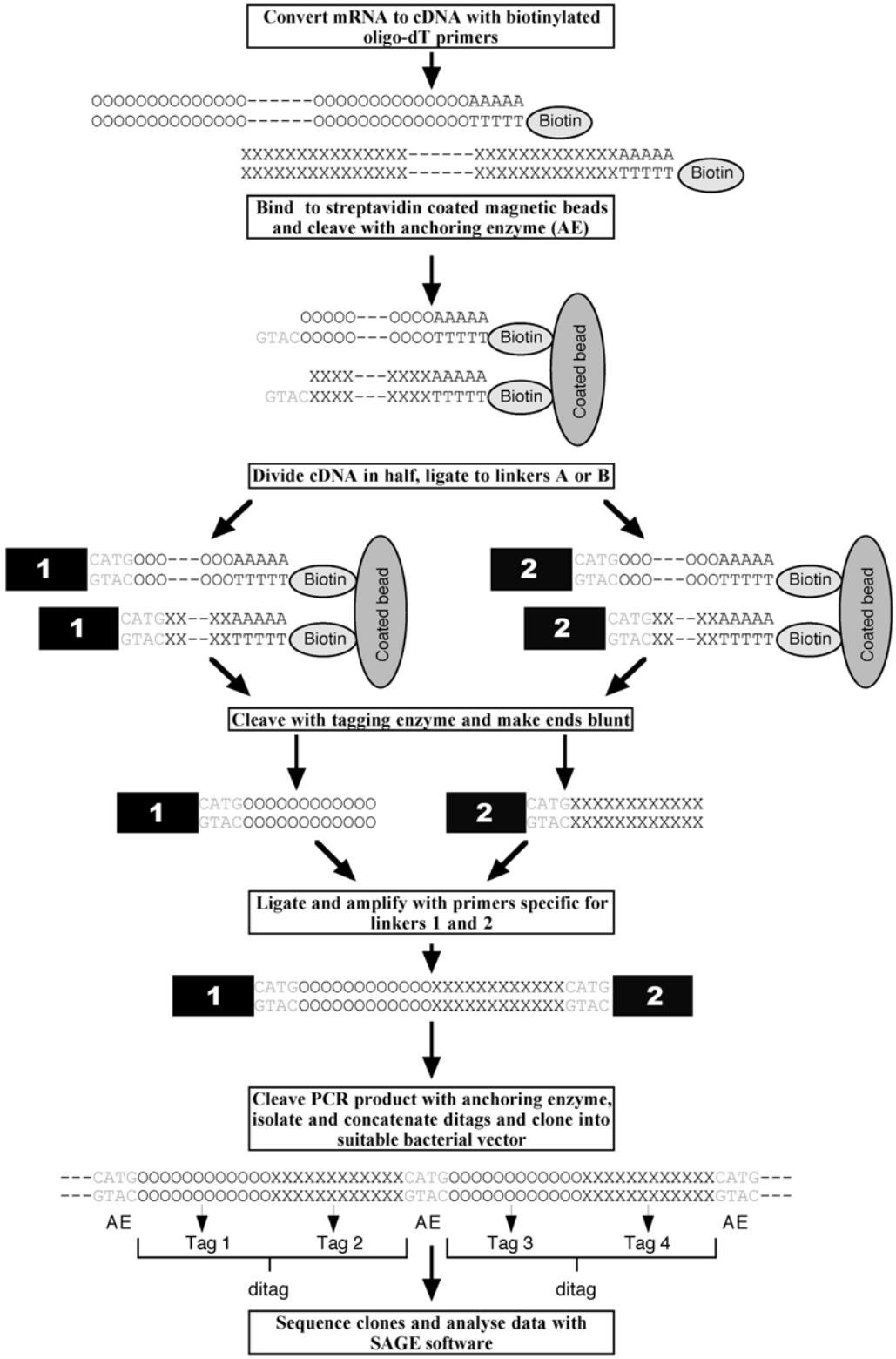
Key Words:

SAGE; MicroSAGE; keratinocyte; epidermis; skin; gene expression.

1. Introduction

Now that the human genome has been sequenced, the interest in methods that allow large-scale analysis of gene expression has risen dramatically. Various technologies are now available, such as complimentary deoxyribonucleic acid (cDNA) microarray analysis and serial analysis of gene expression (SAGE; **ref.** ***1***; *see* **Note 1**). SAGE allows the qualitative as well as quantitative analysis of a large number of genes expressed in a well-defined cell type or tissue and can be used to identify differentially expressed genes in a wide variety of diseases and cell culture conditions ***(2–7)***. Three principles underlie the SAGE methodology: (1) a short 10- to 14-basepair sequence or tag contains sufficient information to uniquely identify a transcript, provided that the tag is obtained from a unique position (3′ of the most distal restriction site of a well-defined endonuclease) within each cDNA corresponding with that particular transcript; (2) sequence tags can be linked together to from long serial molecules that can be cloned and sequenced; and (3) quantitation of the number of times a particular tag is observed provides the expression level of the corresponding transcript. A simplified overview of the technique is shown in **Fig. 1**. Here we concentrate on the application of SAGE to human cultured keratinocytes, and on MicroSAGE, an adapted protocol that allows the use of small amounts of mRNA as input, derived from small specimens such as isolated epidermis from a skin biopsy ***(8–11)***.

From: *Methods in Molecular Biology, vol. 289: Epidermal Cells: Methods and Protocols*
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ



2. Materials

2.1. Solutions

1. 2X B+W buffer: 10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, pH 8.0, 2 *M* NaCl; store at room temperature.
2. LoTE: 3 m*M* Tris-HCl, pH 7.5, 0.2 m*M* EDTA, pH 8.0; store at 4°C.
3. Phenol:chloroform (3 : 4, pH 8): 480 mL phenol (Invitrogen, Carlsbad, CA; cat. no. 15509-037), 320 mL 0.5 *M* Tris-HCl, pH 8.0, 640 mL chloroform. First, warm phenol to 65°C, then add Tris-HCl and chloroform, shake, and place at 4°C; shake again after 2 to 3 h. Check pH of the aqueous layer; if necessary, equilibrate again with 0.5 *M* Tris-HCl; after an additional 2 to 3 h, aspirate aqueous layer; aliquot in 50-mL portions and store at –20°C.
4. 10X PCR buffer: 166 m*M* $(NH_4)_2SO_4$, 670 m*M* Tris-HCl, pH 8.8, 67 m*M* $MgCl_2$, 100 m*M* β-mercaptoethanol; aliquot into 0.5-mL portions and store at –20°C.

2.2. Oligonucleotides and Linkers

High-quality oligonucleotides are absolutely crucial to several steps in SAGE and should therefore be obtained gel-purified from a trusted company.

1. Linker 1A: 5′ TTT GGA TTT GCT GGT GCA GTA CAA CTA GGC TTA ATA GGG ACA TG 3′ (*see* **Note 2**).
2. Linker 1B: 5′ TCC CTA TTA AGC CTA GTT GTA CTG CAC CAG CAA ATC C[Amino mod. C7] 3′.
3. Linker 2A: 5′ TTT CTG CTC GAA TTC AAG CTT CTA ACG ATG TAC GGG GAC ATG 3′ (*see* **Note 2**).
4. Linker 2B: 5′ TCC CCG TAC ATC GTT AGA AGC TTG AAT TCG AGC AG[Amino mod. C7] 3′.
5. Primer 1: 5′ GGA TTT GCT GGT GCA GTA CA 3′.
6. Primer 2: 5′ CTG CTC GAA TTC AAG CTT CT 3′.
7. Biotinylated oligo-d T_{18}: 5′[biotin]dT_{18} 3′.
8. pUC18 Forward: 5′ AAG TTG GGT AAC GCC AGG 3′.
9. pUC18 Reverse: 5′ GGC TCG TAT GTT GTG TGG 3′.
10. M13(-21) Forward: 5′ TGT AAA ACG ACG GCC AGT 3′.

2.2.1. Phosphorylation of Oligos for Linkers

To make linkers, first phosphorylate oligos 1B and 2B as follows:

	Tube 1	Tube 2
Linker 1B (350 ng/µL)	9 µL	–
Linker 2B (350 ng/µL)	–	9 µL
LoTE	6 µL	6 µL
10X kinase buffer (New England Biolabs, Beverly, MA)	2 µL	2 µL
10 m*M* ATP	2 µL	2 µL
T4 polynucleotide kinase (10 U/µL)	1 µL	1 µL

Mix well and incubate at 37°C for 30 min and then heat-inactivate enzyme at 65°C for 10 min.

Fig. 1. *(see opposite page)* An overview of the conventional SAGE method. In MicroSAGE, the sample is already split before cDNA synthesis. Reprinted from **ref. *9***, with permission of Blackwell Publishing, Oxford, UK.

2.2.2. Annealing of Phosphorylated Oligos to Create Double-Stranded DNA Linkers

To make dsDNA linkers, add 9 µL of linker 1A (350 ng/µL) to 20 µL of phosphorylated linker 1B and 9 µL of linker 2A (350 ng/µL) to 20 µL of phosphorylated linker 2B in a 0.2-mL polymerase chain reaction (PCR) tube and mix well; to anneal, heat mixture to 95°C for 2 min in a PCR machine, and slowly cool down to 21°C at 0.1°C/s. The concentration of the linkers is now approx 200 ng/µL. To check for efficient phosphorylation, self-ligate approx 200 ng of each linker pair and check ligation on a 20% Novex gel (Invitrogen; cat. no. EC6315), or on a 12% polyacrylamide gel. Only linkers that self-ligate >70% should be used for SAGE.

2.3. Kits and Reagents

2.3.1. Kits and Reagents for Conventional SAGE

1. Any commercial total RNA isolation kit; alternatively, one can use conventional protocols (**ref. *12***; *see* **Note 3**).
2. Any mRNA isolation kit (such as Oligotex kit from Qiagen, Hilden, Germany).
3. Any cDNA synthesis kit (cDNA Synthesis System, Roche, Basel, Switzerland; cat. no. 1117831; or SuperScript™ Double-Stranded cDNA Synthesis Kit, Invitrogen; cat. no. 11917-010.
4. Magnetic Dynabeads® M-280 Streptavidin (Dynal, Oslo, Norway; cat. nos. 112.05/06 and 602.10).
5. Magnet, Dynal MPC®-S (Dynal; cat. no. 120.20).
6. Restriction enzyme *Nla*III (New England Biolabs; cat. no. R0125S or R0125L; *see* **Note 4**).
7. Restriction enzyme *Bsm*FI (New England Biolabs; cat. no. R0580S or R0580L).
8. Restriction enzyme *Sph*I (New England Biolabs; cat. no. R0182S or R0182L).
9. dNTPs (100 m*M* stock of each dATP, dCTP, dGTP, and dTTP, Amersham Biosciences, Buckinghamshire, United Kingdom; cat. no. 27-2035-02; make 25 m*M* dNTP working solution).
10. DNA polymerase I, Klenow fragment (Roche; cat. no. 1008404).
11. T4 DNA ligase high concentration (5 U/µL, Invitrogen; cat. no. 15224-041).
12. Platinum *Taq* DNA polymerase (5 U/µL, Invitrogen; cat. no. 10966-034).
13. Dimethyl sulfoxide (Sigma Chemicals, St. Louis, MO; cat. no. D2650).
14. 40% Acryl-bisacrylamide (19 : 1 acrylamide : bisacrylamide, Biorad, Hercules, CA; cat. no. 161-0144).
15. 40% Acryl-bisacrylamide (37.5 : 1 acrylamide : bisacrylamide, Bio-Rad; cat. no. 161-0144).
16. 50X TAE: 2*M* Tris/acetic acid (pH 8), 50 m*M* EDTA.
17. 10% Ammonium persulfate (APS, Sigma Chemicals; cat. no. A9164).
18. TEMED (Sigma Chemicals; cat. no. T7024).
19. SuperLadder-low 20-bp/100-bp marker (Eurogentec, Seraing, Belgium; cat. no. MW-0312-05).
20. SmartLadder (Eurogentec; cat. no. MW-1700-10).
21. SYBR Green I (Molecular Probes, Eugene, OR; cat. no. S-7563).
22. Spin-X microcentrifuge tube filters (Corning Costar, Corning, NY; cat. no. 8160).
23. Vector pUC18 (various suppliers; digest 1 µg with *Sph*I, clean up vector over agarose gel, and resuspend in TE to an end concentration of 25 ng/µL).
24. Chemically competent or electrocompetent bacteria (sold by various companies).
25. Sequencing kit and automated sequencer (sold by various companies).
26. Glycogen (20 mg/mL, Roche; cat. no. 901393).
27. 7.5 *M* Ammonium acetate (Sigma Chemicals; cat. no. A2706).
28. Ethanol, molecular biology grade.

2.3.2. Reagents Needed for MicroSAGE

MicroSAGE was developed to accommodate the use of small amounts of total RNA, thus, the use of small biopsies. This requires special reagents that allow the separation of epidermis from the dermis, as well as a different kit that accommodates the isolation of 3′ cDNA ends from total RNA. MicroSAGE requires the use of streptavidin coated 0.2-mL tubes to capture biotin labeled cDNA ends. The following reagents are required:

1. Actinomycin D (Sigma Chemicals; cat. no. A9415).
2. Dispase (Roche; cat. no. 0210455).
3. mRNA Capture Kit (Roche; cat. no. 1787896).

2.4. Software and Supplies for Analysis

For the automated analysis of sequenced clones and the extraction of tags, special software is required. The software, called SAGE2000, can be obtained from the inventors of SAGE ***(1)***, and to obtain a copy, one should download and sign a Material Transfer Agreement at http://www.sagenet.org/ (*see* **Note 1**).

3. Methods

3.1. Preparation of Input Material

This section describes the ways of isolating and generating enough RNA for the construction of a SAGE library. The way of isolating RNA depends on the source and amount of cells or biopsy material. It is absolutely critical to obtain pure RNA, which can best be achieved by using a trusted commercial RNA isolation kit. Conventional methods, however, can be used ***(12)***.

3.1.1. Preparation of Input Material for Conventional SAGE

Various protocols exist on how to culture epidermal keratinocytes. In general, for SAGE to be successful, at least 8×10^6 cultured cells are needed per SAGE library. Although as little as 2.5 µg of polyadenylated mRNA may be sufficient for conventional SAGE, 5 to 10 µg is recommended. Pure messenger RNA is required for the conventional protocol. Use any of the commercially available kits to purify total RNA and subsequently mRNA, following the manufacturer's recommendations. Measure the amount of obtained mRNA spectrophotometrically, and check the integrity of RNA by gel electrophoresis and Northern blotting.

3.1.2. Preparation of Input Material for MicroSAGE

MicroSAGE allows the use of as little as 100 ng total RNA (less than 10 ng mRNA) and can therefore be applied to generate SAGE libraries from skin biopsies. To obtain pure epidermis, it first needs to be separated from the dermis. MicroSAGE requires total RNA as input, to minimize the loss of mRNA in the sample. Since there is very little RNA in the sample after isolation, there is no need to measure the amount spectrophotometrically. If the RNA concentration is known, do not use less than 2 µg of total RNA for MicroSAGE. The use of 10 µg of total RNA should give good results without the need of any further amplification steps as described in the original MicroSAGE protocol ***(8)***.

1. Wash punch biopsy (4 mm) in phosphate buffered saline, pH 7.0, containing 0.9 *M* $CaCl_2$ and 0.49 *M* $MgCl_2$.
2. Incubate biopsy with 4 mL dispase solution (dissolved in PBS, 12 mg/mL) containing actinomycin D (5 µg/mL; to prevent induction of new rounds of transcription in epidermis) for 4 h at 4°C.
3. Separate epidermis from dermis with a pair of tweezers and cut epidermis in two halves.
4. Fix approximately one half of the epidermis in 3.8% formaldehyde for immunohistochemistry, to check for correct separation (*see* **Note 5**).
5. Process the other half for RNA isolation; when under TRIzol or RNAzol, the epidermis can also be stored at –80° at this point; otherwise, disrupt the epidermis as much as possible by means of mechanical force using a plunger or a pipet tip.
6. Proceed with RNA isolation with the protocol of choice.
7. After precipitation according to manufacturer's protocol, resuspend total RNA in 40 µL lysis buffer (mRNA Capture Kit).
8. Split sample: 2 × 20 µL (for the ligation of linker 1 resp. linker 2) into two 0.2-mL tubes, then proceed to cDNA synthesis (**Subheading 3.2.2.**).

3.2. cDNA Synthesis

The synthesis of cDNA is essentially the same for both conventional SAGE and MicroSAGE, although the volumes are different, and the purification of cDNAs for both protocols is completely different.

3.2.1. cDNA Synthesis for Conventional SAGE

Use a commercial cDNA synthesis kit, following the manufacturer's protocol. Use 2.5 µg biotinylated oligo-dT_{18} (*see* **Subheading 2.2.**) to prime cDNA synthesis of approx 5 µg polyadenylated mRNA. Adapt reaction volumes to the amount of RNA used. Check the quality of the cDNA by means of agarose gel electrophoresis. After second-strand synthesis, clean up DNA as follows:

1. Raise the sample volume to 200 µL with LoTE.
2. Extract with equal volume phenol : chloroform (3 : 4, pH 8.0): mix by vortexing for several seconds and spin for 2 min at top speed in microcentrifuge, then transfer aqueous layer to new 1.5-mL microcentrifuge tube.
3. Precipitate DNA, add to 200-µL sample: 1 µL glycogen (*see* **Note 6**); 133 µL 7.5 *M* ammonium acetate, mix well; and 777 µL 100% ethanol.
4. Spin sample at top speed for 10 min at 4°C.
5. Wash pellet twice with 1 mL of ice-cold 70% ethanol; spin at 4°C full speed.
6. Dry pellet in SpeedVac, then resuspend pellet in 20 µL LoTE.

3.2.2. cDNA Synthesis for MicroSAGE

For MicroSAGE, mostly reagents from the mRNA Capture Kit, which has specifically been developed to capture mRNA from small amounts of total RNA, are used; carefully read the manufacturer's protocol before using this kit.

1. Add 4 µL of biotinylated oligo-dT_{20} (present in kit; 5 pmol/µL) to each of two 0.2-mL tubes containing 20 µL of lysis buffer containing RNA; mix by pipetting.
2. Incubate 5 min at 37°C, preferably in a polymerase chain reaction (PCR) machine.
3. Transfer mixture to streptavidin-coated 0.2-mL tube (mRNA Capture Kit).
4. Incubate 3 min at 37°C, preferably in a PCR machine.

5. Carefully wash captured mRNA three times with 100 µL of wash buffer supplied with mRNA Capture Kit, by pipetting slowly up and down three to five times; remove wash buffer quantitatively, as the mRNA annealed to the biotinylated oligo-dT_{18} is now attached to the streptavidin-coated wall of the 0.2-mL tube.
6. Synthesize cDNA with a kit of choice; do first-strand synthesis in a volume of 20 µL, and second-strand synthesis in a maximum volume of 100 µL, preferably in a PCR machine; the cDNA can be stored at –20°C until further use.
7. After cDNA synthesis, remove reaction buffer and carefully wash cDNA three times with 100 µL wash buffer supplied in the mRNA Capture Kit; immediately proceed to digestion with *Nla*III (**Subheading 3.3.2.**).

3.3. Digestion of cDNA With NlaIII (see Note 7)

In this step, the 3′ biotinylated cDNA is digested with an "anchoring" enzyme, *Nla*III, resulting in the generation of 3′ biotinylated cDNA ends that can be captured with magnetic beads (in the case of conventional SAGE) or are attached to the wall of a streptavidin-coated 0.2-mL tube (in the case of MicroSAGE). Obviously, the cDNA ends are characterized by the presence of the most 3′ *Nla*III restriction site in the cDNA at their 5′ ends, and therefore this *Nla*III recognition sequence will later serve as an anchor in the analysis of the SAGE library.

3.3.1. Digestion of cDNA With NlaIII in Conventional SAGE

1. The following components are mixed and incubated at 37°C for 1 h: 10 µL cDNA (half of the sample); 163 µL LoTE; 2 µL 100X BSA*; 20 µL 10X *Nla*III buffer (Both 100X BSA and 10X NlaIII buffer are supplied with the enzyme); 5 µL of *Nla*III (10 U/µL).
2. Extract cDNA with phenol : chloroform and precipitate as indicated in **Subheading 3.2.1.**
3. Resuspend pellet in 20 µL LoTE; keep the digested DNA (The 3′ cDNA ends still need to be captured from the mixture of fragments; proceed to **Subheading 3.4.**) on ice to prevent denaturation or store at –20°C until further use.

3.3.2. Digestion of cDNA With NlaIII in MicroSAGE

1. Carefully wash cDNA, attached to the streptavidin-coated 0.2-mL tubes, three times with 100 µL 1X *Nla*III buffer containing 1X BSA, by slowly pipetting up and down three to five times.
2. Incubate attached cDNA with 20 U *Nla*III in 1X *Nla*III buffer containing 1X BSA in a volume of 25 µL for 1 h at 37°C.
3. Remove digestion buffer and wash remaining 3′ cDNA ends (The 3′ cDNA ends are now ready for ligation of linkers 1 and 2; proceed to **Subheading 3.5.2.**) on the wall of the tube three times with 100 µL wash buffer (mRNA Capture Kit) as indicated previously; then proceed to next step immediately.

3.4. Capture of 3′ cDNA Ends Using Streptavidin-Coated Magnetic Beads

In conventional SAGE, the cDNA ends have to be isolated to get rid of the 5′ sequences, and to enrich for the 3′ ends that have an overhang at their 5′ ends, generated by *Nla*III. Magnetic streptavidin-coated beads are used in this procedure, which can be immobilized with the Dynal magnet.

1. Add 100 µL of the streptavidin-coated magnetic slurry to each of two 1.5-mL microcentrifuge tubes (one for the isolation of 3′ ends for ligation with Linker 1, the other for Linker 2).

2. Immobilize slurry with magnet.
3. Remove supernatant and add 200 µL 1X B+W buffer.
4. Mix, immobilize the beads with the magnet, and remove buffer.
5. Add 100 µL of 2X B+W buffer, 90 µL dH_2O and 10 µL cDNA to each tube.
6. Incubate 15 min at room temperature while mixing every few minutes to ascertain binding of the cDNA ends to the beads.
7. Wash beads by immobilization with magnet, remove supernatant, and add 200 µL 1X B+W buffer and mix.
8. Wash beads twice more.
9. Wash beads twice with 200 µL LoTE.
10. Immediately proceed to linker ligation.

3.5. Ligation of Linkers 1 and 2

Linkers 1 and 2 contain annealing sites for primers 1 and 2, respectively, as well as a recognition sequence for the type IIS enzyme *Bsm*FI, which cuts dsDNA 10 to 14 base pairs from its recognition sequence, and will generate the actual unique tags corresponding with unique transcripts. This enzyme is therefore being referred to as "tagging" enzyme. The linkers also contain an overhang at their 3′ ends as to accommodate ligation to the *Nla*III overhang that has been generated at the 5′ ends of the cDNA ends.

3.5.1. Ligation of Linkers in Conventional SAGE

Because the cDNA sample has been split before binding of the cDNA ends to the beads, there are now two samples: one for ligation to linker 1, and the other for ligation to linker 2.

1. Add following to each microcentrifuge tube as follows: 25 µL LoTE; 5 µL of linker (200 ng/µL; linker 1 to one tube, linker 2 to the other); and 8 µL 5X ligase buffer (provided with commercial kit; *see* Reagents, **Subheading 2.3.1.**)
2. Mix well and heat samples at 50°C for 2 min, then cool to room temperature for 15 min.
3. Add 2 µL of T4 DNA ligase (5U/µL) to each sample and mix by slowly pipetting up and down.
4. Incubate for 2 h at 16°C while mixing gently intermittently, to accommodate ligation of linkers to the bound cDNA ends.
5. Wash both samples four times with 1 mL of 1X B+W buffer using the magnet to immobilize the beads, as described before, and proceed to digestion of linker-cDNA molecules with the "tagging" enzyme.

3.5.2. Ligation of Linkers in MicroSAGE

1. Wash captured cDNA on the walls of the two 0.2-mL tubes three times with 100 µL of 1X ligase buffer; then add the following to each tube: 1.25 µL linker (200 ng/µL linker 1 to one tube, and 1.25 µL of 200 ng/µL linker 2 to the other); 5 µL 5X ligase buffer; and 16.75 µL LoTE.
2. Mix well by slowly pipetting up and down.
3. Heat samples 2 min at 50°C, then slowly cool (0.1°C/s) to room temperature in a PCR machine.
4. Add 2 µL of T4 DNA ligase and incubate 2 h at 16°C in a PCR machine; do NOT use a heated lid.
5. Remove ligation mix quantitatively.
6. Wash attached linker-cDNA molecules on the wall of each tube three times with wash buffer (mRNA Capture Kit) as indicated before.
7. Proceed to digestion with the "tagging" enzyme, to generate unique tags.

3.6. Release of cDNA Tags by Digestion With BsmFI (see Note 8)

In this step, cDNA tags are generated from the linker-cDNA molecules by digestion with *Bsm*FI, a recognition sequence of which is present in the linker moiety. This enzyme cuts dsDNA 10 to 14 bp downstream of its recognition sequence, well into the cDNA moiety, thereby generating cDNA tags.

3.6.1. Release of cDNA Tags in Conventional SAGE

1. After extensive washing of the linker-cDNAs attached to the magnetic beads, add the following to each of the two microcentrifuge tubes containing beads: 86 µL LoTE; 10 µL 10X *Bsm*FI buffer (provided with enzyme); 2 µL 100X BSA (provided with enzyme); and 2 µL *Bsm*FI (2 U/µL).
2. Mix contents and incubate at 65°C for 2 h; mix intermittently as to keep the beads floating and to accommodate efficient release of cDNA tags.
3. Add 100 µL LoTE and extract samples with equal volume of phenol : chloroform (3 : 4, pH 8.0).
4. Transfer aqueous phase (200 µL) to new tube, and precipitate tags with 133 µL 7.5 *M* ammonium acetate, 1 µL glycogen, mix, and add 1000 µL ice-cold ethanol and mix well again.
5. Spin for 30 min top speed at 4°C.
6. Wash pellets twice with 1 mL of ice-cold 70% ethanol.
7. Dry pellets and resuspend each in 10 µL LoTE and keep samples on ice or store at –20°C.

3.6.2. Release of cDNA Tags in MicroSAGE

1. Wash linker-cDNA molecules, which are attached to the wall of the 0.2-mL tubes, two or three times with 100 µL 1X *Bsm*FI digestion buffer.
2. Add 25 µL of 1X *Bsm*FI digestion buffer containing 2 U of *Bsm*FI, using the buffer stocks provided with the enzyme.
3. Incubate reactions 1 h at 65°C in PCR machine, if possible, with the lid heated to 65°C to prevent excessive aspiration of digestion mixtures.
4. After digestion, transfer both mixes, which now contain the cDNA tags, to other 1.5-mL microcentrifuge tubes.
5. Increase volume of each sample to 200 µL with LoTE.
6. Extract samples once with an equal volume of phenol:chloroform (3:4, pH 8), spin 2 min at 4°C, and transfer aqueous phase to new 1.5-mL tube.
7. Add to 200 µL sample: 133 µL 7.5 *M* ammonium acetate, 1 µL glycogen; mix by pipetting, then add 777 µL ice cold ethanol and mix well.
8. Precipitate tags by spinning down for 30 min at 4°C.
9. Wash samples twice with 1 mL ice cold 70% ethanol.
10. Dry pellets, and resuspend each in 10 µL LoTE; leave on ice for next step or store at –20°C.

3.7. Generating Blunt-Ended cDNA Tags

This step is essentially the same for both conventional and MicroSAGE.

1. To each sample, add the following to an end volume of 50 µL: 10 µL 5X second-strand buffer (supplied with cDNA synthesis kit); 1 µL 100X BSA (supplied with *Nla*III and *Bsm*FI); 1 µL 25 m*M* dNTPs; 26.5 µL dH_2O; and 1.5 µL Klenow polymerase (2 U/µL). Mix well but carefully.
2. Incubate reactions for 30 min at 37°C.
3. Then increase volume to 200 µL with LoTE.
4. Extract once with an equal volume of phenol : chloroform (3 : 4, pH 8.0).

5. Precipitate blunted fragments: add 133 µL of ammonium acetate and 1 µL of glycogen, mix by pipetting, then add 1000 µL of ice-cold ethanol and mix thoroughly.
6. Spin down precipitate for 30 min at 4°C at top speed.
7. Wash pellets twice with 1 mL ice-cold 70% ethanol.
8. Dry pellets and resuspend each in 6 µL (conventional SAGE) or 4 µL LoTE (MicroSAGE).

3.8. Ligation of cDNA Tags to Generate Ditags

In this step, the linker-cDNA tag molecules are combined to generate molecules that consist of two ligated tags, each corresponding to a unique transcript, flanked by linker 1 and linker 2 on each side. The resulting ligation product, or ditag, will serve as a template in a PCR with primers specific for each linker.

For conventional SAGE, proceed as follows:

1. Pool in one tube: 2 µL linker 1-cDNA and 2 µL linker 2-cDNA.
2. Add 1.2 µL 5X ligase buffer and mix.
3. Add 0.8 µL T4 DNA ligase (5U/µL); mix gently.
4. Incubate ligation overnight at 16°C.
5. Increase volume to 20 µL with LoTE, and proceed to next step.

Include a negative, "no ligase" control ligation that will serve as a negative control in the following PCR amplification. In case of MicroSAGE, use all the linker-cDNA collected for the ligation, and it is essential to include a negative control in order to exclude contamination of your sample with a 102-bp fragment from another source; add to the linker-tag mixture (a total volume of 8 µL) 2.2 µL 5X ligase buffer and 0.8 µL T4 DNA ligase (5U/µL) and proceed from there as with conventional SAGE.

3.9. Amplification of Ditags Using Linker-Specific Primers

Amplify ditags using primers 1 and 2 (*see* **Subheading 2.2.**). Optimize PCR by using different dilutions of the template (use dilutions 1:10 to 1:1000 of the ligation product) per PCR and by using different numbers of cycles (use 26, 28, and 30 cycles).

1. Add following reagents to a 0.2-mL PCR tube, to an end volume of 50 µL: 5 µL 10X PCR buffer (*see* **Subheading 2.1.**); 3 µL dimethyl sulfoxide; 3 µL 25 m*M* dNTPs (*see* **Note 9**); 1 µL primer 1 (350 ng/µL); 1 µL primer 2 (350 ng/µL); 35 µL dH_2O; 1 µL Platinum *Taq* polymerase (5 U/µL) (*see* **Note 10**); and 1 µL ligation product (various dilutions).
2. Perform PCR with following conditions:

   to 95°C, 1°C/s
   95°C for 4 min
   to 95°C, 1°C/s
   95°C for 30 s
   to 55°C, 1°C/s
   55°C for 1 min
   to 66°C, 1°C/s
   66°C for 1 min
   } 26, 28, and 30X
   66°C for 5 min
   to 4°C, 1°C/s
   4°C to end
3. Analyze 10 µL of the PCR products on a 12% polyacrylamide gel (19:1 acrylamide:bisacrylamide) in 1X TAE.

Per 35-mL gel (needed for use in the Bio-Rad Protean system), use 10.5 mL polyacrylamide (*see* **Subheading 2.3.1.**), 23.5 mL dH_2O, 700 µL 50X TAE, 350 µL 10% ammonium persulfate, and 35 µL TEMED; adjust volumes accordingly for your preferred gel system; use a standard sample buffer ***(12)***, and take SuperLadder low 20/100-bp marker along on the gel; amplified ditags should run at 102 bp; a background band at 80 bp may be visible.

If yields are generally low, increase the number of cycles to 28 but be careful not to generate too much background (*see* **Note 11**). For isolation of ditags, use the dilution of ligation mix that gives the most robust amplification of the 102-bp fragment, while generating the least amount of background possible. Perform a large-scale PCR, two to three 96-well plates using the optimal dilution with the parameters that generate the most ditags.

3.10. Isolation of Ditags

Although the large-scale PCR can be aliquoted over microcentrifuge tubes, collection of the material in one single 50-mL tube is recommended because it will speed up phenol extraction.

1. Pool large-scale PCR product into one 50-mL tube (~15 mL).
2. Extract with equal volume phenol : chloroform (3 : 4, pH 8) and spin at 4000*g* for 10 min at 4°C.
3. Split the aqueous supernatant (~13.5 mL) in portions of 500 µL in 2-mL microcentrifuge tubes, and add to each 165 µL 7.5 *M* ammonium acetate and 2 µL glycogen, mix, and add 1500 µL ice-cold ethanol.
4. Spin DNA at top speed for 30 min at 4°C.
5. Wash pellets three times with ice cold 70% ethanol; spin for 5 min at 4°C between each wash.
6. Resuspend all pellets in a total volume of 243 µL of LoTE.
7. Dot quantitate the amount of DNA recovered ***(13)***: first, make a concentration range of a known amount of pure DNA of 0 ng/µL, 1 ng/µL, 2.5 ng/µL, 5ng/µL, 7.5 ng/µL, 10 ng/µL, and 20 ng/µL; then, dilute sample DNA 1/5, 1/25, and 1/125; add 4 µL of each standard or 4 µL of each sample dilution to 4 µL of a 1 µg/mL ethidium bromide and mix well; place sheet of parafilm or plastic wrap on an ultraviolet (UV) transilluminator and spot each 8 µL mix on the plastic; make a picture under UV irradiation and estimate the DNA concentration in the sample; at this step, 60 to 100 µg of DNA should be recovered.
8. Add 27 µL of a 10X DNA sample buffer ***(12)*** and load in portions (10 to 20 µL, depending on gel system used) in each slot of a 12% polyacrylamide gel (19 : 1 acrylamide : bisacrylamide) in 1X TAE and run gel, with SuperLadder low 20/100-bp marker alongside.
9. After run, stain gel in SYBR Green I, diluted 1 : 10,000 in 1X TAE, for 15 min at room temperature.
10. Visualize DNA on a UV transilluminator with a SYBR Green filter or on a DarkReader with an amber screen (*see* **Note 12**).
11. Cut out amplified ditags, and pool two to three pieces of gel in one 0.5-mL tube that is pierced at the bottom with a 21-gauge needle.
12. Place pierced 0.5-mL tubes in 2-mL tubes and spin down for 2 min at top speed; this causes the grinding of the polyacrylamide gel.
13. Discard the 0.5-mL tubes and add 250 µL LoTE and 50 µL 7.5 *M* ammonium acetate to fragmented gel; vortex and incubate at 65°C for 15 min.
14. Transfer contents of each 2-mL tube to a Spin-X microcentrifuge tube filters, prewet with 5 µL LoTE.

15. Spin Spin-X tubes at full speed for 5 min.
16. Transfer eluate to new 2 mL microcentrifuge tube and precipitate DNA by adding 2 µL glycogen, 133 µL 7.5 *M* ammonium acetate, mix well, and add 1000 µL ice cold ethanol.
17. Spin 15 min at top speed, 4°C.
18. Wash pellets three times with ice cold 70% ethanol, remove ethanol, and dry to air.
19. Dissolve all pellets in a total volume of 162 µL LoTE.
20. Dot quantitate the amount of DNA; yield should now be 10 to 20 µg.
21. Digest PCR products with 200 U *Nla*III in 200 µL 1X *Nla*III buffer, for 1 h at 37°C.
22. Extract digestion mix with phenol:chloroform (3:4, pH 8) and precipitate by adding 67 µL 7.5 *M* ammonium acetate, 3 µL glycogen, mix, and add 733 µL ice-cold 100% ethanol.
23. Mix and precipitate at –80°C for at least 10 min.
24. Spin at 4°C for 15 min at top speed.
25. Wash pellet once with 70% ethanol and dry to air.
26. Resuspend pellet in 36 µL LoTE with 5 m*M* $MgCl_2$ (*see* **Note 13**).
27. Add 4 µL of a 10X loading buffer, and run in four lanes (10 µL/lane sample) on a 12% polyacrylamide gel (19:1 acrylamide:bisacrylamide) in 1X TAE, alongside SuperLadder low 20/100-bp marker.
28. After run, stain gel with SYBR Green I as indicated earlier, and cut out 24–26 bp fragments.
29. Combine two pieces of gel in one 0.5-mL tube, pierced at the bottom with a 18-gauge needle, and place these in a 2-mL microcentrifuge tube.
30. Spin tube at full speed, 4°C.
31. Add 300 µL of LoTE with 5 m*M* $MgCl_2$ to each 2-mL tube, vortex, and incubate at 37°C for 15 min.
32. Separate solution containing DNA from slurrie with Spin-X microcentrifuge tube filters as indicated above; spin the samples at 4°C.
33. Transfer eluate to three 1.5-mL microcentrifuge tubes (200 µL each) and precipitate ditags with 67 µL 7.5 *M* ammonium acetate and 2 µL glycogen, mix well, and add 733 µL ice-cold 100% ethanol.
34. Incubate at –80°C for at least 10 min.
35. Spin at 4°C for 15 min at top speed.
36. Wash pellets three times with ice cold 70% ethanol.
37. Resuspend pellets in a total volume of 10 µL 1X ligation buffer.
38. Dot quantitate the amount of ditags (use 1 µL of total to make serial dilutions), which should be several hundreds of nanograms.

3.11. Ligation of Ditags to Generate Concatemers

Concatemers are chains of serially ordered, similar entities. In the case of SAGE, ditags are ligated to generate a chain of serially ordered ditags, which are separated by an *Nla*III site. This site allows for the determination of the correct orientation of the tag, and serves as a boundary between two ditags.

1. Add to 9 µL of ditags 1 µL of T4 DNA ligase (5 U/µL).
2. Ligate for 30 min to 2 h at 16°C.
3. Add 1 µL of 10X sample buffer, and load sample in one lane of an 8% polyacrylamide gel (39.5:1 acrylamide:bisacrylamide) in 1X TAE, alongside SuperLadder low 20/100-bp marker.
4. Run gel for several hours, following manufacturer's recommendations.
5. Stain gel with SYBR Green, diluted 1:10,000 in 1X TAE for 15 min at room temperature.
6. Visualize DNA using an UV transilluminator or the DarkReader; concatemers will appear as a smear ranging from several hundred basepairs to several kilobases.

7. Cut out smears in range from 500 to 1000 bp and from 1000 to 2500 bp.
8. Place these gel fragments each in a 0.5-mL tube pierced with a 21-gauge needle and place in a 2-mL microcentrifuge tube.
9. Spin in microcentrifuge for 5 min at top speed.
10. Discard 0.5-mL tubes and add 300 µL LoTE with 5 m*M* $MgCl_2$ to each gel slurry.
11. Vortex tubes vigorously and place at 65°C for 15 min.
12. Transfer the contents of each 2-mL tube to two Spin-X microcentrifuge tube filters and spin for 5 min at full speed.
13. Pool eluate, containing concatemers, from 2 Spin-X tubes in one 1.5-mL microcentrifuge tube.
14. Precipitate concatemers by adding to approx 300 µL of eluate: 1 µL glycogen, 133 µL 7.5 *M* ammonium acetate, then mix and add 1000 µL ice-cold 100% ethanol.
15. Spin for 15 min at 4°C, top speed.
16. Wash the pellets two to three times with ice-cold 70% ethanol.
17. Dry pellets and resuspend both in a total volume of 5 µL LoTE.

3.12. Cloning of Concatemers Into pUC18 (see Note 14)

Clone both the fraction with the small (500 to 1000 bp) as well as the large (1000 to 2500 bp) concatemers into pUC18 and proceed as follows for each of the two.

1. Make the following mix on ice: 5 µL purified concatemers; 1 µL pUC18 (25 ng/µL, digested with *Sph*I); 2 µL 5X ligase buffer; 1 µL dH_2O. Mix components by pipetting, then add 1 µL T4 DNA ligase (5 U/µL). Mix enzyme by slowly pipetting up and down, then incubate ligation for 2 h to overnight at 16°C.
2. Optional: clean ligation by increasing volume to 200 µL with LoTE and performing phenol:chloroform extraction followed by an ethanol precipitation; resuspend pellet in appropriate volume for either electro- or chemical transformation, following the recommendations of supplier of competent bacteria.
3. Perform heat-shock transformation or electroporation according to manufacturer's recommendations with 1–2 µL of ligation reaction.
4. Plate 1/10 and 1/100 of the resulting tranformation mix onto LB agar plates (10 cm diameter) containing 1 m*M* isopropylthiogalactoside, 50 µg/mL X-Gal, and 100 µg/mL ampicillin ***(12)***, according to the manufacturer's recommendations; store remaining mix at 4°C no longer than one night.
5. First assess the insert lengths by means of colony PCR on 96 colonies (see following subsection; more than 80% of PCRs should yield fragments of 600 bp or longer), before plating the remainder of the transformation mix the next day. The total number of colonies should be in the range of 1000 to 1500 to be able to create a medium-sized (~25,000 tags) library. If larger libraries are desired, simply perform more transformations to increase the amount of colonies. The number of colonies derived from the large concatemers may be lower, but also provide much larger inserts that can be sequenced bi-directionally.

3.13. Colony PCR of Transformed Bacteria

In this step, primers flanking the multiple cloning site of pUC18 are used to generate a PCR product that contains approx 185 bp of total flanking vector sequences.

1. Pick colonies from an LB agar plate and smear a little in the bottom/on the wall of a 0.2-mL PCR tube in a 96-well PCR plate; for the following steps, use multichannel pipets if possible, as this will considerably speed up the preparation and clean up of the PCRs.
2. Make a PCR mix, from which 25 µL can be aliquoted into each tube containing a bacterial smear; per 25-µL reaction, use the following ingredients: 2.5 µL 10X Platinum *Taq* PCR

buffer (supplied with enzyme); 1.5 µL 25 m*M* $MgCl_2$; 2.5 µL 2 m*M* dNTPs; 2.5 µL 2.5 µ*M* primer pUC18-F; 2.5 µL 2.5 µ*M* primer pUC18-R; 0.2 µL 5U/µL Platinum *Taq* polymerase; and 13.3 µL dH_2O.

3. Run the colony PCR with following program: 1 cycle at 94°C for 4 min; 35 cycles at 94°C for 1 min; 54°C for 1 min, and 72°C for 1.5 min; 1 cycle at 72°C for 10 min; and then 4°C to the end.
4. Afterward, clean up each colony PCR by adding to each 25-µL reaction 42 µL TE, pH 7.5, 3 µL glycogen, and 20 µL 7.5 *M* ammonium acetate (*see* **Note 15**); mix end over end and spin briefly in centrifuge capable of carrying 96-well plates, then add 60 µL 100% ethanol (*see* **Note 16**).
5. Mix end over end and keep on ice for 30 min.
6. Spin at 2500*g* for 45 min at 4°C.
7. Carefully remove caps to prevent spills from one well to the other.
8. Remove supernatant by inverting tray on paper towel.
9. Remove traces of ethanol by spinning the tray upside down on three layers of similarly sized Whatman 3MM at 160*g* for 3 min at 4°C.
10. Wash pellets twice by adding 150 µL of 70% ethanol; remove supernatants as described above.
11. Dry pellets for 10 min to the air, then resuspend each in 25 µL LoTE or TE.
12. Check 5 µL of the PCR product on 1% agarose gel in 1X TAE ***(12)*** alongside Smartladder marker.
13. Estimate the amount of PCR product; store remaining PCR product at –20°C.
14. Products longer than 600 bp are suitable for sequencing (*see* **Note 17**).

3.14. Sequencing and Analysis of Sequence Data

Sequencing can be performed with any sequencing kit that is recommended for use with the preferred automated sequencer (for example, ABI3100/3700/3730 or LiCor sequencers), strictly following the manufacturer's recommendations. For pUC18 derived vectors, use the M13(-21) Forward primer. One could also sequence clones from both sides, but this will double the cost of sequencing, while providing little extra information. Before proceeding to sequence analysis and tag extraction, it is advised to remove vector sequences. This can usually be done with software that is supplied with the automated sequencer.

For tag sequence extraction, use the SAGE software package, to be downloaded after signing a Materials Transfer Agreement from http://www.sagenet.org/ (*see* **Note 1**). The software not only allows one to extract tags, but also to compare different libraries, thus to identify differentially regulated genes in different conditions or disease. The software also removes contaminating linker sequences and duplicate dimers, which occur because of PCR bias. Other SAGE data are also publicly available from the Gene Expression Omnibus at the NCBI (*see* http://www.ncbi.nlm.nih.gov/GEO/) and SAGE archives at the NCBI (*see* http://www.ncbi.nlm.nih.gov/SAGE/), and at the currently most reliable website for analysis and presentation of SAGE data, SAGEGenie (see http://cgap.nci.nih.gov/SAGE). The available data can easily be incorporated in one's own analysis.

4. Notes

1. It should be noted that the SAGE protocol and variations thereof are freely available for academic researchers at http://www.sagenet.org/; those who wish to use SAGE for

commercial purposes, should contact Genzyme Molecular Oncology at http://www.genzyme.com/; furthermore, a commercial I-SAGE kit is available from Invitrogen; see http://www.invitrogen.com/ for details.

2. Linkers 1A and 2A can be ordered with a biotin moiety at their 5′ ends, which will facilitate the removal of linkers after digestion with a tagging enzyme, and thus linker contamination in the eventual library; the procedure to remove these has been described elsewhere ***(14)***.
3. Although conventional methods can be used, it is advised use commercially available RNA isolation kits, to minimize contamination of the library.
4. The restriction enzyme *Nla*III should always be stored at –70°C to –80°C, and should be aliquoted upon arrival, as the enzyme is not very stable at –20°C and is sensitive to frequent freeze-thaw cycles.
5. Integrity of the separated epidermis should be checked by immunohistochemical staining for cytokeratin 5, which is expressed in the basal layer of the epidermis; when separation shows a damaged (i.e., partly absent) basal layer, part of this layer is still attached to the underlying dermis; if this is the case, do not proceed with MicroSAGE. To check for possible RNA degradation during dispase treatment of the biopsy, one can perform a Northern blot analysis of a whole 4-mm biopsy and a separated, intact epidermis from a 4-mm biopsy from the same body area, and check whether the mRNA expression levels and ratio of cytokeratins 5 and 10 are unaffected; if levels and ratios are unaffected, one can proceed with the protocol.
6. Although the effect of glycogen on ligations, digestions and amplifications is considered minimal, one should realize that subsequent precipitations of the same sample without gel purification will lead to an accumulation of glycogen in the sample; for ligations and digestions to be successful, it is advised to keep the glycogen concentration as low as possible, and not higher than 7 µg/µL in digestions and ligations.
7. The anchoring enzyme *Nla*III can also be replaced by other enzymes that generate overhangs, provided that the replacement enzyme only recognizes 4 basepairs and thus theoretically only cuts cDNA once every 256 bp; be aware that linkers have to be modified to accommodate digestion by other anchoring enzymes.
8. The tagging enzyme *Bsm*FI can also be replaced with *Fok*I or *Mme*I (the latter generates tags of approx 17 bp, and is used in the LongSAGE protocol; *see* http://www.sagenet.org/ for details), but be aware that linkers have to be modified to accommodate digestion by other tagging enzymes.
9. As the concentration of dNTPs and primers is relatively high and may in fact inhibit the amplification of linker-ditags, it may be necessary to optimize their concentration as to increase yield and decrease background.
10. Platinum *Taq* polymerase is an enzyme formulation that allows for a hot start of the PCR, meaning that one can safely prepare the PCR on the lab table, and that the PCR will only start after the sample has been heated to 95°C for more than 3 min.
11. It is best to immediately check the yields of ditags after 26, 28, and 30 cycles before proceeding to a large-scale amplification.
12. Because UV irradiation may cause DNA damage, it is advised to use SYBR Green I in combination with a DarkReader illuminator and the appropriate shield, although one can also use ethidium bromide for staining of the gels.
13. $MgCl_2$ is added to increase the amount of salt in the sample to prevent denaturation of the ditags; also, keep ditags on ice as much as possible.
14. Any suitable sequencing vector can be used, but be aware this may also require different primers for colony PCR and sequencing; the use of pZero-1 facilitates the screening of the clones and gives good cloning efficiencies, as cloned concatemers disrupt a suicide gene, leading to a very low background.

15. When having to perform a large number of precipitations, it is possible to make a mix of TE, glycogen and ammonium acetate, and add 65 µL of this mix to each colony PCR; then mix your samples, spin briefly and then add ethanol as indicated.
16. The colony PCR products are precipitated in only 40% ethanol; this will almost completely prevent the co-precipitation of nucleotides and primers, which may negatively affect the subsequent sequence reaction.
17. It should be noted that, when using pUC18 as the vector and primers pUC18-F and pUC18-R for colony PCR, PCR products will contain vector sequences at both ends to a total of 185 bp; this means that a PCR product of 600 bp contains a concatemer of slightly over 400 bp long, which in turn corresponds to roughly 14 ditags, which in turn corresponds to approx 28 tags or transcripts.

References

1 Velculescu, V. E., Zhang, L., Vogelstein, B., and Kinzler, K. W. (1995) Serial analysis of gene expression. *Science* **270,** 484–487.
2 Caron, H., van Schaik, B., van der Mee, M., et al. (2001) The human transcriptome map: clustering of highly expressed genes in chromosomal domains. *Science* **291,** 1289–1292.
3 Hermeking, H., Lengauer, C., Polyak, K., et al. (1997) 14-3-3 sigma is a p53-regulated inhibitor of G2/M progression. *Mol. Cell. 1,* 3–11.
4 Polyak, K., Xia, Y., Zweier, J. L., Kinzler, K. W., and Vogelstein, B. (1997) A model for p53-induced apoptosis. *Nature* **389,** 300–305.
5 Velculescu, V. E., Zhang, L., Zhou, W., et al. (1997) Characterization of the yeast transcriptome. *Cell* **88,** 243–251.
6 Zhang, L., Zhou, W., Velculescu, V. E., et al. (1997) Gene expression profiles in normal and cancer cells. *Science* **276,** 1268–1272.
7 Zhou, W., Sokoll, L. J., Bruzek, D. J., et al. (1998) Identifying markers for pancreatic cancer by gene expression analysis. *Cancer Epidemiol. Biomarkers Prev.* **7,** 109–112.
8 Datson, N. A., van der Perk-de Jong, J., van den Berg, M. P., de Kloet, E. R., and Vreugdenhil, E. (1999) MicroSAGE: a modified procedure for serial analysis of gene expression in limited amounts of tissue. *Nucleic Acids Res.* **27,** 1300–1307.
9 Jansen, B. J. H., van Ruissen, F., de Jongh, G. J., Zeeuwen, P. L. J. M., and Schalkwijk, J. (2001) Serial analysis of gene expression in differentiated cultures of human epidermal keratinocytes. *J. Invest. Dermatol.* **116,** 12–22.
10 van Ruissen, F., Jansen, B. J. H., de Jongh, G. J., Zeeuwen, P. L. J. M., and Schalkwijk, J. (2002) A partial transcriptome of human epidermis. *Genomics* **79,** 671–678.
11 van Ruissen, F., Jansen, B. J. H., de Jongh, G. J., van Vlijmen-Willems, I. M., and Schalkwijk, J. (2002) Differential gene expression in premalignant human epidermis revealed by cluster analysis of serial analysis of gene expression (SAGE) libraries. *FASEB J.* **16,** 246–248.
12. Sambrook, J. and Russell, D. W. (2001) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
13. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., and Struhl, K., eds. (1988) *Current Protocols in Molecular Biology.* John Wiley & Sons, Inc., Hoboken, NJ.
14. Powell, J. (1998) Enhanced concatemer cloning-a modification to the SAGE (serial analysis of gene expression) technique. *Nucleic Acids Res.* **26,** 3445–3446.

38

Methods for Gene Expression Profiling in Dermatology Research Using DermArray® Nylon Filter DNA Microarrays

Richard L. Davis, Jr., Rusla M. DuBreuil, Shanker P. Reddy, and Thomas P. Dooley

Summary

Here we present methods of gene expression profiling using nylon filter deoxyribonucleic acid (DNA) microarrays and radiolabeled and nonradiolabeled hybridization probes. DermArray® nylon filter DNA microarrays were designed specifically for use in dermatology research. A patent-pending method was used to select approx 4400 highly informative, sequence-verified human cDNA clones for this DNA microarray. Using DermArray® filters, biomarkers have been discovered for normal and pathologic cells from skin, and for responses to dermatologic drugs. As an example, gene expression profiling was performed with hydroquinone-treated SKMel-28 cells, a melanoma cell line. Also included are the methods for bioinformatic analysis using Pathways™ software.

Key Words:

Biomarkers; hydroquinone; melanocyte; pigmentation; hybridization; DNA microarray; bioinformatics.

1. Introduction

IntegriDerm Inc. (www.integriderm.com) has developed DermArray® nylon filter DNA microarrays specifically for use in the field of dermatological research. This product contains approx 4400 sequence-verified human complimentary deoxyribonucleic acid (cDNA) clones (polymerase chain reaction amplimers) spotted on a 5- × 7-cm nylon membrane by physical contact using a Cartesian robotic instrument. These genes were chosen from a pool of 26,000 human cDNA clones using a patent-pending method to select genes that are differentially expressed in human skin cells. Included on DermArray filters are 383 triplicated genes of relevant dermatological interest, thus allowing the investigator to assess statistical variation in hybridization intensities on either one filter (in cases where one filter is subjected to multiple stripping/reprobing cycles) or on multiple filters (in cases where multiple filters, each hybridized to a unique probe, are being simultaneously compared).

We have recently reported on the use of DermArray technology and new bioinformatic algorithms related to likelihood ratios to identify biomarkers for cultured human melanocytes, keratinocytes, and fibroblasts ***(1)***. We identified 136 genes that are robustly

From: *Methods in Molecular Biology, vol. 289: Epidermal Cells: Methods and Protocols*
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

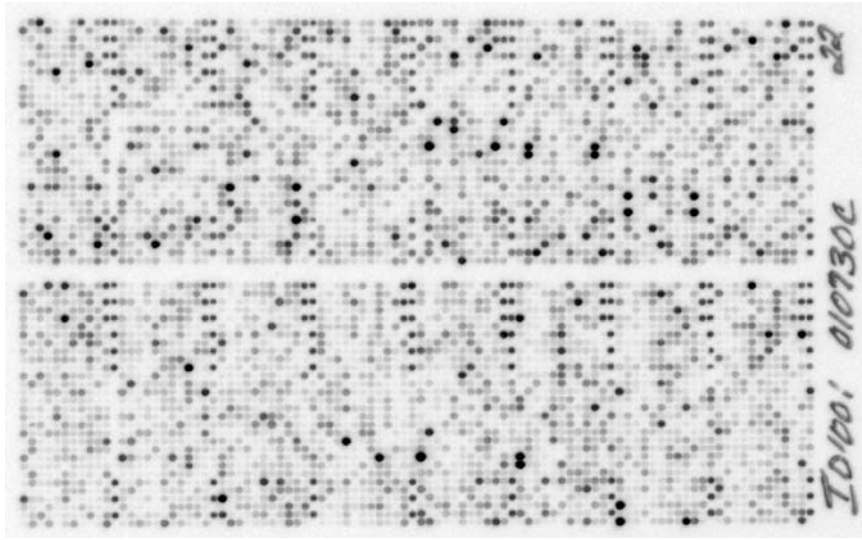


Fig. 1. An example of a DermArray microarray hybridization experiment. Three micrograms of human total RNA were converted to cDNA by reverse transcriptase in the presence of ^{33}P-dCTP, denatured, and added to a hybridization roller bottle containing a DermArray® microarray membrane. Hybridization proceeded for 16 h at 42°C. The membrane was washed as described in this paper and was exposed to a phosphor imager screen for 16 h at room temperature. The image shown was generated in a Packard Cyclone® phosphor imager, and was saved in TIFF file format.

upregulated in one of the three cell types compared to the other two cell types, which are referred to as "signature biomarker genes," and 22 genes that are downregulated in one of the three cell types compared with the other two cell types, which are referred to as "anti-signature biomarker genes." Approximately 41% of the biomarkers we identified in a given cell type have been previously described in the literature as being present in that cell type or human skin, thereby supporting the accuracy of the array experiments and analyses. We identified many other genes that were not formerly noted as being related to cell biology of the skin, including 17 signature genes identified as ESTs of unknown functions and 22 antisignature biomarkers ***(1)***. We also have used DermArray technology for gene expression profiling of melanoma, squamous cell carcinoma, and drug treatments of skin-derived cells (Dooley and co-workers, unpublished results). **Figure 1** shows a typical DermArray hybridization experiment.

We also have developed PharmArray® DNA microarrays intended for use in pharmacology and toxicology research. This array allows the researcher to limit the choice of genes or molecular targets by eliminating genes that are coincidentally upregulated in ADME (absorption, distribution, metabolism, excretion)-related organs representing colon, kidney, and liver (Dooley and coworkers, unpublished results). PharmArray contains approx 4400 human cDNA clones, which were chosen from a pool of 35,000 cDNA clones, and also contains 384 individual genes in triplicate, which are of pharmacological relevance.

In this chapter, we present a series of protocols designed to demonstrate the methods and utility of our nylon filter microarray products. Complete protocols covering total

RNA isolation, radioactive, and nonradioactive probe preparation, DermArray hybridizations, autoradiograph preparation, and data retrieval and analysis in Pathways™ software are included.

Then, as a specific example of the application of our microarray technology, consider hydroquinone, an agent that affects skin pigmentation. Pigmentation is a complex multi-step process that occurs as a result of the enzymatic conversion of tyrosine into melanin within the melanosomes of melanocytes, followed by transfer of the melanosomes into the keratinocytes of the epidermis ***(2)***. Abnormalities of human skin pigmentation, both hyperpigmentation and hypopigmentation (e.g., vitiligo), can occur as a result of both genetic and environmental factors.

There are a number of over-the-counter topical skin care products that can be used to treat hyperpigmentation, that is, age spots or melasma. Many of these products contain hydroquinone (HQ) in concentrations ranging from 1.5 to 4%. Although HQ has been shown to be clinically efficacious for the treatment of hyperpigmentation disorders, it is also highly reactive, mutagenic, and cytotoxic to melanocytes ***(3,4)***. It was originally thought that HQ exerts its effects by directly inhibiting the activity of tyrosinase, a key enzyme involved in the biochemical pathway leading to melanin production. However, more recent evidence suggests that HQ may act either by exerting a cytotoxic effect on melanocytes and/or acting as a nonenzymatic chemical bleaching agent. Regardless of the mechanism of action of compounds such as HQ, it is now possible to determine the global gene expression effects of topical skin lighteners on in vitro cultured cells utilizing modern post-genomic tools such as DNA microarrays ***(5–7)***.

The following protocols demonstrate how nylon filter DNA microarrrays, such as DermArray, can be used to examine the dose-response effects of HQ on gene expression in SKMel-28 cells, and are intended as a specific example of the application of microarray technologies to answer complex biological questions. In this case, expression profiles of ca. 4400 genes in response to various doses of HQ were analyzed and categorized as over- or underexpressed compared with the untreated cultured cells. The upregulated genes (typically 2.5X or greater) in response to drug treatment are referred to as sentinel biomarkers, whereas downregulated genes are referred to as antisentinel biomarkers. **Figure 2** displays a dose–response plot of HQ influence on gene expression in human cultured SKMel 28 cells. Both sentinel and antisentinel biomarker responses were noted.

We have also included two nonradioactive labeling and detection methods, such as digoxigenin-dUTP labeling, followed by chemiluminescent detection and NBT/BCIP color precipitation method. In **Fig. 3**, nylon filters (similar to DermArray) were subjected to non-radioactive labeling and detection methods.

2. Materials

2.1. Sample Handling

DermArray and PharmArray DNA microarray experiments involve the use of ^{33}P-labeled probes and thus require the safe handling of radioactive materials. Radioisotope use should be logged according to state and federal regulations and experiments using radioactive materials should be conducted in separate laboratory areas that are monitored for spills. Gloves and labcoats should be worn whenever radioisotopes are in use. Pipettors used in handing radioisotopes should be kept separate from other lab activities.

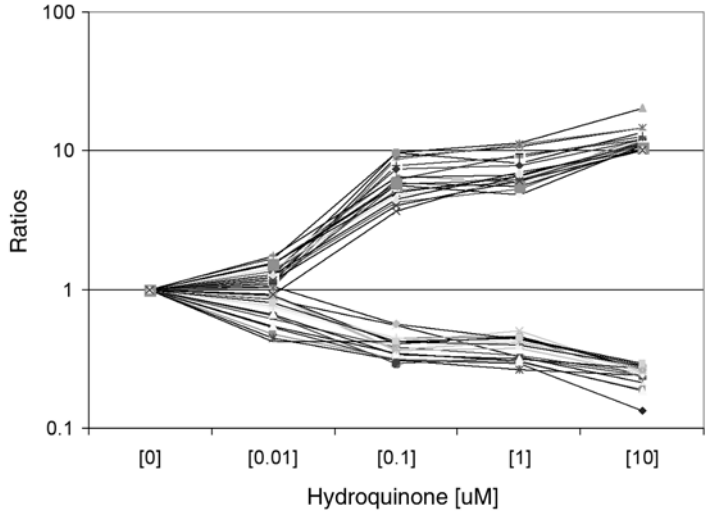


Fig. 2. Dose–response plot for hydroquinone on cultured human SKMel 28 cells. Examples of individual genes that are substantially over (sentinel) or underexpressed (antisentinel) compared with untreated control cells are displayed.

2.2. Total RNA Isolation

RNeasy Midi Kit (Qiagen, Valencia, CA; cat. no. 75144).

2.3. cDNA Probe Preparation (Radioactive Labeling)

1. Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA; cat. no. 18064-014).
2. 5X First strand buffer (Invitrogen; cat. no. 18064-014).
3. 0.1 *M* DTT (Invitrogen; cat. no. 18064-014).
4. ^{33}P-dCTP (10 mCi/ml, Perkin/Elmer Life Sciences, Boston, MA; cat. no. NEG613H).
5. Nuclease-free water (Promega, Madison, WI; cat. no. P119C).
6. 20 m*M* dNTP mixture (Amersham Biosciences, Piscataway, NJ; cat. no. 27-2035-02) prepare a fresh 20 m*M* mixture of dATP, dGTP, and dTTP from 100 m*M* stocks and nuclease-free water.
7. Bio-Spin 6 chromatography columns (Bio-Rad, Hercules, CA; cat. no. 732-6002).

2.4. cDNA Probe Preparation (Nonradioactive Labeling: See *Subheading 3.3.)*

1. 1 m*M* DIG-11-dUTP (Roche, Indianapolis, IN; cat. no. 1573152).
2. Nuclease-free water (Promega, Madison, WI; cat. no. P119C).
3. DIG DNA labeling and detection kit (Roche; cat. no. 1093657).
4. CDP-Star Substrate System (Roche; cat. no. 2041677).
5. NBT/BCIP (Roche; cat. no. 1681451).
6. 20 m*M* dNTP mixture of dATP, dGTP, and dCTP (Amersham Biosciences, Piscataway, NJ; cat. no. 27-2035-02).
7. 20 m*M* dTTP (Amersham Biosciences, Piscataway, NJ; cat. no. 27-2035-02).
8. Sephadex G-50 spin columns (Roche; cat. no. 1273973).
9. mRNA Capture Kit (Roche; cat. no. 1787896).

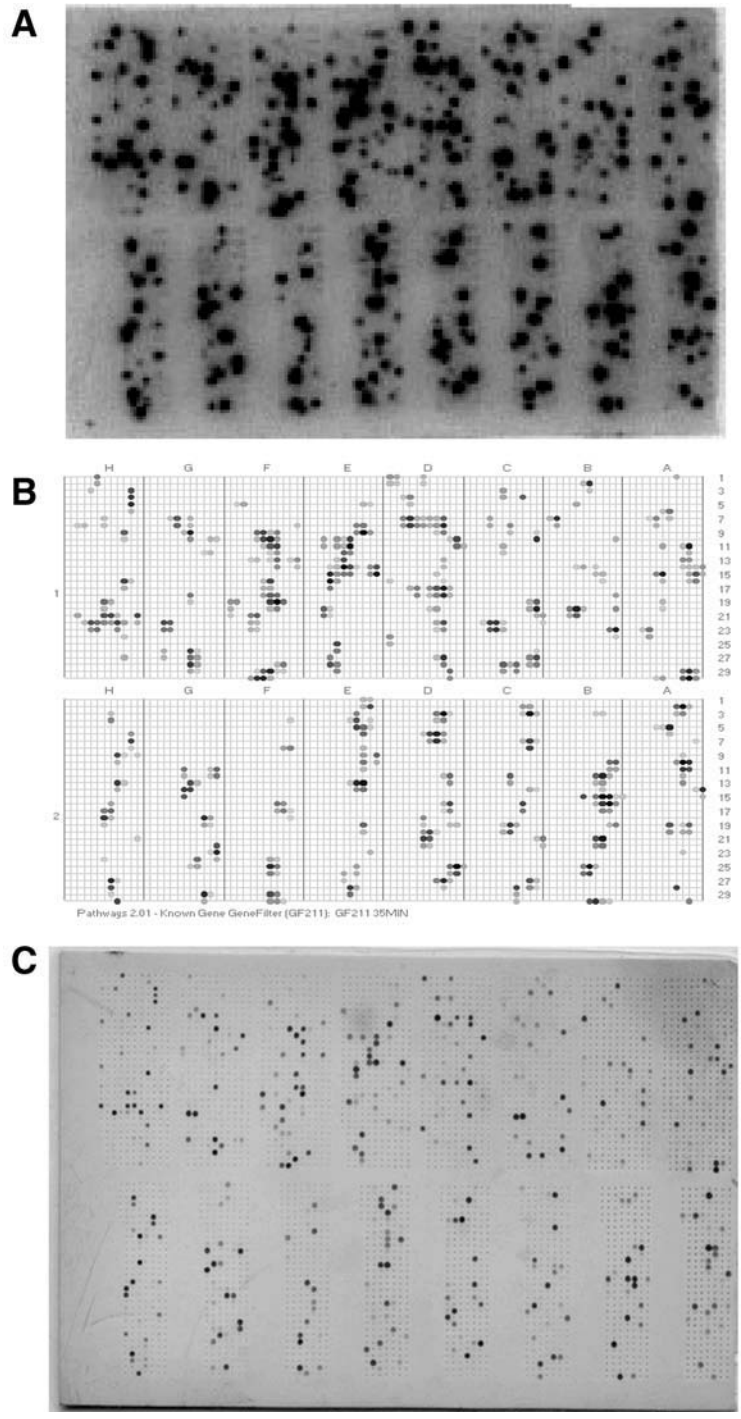


Fig. 3. A nylon filter (GF 211; Invitrogen) depicting the gene expression profile of a sample labeled with digoxigenin. **(A),** The CDP star chemiluminescent output was detected on a Lumi-Imager™ and an overexposed image was produced. **(B),** This image was then processed using Pathways software to produce a computer generated synthetic image. **(C),** Finally, a nylon filter was processed with NBT/BCIP color reaction to generate the image that could be analyzed using Pathways software.

2.5. Microarray Hybridizations and Washes

1. MicroHyb hybridization solution (Invitrogen; cat. no. HYB250.GF).
2. Human Cot-1 DNA (Invitrogen; cat. no. 15279-011).
3. Poly A (Invitrogen; cat. no. POLYA.GF).
4. Oligo dT (Invitrogen; cat. no. POLYT.GF).
5. 20X standard saline citrate (SSC) solution (Invitrogen; cat. no. 15557-044).
6. 10% sodium dodecyl sulfate (SDS) solution (Invitrogen; cat. no. 24730-020).
7. Model 5420 Hybridization Oven (VWR Scientific; cat. no. 25000-000).
8. 35 × 150-mm hybridization roller bottles (VWR Scientific; cat. no. 16300-304).
9. Opti-Flour® liquid scintillant (Packard Bioscience; cat. no. 6013199).

2.6. Image Capture and Microarray Data Analysis

1. Cyclone® storage phosphor system (Perkin/Elmer Life Sciences, Meriden, CT) with OptiQuant® software (can be used for both radioactive and nonradioactive labeling methods).
2. Cyclone® multipurpose storage phosphor screen (Perkin/Elmer Life Sciences; cat. no. 70014809902).
3. Lumi-Imagers® (Roche; cat. no. 2012847; an alternative device capable of capturing chemiluminescent probe images; *see* **Subheading 3.3.** below).
4. Pathways™ 4-Universal Software (Invitrogen; cat. no. PW4.UC).

3. Methods

3.1. Radioactive Probe Labeling and Microarray Hybridization

This section describes the preparation of a radiolabeled cDNA probe using a total RNA template prepared from cultured cells (SKMel-28 cells as the specific example), the procedure used to hybridize the labeled probe to a DermArray DNA microarray nylon filter, the wash steps following hybridization, exposure of the washed filter to a storage phosphor screen, and finally image capture of the exposed filter.

3.1.1. Prehybridization of DermArray® Microarray

If using a DermArray microarray for the first time, place the membrane in a plastic box and pour boiling 0.5% SDS over it. Gently shake the plastic box for 5 min on a rotary shaker. This step removes undesirable residuals from the microarray production process that may have been carried over with the membrane (*see* **Note 1**).

1. Place the DermArray membrane in a 35- × 150-mm hybridization roller bottle with the DNA spot side of the membrane facing toward the interior of the bottle such that the DNA spots do not touch the glass (*see* **Note 2**).
2. Add 5 mL of MicroHyb hybridization solution to the bottle containing the microarray and replace the roller bottle cap tightly. Roll the bottle horizontally using both hands so that the microarray membrane adheres to the side of the bottle without any air bubbles being trapped between the membrane and the glass.
3. Add the following nucleic acid reagents to the roller bottle: 5 µL of Cot-1 DNA (5 µg total) (*see* **Note 3**) and 5 µL of Poly A (5 µg total).
4. Replace roller bottle cap tightly and rotate tube several times with the bottle in a vertical position to ensure that the components are thoroughly mixed. Place the bottle horizontally in a hybridization oven that has been set to 42°C and its rotation speed set to one third of the maximum. Allow prehybridization to continue for at least 2 h.

3.1.2. Isolation of Total RNA and Preparation of Radiolabeled Probe

Total RNA from cultured cells can be prepared using a commercially available RNA isolation kit (*see* **Note 4**). As an example, RNA can be harvested from SKMel-28 cells treated with 10-fold dilutions of hydroquinone ranging in concentration from 0.01 μ*M* to 100 μ*M*, and RNA harvested from untreated SKMel-28 cells can be compared with RNAs derived from the HQ-treated cell lines in subsequent microarray analyses.

1. Disrupt the cultured cells directly by addition of 2 mL of Qiagen buffer RLT (add 10 μL β-mercaptoethanol per 1 mL of buffer RLT prior to use) to the cell-culture vessel. Rock the vessel to ensure complete lysis of the cells. Transfer the 2 mL of lysed cells to a sterile 50-mL conical centrifuge tube.
2. Add 1 vol of 70% ethanol to the lysate and mix by inversion.
3. Apply the sample, including any precipitate that may have been formed, to an RNeasy midi column placed in a 15-mL centrifuge tube (supplied with the Qiagen kit). Close the tube gently, and centrifuge for 5 min at 3000–5000*g*. Discard the flow-through.
4. Add 4 mL of Qiagen Buffer RW1 to the RNeasy column. Close the centrifuge tube gently, and centrifuge for 5 min at 3000–5000*g* to wash the column. Discard the flow-through.
5. Add 2.5 mL of Qiagen Buffer RPE to the RNeasy column. Close the centrifuge tube gently, and centrifuge for 2 min at 3000–5000*g* to wash the column. Discard the flow-through.
6. Add another 2.5 mL of Qiagen Buffer RPE to the RNeasy column. Close the centrifuge tube gently, and centrifuge for 5 min at 3000–5000*g* to dry the RNeasy silica-gel membrane.
7. To elute the total RNA, transfer the RNeasy column to a new 15 mL collection tube (supplied with the Qiagen kit). Pipet 250 μL RNase-free water directly onto the center of the RNeasy silica-gel column membrane. Close the tube gently. Let it stand for 1 min, and then centrifuge for 3 min at 3000–5000*g*.
8. Repeat the above elution step with a second 250 μL RNase-free water.
9. Combine the two 250 μL eluates into a single sterile RNase-free microfuge tube and determine the total RNA concentration of the sample by measuring the optical density of a 1 : 20 dilution of the RNA at 260 nm in a UV spectrophotometer.
10. Concentrate the total RNA sample in a SpeedVac concentrator until the RNA concentration is between 0.5–1 μg/μL.
11. Assemble the following in a sterile RNase-free 1.5-mL microfuge tube: 3 μg total RNA in 8 μL or less nuclease-free water; x μL nuclease-free water to bring total RNA vol to 8 μL; and 2 μL oligo dT. Close microfuge caps tightly.
12. At the total vol 10 μL, place tubes at 70°C for 10 min, then quick chill on ice for 2 min; quick spin tube contents to collect samples at the bottom of each tube.
13. Add the following components to each tube: 6 μL 5X first-strand buffer; 1 μL 0.1 *M* dithiothreitol; 1.5 μL 20 m*M* dNTP mixture; 1.5 μL Superscript II reverse transcriptase; and 10 μL ^{33}P-dCTP for a total vol 30 μL in each sample tube. Cap microfuge tubes tightly, quick spin samples in a microfuge, and incubate samples at 37°C for 90 min.
14. For each sample take one Bio-Rad Bio-Spin 6 purification column and invert it to resuspend the column gel matrix uniformly in its suspension buffer.
15. Place a 2-mL collection tube (supplied with Bio-Spin columns) at the bottom of a sterile 15-mL centrifuge tube, insert the Bio-Spin column into the 15-mL tube so that the tip of the column fits within the 2-mL collection tube, replace the cap to the 15 mL centrifuge tube and tighten, and spin the tube at 1800*g* for 5 min at room temperature in a swinging bucket centrifuge.
16. Cut the top off of a sterile nuclease-free 1.5-mL microfuge tube and place the tube into another sterile 15-mL centrifuge tube. Place the packed Bio-Spin column into this tube so that the tip of the Bio-Spin column fits within the 1.5-mL collection tube.

17. Add 70 µL nuclease-free water to each probe labeling reaction from above, mix, quick spin the sample, and transfer the entire contents of the tube to the center of the packed Bio-Spin column, taking care not to touch the packed column with the end of the transfer tip.
18. Spin the tube at 1800*g* for 5 min at room temperature. Remove the column from the 15-mL centrifuge tube and place the column into the radioactive solid waste container.
19. Transfer the labeled cDNA probe that was collected at the bottom of the 15-mL centrifuge tube to another sterile nuclease-free microfuge tube.
20. Count 1 µL of each labeled probe mixed with 3 mL scintillant cocktail in a liquid scintillation counter and record the results (*see* **Note 5**).
21. Transfer the entire probe solution to a screw-capped 2 mL microfuge tube and screw the cap on tightly. Transfer the tube to a boiling water bath and denature the probe for 3 min, then quick chill the probe on ice. Quick spin the tube containing the probe to collect contents at the bottom of the tube.

3.1.3. Hybridization of Probe to a DermArray Microarray

1. Stop the rotation on the hybridization oven and remove the roller bottle containing the prehybridized microarray. Remove the bottle cap.
2. With the roller bottle held vertically, carefully add the entire probe solution to the hybridization solution in the roller bottle using a P-200 pipettor. Be sure not to allow any probe solution to splash on the microarray on the side of the roller bottle. Replace the roller bottle cap tightly and, with the bottle still held vertically, shake the bottle counterclockwise in order to ensure that the hybridization solution is thoroughly mixed.
3. Place the roller bottle back into the hybridization oven, keep the temperature set to 42°C, and set the rotation speed to one third of the maximum.
4. Approximately 10 min after placing the roller bottle in the hybridization oven, stop the rotation, remove the bottle from the oven, and retighten the cap on the roller bottle (*see* **Note 6**).
5. Place the roller bottle back into the oven and continue rotation of the bottle overnight at 42°C with the rotation speed remaining at one third of the maximum.

3.1.4. Microarray Washes

1. Remove the hybridization roller bottle(s) from the hybridization oven and place the bottle upright in a rack. Remove the bottle cap carefully and decant the hybridization solution into a liquid radioactive waste container. Set the temperature on the hybridization oven to the wash temperature of 50°C.
2. Add 30 mL of a 2X SSC, 1% SDS solution to the roller bottle, replace the bottle cap tightly, and rotate the bottle horizontally by hand to perform a prewash of the hybridized filter. Remove the bottle cap and decant the prewash solution into the radioactive liquid waste container.
3. Add 30 mL of 2X SSC, 1% SDS to the bottle and place the bottle back into the hybridization oven. Set rotation speed to maximum and wash for 20 min at 50°C.
4. Take the roller bottle out of the oven and carefully decant the wash solution into the liquid waste container. Add a second 30-mL aliquot of 2X SSC, 1% SDS wash solution to the roller bottle and continue washing at 50°C for 20 min as previously.
5. Remove the roller bottle from the oven and decant the second wash solution into the liquid waste container. Add 30-mL of 0.5X SSC, 1% SDS wash solution to the bottle and continue washing the filter at 50°C for 20 min.
6. Remove the roller bottle from the oven and decant the third wash solution into the radioactive liquid waste container.

3.1.5. Exposure of Hybridized Microarray Filter

1. After the washing steps are completed cut out a 8″ × 10″ piece of Whatman 3 MM paper and wet it with distilled water and place it on a slightly larger piece of 3 MM paper.
2. Place the array(s) (DNA-side up) on the wet 3 MM paper with the notched corner of the membrane oriented toward the top and the right.
3. Place another piece of plastic wrap on top of the membrane and, using a cookie roller, gently smooth out any creases in the plastic wrap and then use Kimwipes® to squeeze out any bubbles that may remain trapped between the plastic wrap and the membrane.
4. Place the wrapped membrane(s) in an 8″ × 10″ X-ray exposure cassette. With the room lights turned off place a medium-sized multipurpose phosphor imaging screen (phosphor side of screen facing down toward the membrane) such that the membrane is either perfectly parallel or perfectly perpendicular to the phosphor imaging screen (in order to ensure that the resulting autoradiographic images are not skewed prior to importing into the microarrray analysis software).
5. Place another 8″ × 10″ imaging screen (or alternatively an 8″ × 10″ piece of cardboard) on top of the multipurpose screen to ensure that the multipurpose screen presses tightly against the wrapped microarray membranes. Latch the cover of the X-ray cassette on top of the wrapped membrane sandwich in order to form a light tight seal.
6. Expose the wrapped microarray membranes to the phosphor imaging screen for either 6 h or overnight, depending on the specific activity of the probes used in the hybridization experiment.

3.1.6. Stripping Probe Off of Hybridized Microarrray Filters (see ***Note 7***)

1. After the microarray(s) have been exposed to the phosphor imaging screen, place each microarray into a plastic tray and pour boiling 0.5% SDS on top of the filters in order to strip the probe off of the membrane.
2. Rotate the plastic tray on a rotary shaker for 1 h at room temparature.

3.1.7. Image Capture by Cyclone® Phosphorimager

1. After the exposure of the hybridized membrane(s) is complete, open the X-ray cassette cover with the room lights turned off. Place the exposed multipurpose screen on the Packard Cyclone phosphor imager carousel unit with the exposed side of the screen facing outside (lock both ends of the multipurpose screen carefully onto the carousel). Place the carousel into the Cyclone imaging chamber and make sure that the chamber cover securely covers the chamber to prevent ambient light from leaking in.
2. Click on the OptiQuant™ application icon, and click “Instrument.” Set “Protocol Settings Resolution” to 600 dpi and “Carousel Type” to medium.
3. Press “Start” and the Cyclone® will start to acquire the image (this process takes approx 10 min for the medium-sized multipurpose screens).
4. After the image capture process has been completed the autoradiograph image will be displayed by the Optiquant software on the screen. Click on the “Transform” file menu, then click “Crop Image” and hold down the left mouse button to enclose the captured image within the cropping toolbox. Release the left mouse button and click “Yes” to crop the selected area.
5. Click on the “magnify” icon, position the resulting magnify tool over the cropped image, and click on the left mouse button to magnify the cropped image 1X.
6. If necessary click on the “Transform” file menu, click on “Rotate Image” and then select either 90° or 180° in order to rotate the image such that the DermArray lot number visible on the microarray image is positioned vertically and on the right.

7. Click on the "File" menu, then "Save As" and choose an appropriate directory on your computer's hard drive in which to store the captured image. These images are automatically saved in the TIFF file format.

3.2. Microarray Analysis in Pathways™ and in Microsoft™ Excel Spreadsheets

This section describes the analysis of microarray data files in Pathways™ software Version 4.0, which is a comprehensive software tool that allows one to do statistical analysis of data sets, view microarray data in a variety of ways including scatter plots as well as tables, and to isolate subsets of larger data sets by means of filters based on user-defined criteria. In addition the software includes multiple clustering algorithms which allow for expanded data mining capabilities. These protocols cover the basics of importing raw microarray TIFF images into Pathways and performing data analysis, as well as the process used to export CSV-formatted data sets from Pathways into Microsoft Excel spreadsheets for further data manipulations.

3.2.1. Importing Microarray TIFF Images Into Pathways

1. Double click on the Pathways version 4.0 desktop icon to launch the application.
2. In the Pathways Quick-Start dialog box click on "Import a new microarray image".
3. In the Image Import dialog box click on the "directory up" icon until the directory containing your TIFF microarray image has been located.
4. In the Image Import file window click on the TIFF file that you want to import into Pathways. Repeat for all other files to be imported.
5. Under Options make certain that "TIFF image" is selected for image format, "GeneFilter" is selected for microarray brand, "ID1001" (product number of DermArray) or "ID1002" (product number for PharmArray if using that product), "Geometrical" is selected for sampling type, and that "Trim Image" box is checked.
6. Once all TIFF files to be imported have been added to the file window click "OK" to begin the file import process.
7. Move the contrast slider to the right to darken and enhance the displayed image.
8. Click on the template button and then click anywhere on the image and drag in order for the cropping rectangle to appear.
9. Position the cropping rectangle so that the top right red square is near the top right total genomic DNA (tgDNA) control spot (see the documentation that ships with DermArray for a diagram of the microarray that displays the organization of gene spot clusters into fields and also shows the locations of the tgDNA and housekeeping genes on the array) and that the bottom right red square is positioned near the bottom right tgDNA control spot.
10. Uncheck the Adjust Global box and make sure that the Use Magnifier box is checked.
11. Press the up key to increase the magnification of the upper right tgDNA control spot and, with the left mouse button continually pressed, drag the red square until it is perfectly centered on the top right tgDNA control spot. Repeat this process for the remaining 7 grids in field 1.
12. In field 2 grid A position the red square so that the square is perfectly centered on top of the bottom right tgDNA control spot. Repeat this process for the remaining 7 grids in field 2.
13. Press "done" to complete the file import process.
14. In order to begin a Pathways comparison of two different filters click on the "Compare two microarrays using intensity ratios and differences" button on the Pathways Quick-Start dialog box.
15. Enter a project name and researcher name in the New Project box and click "Next."

16. In the Library box click on the file that represents your control filter and click "Add" to add the selected file to the "First Array" box.
17. In the Library box click on the file that represents your experimental filter and click "Add" to add the selected file to the "Second Array" box.
18. Click "Next" and select "Data Point Normalization," then click "Next" followed by "Finish." These steps result in the execution of the microarray comparison routine.
19. In the "Comparison:Microarray pair (by address)" window on the top right of the screen click on X:log and Y:log in order to see a log plot distribution of the comparison data.
20. In the "Comparison:Microarray pair (by address)" window click on the "Report" window.
21. In the Report Wizard dialog box under "Data" check the following boxes: clone number, Intensity I, Intensity II, and Ratio. Under Meta-Data check the following boxes: acc (accession number), cDNA_id, cluster_id, and title.
22. Click save and choose an appropriate directory in which to store the csv (comma separated file)-formatted data output file.

3.2.2. Importing Microarray CSV Files Into Microsoft® Excel

1. Click on the Microsoft Excel program icon.
2. Click on "File," then "Open." Select the directory containing the microarray csv file exported from Pathways™, select "All Files" in the "Files of Type" window, and select the csv file in the "Open" window. Click "Open" to import the csv file into Excel.
3. At this point data can be sorted according to standard Excel procedures and the imported data file can be saved in Excel file format for later analysis.

3.3. Nonradioactive Detection Method

This section describes the method used to label RNA with nonradioactive digoxigenin-dUTP (DIG-dUTP) as well as a discussion of both chemiluminescent and color precipitate detection of the labeled cDNA products ***(8–10)***. Either mRNA or total RNA can be used with this labeling procedure. In this example we will use mRNA as the RNA starting material, which can be extracted from human cells using an mRNA extraction kit that incorporates a biotin-labeled oligo-dT probe and streptavidin magnetic particles (Roche). The protocols listed above for the radioactive probe labeling method are mainly adhered to, with modifications discussed below.

3.3.1. Prehybridization of DermArray Microarray

1. Prior to proceeding with the following protocol, cut off the portion of the microarray membrane that contains ink-written information, as the ink has been shown to generate nonspecific signals during detection.
2. Prewash each microarray in 0.5% SDS as described in the radioactive labeling protocol. Place the membrane in a 35 × 150-mm hybridization roller bottle with the DNA side of the membrane facing the interior of of the bottle such that it is not touching the glass.
3. Add the following components to the roller bottle: 5 mL DIG Easy Hyb hybridization solution (Roche); 5 µL denatured Cot-1 DNA (1 µg/µL); and 5 µL Poly A (1 µg/µL).
4. Prehybridize the membrane at 42°C for 2 h.

3.3.2. Labeling of mRNA With DIG-dUTP

1. Add 2 µL Oligo dT to 200 ng mRNA in a total volume of 8 µL to prime the reverse transcriptase reaction. Mix, quick spin, and denature the mixture at 70°C for 10 min, followed by quick chilling on ice.

2. To the primed mRNA, add the following (Superscript II kit; Invitrogen): 6 µL 5X first-strand buffer; 1 µL 0.1 *M* DTT; 1 µL dNTP mix containing dATP, dGTP, and dCTP at 20 m*M*; 0.25 µL dTTP at 20 m*M*; 6 µL DIG-11-dUTP at 1 m*M*; 300 units Superscript Reverse Transcriptase II; and Nuclease-free water to bring the total volume of the reaction to 30 µL.
3. Mix components thoroughly, quick spin and incubate reaction at 37°C for 90 min followed by incubation at 70°C to stop the reaction
4. Remove unincorporated nucleotides from the labeling reaction by passage through a Sephadex G-50 spin column.
5. Remove 1 µL of the purified probe and spot onto a nylon membrane, and incubate with anti-DIG antibody conjugated to alkaline phosphatase, followed by detection with NBT/BCIP according to manufacturer instructions (Roche) to confirm label incorporation.

3.3.3. Hybridization of DermArray With Nonradioactive Probe

1. Denature the DIG-dUTP probe in boiling water bath for 3 min, followed by quick chilling on ice.
2. Transfer the entire amount of the probe to the roller bottle containing the membrane and the prehybridization solution, taking care not to pipet the probe directly onto the membrane. Retighten the roller bottle and swirl the bottle contents by hand prior to addition of the roller bottle to the hybridization oven.
3. Rotate the roller bottle in a VWR hybridization oven overnight at 42°C and approx one-third maximum speed.

3.3.4. Microarray Washes

1. After overnight incubation of the membrane with the DIG-dUTP probe, wash the filter twice in 30 mL of 2X SSC, 1% SDS at 50°C for 20 min each time.
2. Carry out a final wash in 100 mL of 0.5X SSC, 1% SDS at room temperature for 15 min in a plastic container.
3. After washing, place the membrane on a moistened piece of Whatman 3MM paper and wrap in plastic wrap. Smooth out any bubbles or creases as these will interfere with the imaging process.

3.3.5. Chemiluminescent Detection

1. Chemiluminescent detection is conducted using the CDP-Star substrate system (Roche). This system generates light signals which allows the detection of biomolecules on X-ray film, phosphor imagers, or dedicated chemiluminescent detection instrumentation. After completion of the hybridization and stringency washes as described in **Subheading 3.3.4.**, the membrane is subjected to detection using anti-DIG antibody conjugated to alkaline phosphatase (Roche), followed by CDP-Star. The membrane is rinsed and 20 mL of dilute anti-DIG-AP conjugate (37.5 mU/mL, 1 : 10,000) is added and incubated for 30 min.
2. Wash the membrane and incubate it in a sealed hybridization bag for 5 min in 1–2 mL of dilute CDP-Star (25 m*M*, 1 : 200) solution.
3. Drain off excess liquid and blot the membrane (DNA-side up) on Whatman 3MM paper. Do not allow the membrane to dry completely. Place the damp membrane into a new hybridization bag and expose it to X-ray film for 15 s to 15 min, or place the membrane in a Lumi-Imager (Roche) for 15–35 min.
4. Luminescence continues for several hours, thus allowing multiple exposures to be taken of the membrane in order to achieve the desired level of exposure. If the Cyclone phosphor imager or the Roche Lumi-Imager is used for detection the resulting images can be stored directly as 16-bit TIFF files without alteration. The images obtained from chemiluminescent

detection are comparable to those obtained from radioactive ^{33}P images, and are analyzed with Pathways software.

3.3.6. Colored Precipitate Detection

An alternate method of visualizing spots on the microarray membrane involves treatment of the membrane with an NBT/BCIP solution (Roche), which forms a colored precipitate, with no further treatment of the membrane required following the chemiluminescent detection procedure. Colored precipitate detection is not as sensitive as the chemiluminescent method but it does allow gross visual inspection of the hybridized microarray spots as well as confirmation of the results obtained from chemiluminescent detection. In addition the NBT/BCIP treated membrane can be scanned with a conventional inexpensive flatbed scanner at 800 dpi and the resulting eight-bit grayscale image of the filter can be analyzed with the Pathways program.

3.3.7. Stripping Microarrays Hybridized to DIG-dUTP-Labeled Probes

1. If alkaline labile DIG-dUTP was used to label the cDNA probes, then the microarray can be stripped of the probe by rinsing the membrane briefly in sterile distilled water, washing the membrane twice for 15 min each time in 0.2 *M* NaOH, 0.1% SDS at 37°C, followed by a final wash in 2X SSC for 5 min. Membranes stripped by this method can be reprobed provided they are never allowed to dry to completion during the entire procedure.
2. For membranes visualized by means of the colored precipitate method, the precipitate can be stripped off the membrane by first shaking the membrane in dimethylformamide as recommended by Roche, followed by stripping of the probe in 0.5% boiling SDS for 1 h.

4. Notes

1. Treatment of DermArray or PharmArray DNA microarrays with boiling 0.5% SDS is only necessary the first time each filter is used.
2. The side of the microarray with the DNA spots on them is always the side that that has been labeled with the catalog number and lot number.
3. Cot-1 DNA should be boiled at 100°C for 3 min and quick chilled on ice for 2 min prior to addition to the roller bottle.
4. We recommend using 3 µg of total RNA in each probe labeling reaction, but a range of 1–10 µg of total RNA can be used successfully. Alternatively purified mRNA can be used as a probe in a concentration range from 100 ng to 1 µg.
5. Generally a total of 10^7 cpm/µL incorporated for each labeled probe is required to generate a satisfactory image for subsequent analysis in the Pathways™ software program.
6. Retightening of the hybridization bottle caps after 10 min is necessary because of the tendency of the Teflon cap seals to expand after heating to 42°C. Retightening of the caps lessens the chance of probe leaking into the hybridization oven during the overnight incubation.
7. DermArray and PharmArray microarray filters can be stripped and reprobed a total of five times while still yielding excellent results.

References

1 Curto, E. V., Lambert, G. W., Davis, R. L., Wilborn, T. W., and Dooley, T. P. (2002) Biomarkers of human skin cells identified using dermArray DNA arrays and new bioinformatics methods. *Biochem. Biophys. Res. Commun.* **291,** 1052–1064.

2 Dooley, T. P. (1997) Topical skin depigmentation agents: current products and discovery of novel inhibitors of melanogenesis. *J. Dermatol. Treat.* **8,** 275–279.

3. Dooley, T. P., Gadwood, R. C., Kilgore, K., and Thomasco, L. M. (1994) Development of an in vitro primary screen for skin depigmentation and antimelanoma agents. *Skin Pharmacol.* **7,** 188–200.
4 Smith, C. J., O'Hare, K. B., and Allen, J. C. (1988) Selective cytotoxicity of hydroquinone for melanocyte-derived cells is mediated by tyrosinase activity but independent of melanin content. *Pigment Cell Res.* **1,** 386–389.
5 Zhao, N., Hashida, H., Takahashi, N., Misumi, Y., and Sakaki, Y. (1995) High-density cDNA filter analysis: a novel approach for large-scale, quantitative analysis of gene expression. *Gene* **156,** 207–213.
6 Schena, M., Shalon, D., Davis, R. W., and Brown, P. O. (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* **270,** 467–470.
7 Cole, J., Tsou, R., Wallace, K., Gibran, N., and Isik, F. (2001) Comparison of normal human skin gene expression using cDNA microarrays. *Wound Repair Regen.* **9,** 77–85.
8 Ross, R., Ross, X. L., Rueger, B., Laengin, T., and Reske-Kunz, A. B. (1999) Nonradioactive detection of differentially expressed genes using complex RNA or DNA hybridization probes. *Biotechniques* **26,** 150–155.
9 Adryan, B., Carlguth, V., and Decker, H. J. (1999) Digital image processing for rapid analysis of differentially expressed transcripts on high-density cDNA arrays. *Biotechniques* **26,** 1174–1179.
10. Engler-Blum, G., Meier, M., Frank, J., and Muller, G. A. (1993) Reduction of background problems in nonradioactive Northern and Southern blot blot analyses enables higher sensitivity than ^{32}P-based hybridizations. *Anal. Biochem.* **210,** 235–244.

39

Two-Photon Fluorescence Imaging and Reactive Oxygen Species Detection Within the Epidermis

Kerry M. Hanson and Robert M. Clegg

Summary

Two-photon fluorescence microscopy is used to detect ultraviolet-induced reactive oxygen species (ROS) in the epidermis and the dermis of ex vivo human skin and skin equivalents. Skin is incubated with the nonfluorescent ROS probe dihydrorhodamine, which reacts with ROS such as singlet oxygen and hydrogen peroxide to form fluorescent rhodamine-123. Unlike confocal microscopic methods, two-photon excitation provides depth penetration through the epidermis and dermis with little photodamage to the sample. This method also provides submicron spatial resolution such that subcellular areas that generate ROS can be detected. In addition, comparative studies can be made to determine the effect of applied agents (drugs, therapeutics) upon ROS levels at any layer or cellular region within the skin.

Key Words:

Reactive oxygen species; two-photon; fluorescence; microscopy; antioxidant.

1. Introduction

Reactive oxygen species (ROS) are highly reactive, short-lived derivatives of molecular oxygen. Superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), as well as free radicals like singlet oxygen ($^1O_2$) and hydroxyl radical ($^{\bullet}OH$) are common ROS with biological significance on the cellular level. ROS can play a critical role in normal cell activities as exemplified by $O_2^{\bullet -}$ and H_2O_2, which are part of normal mitochondrial aerobic respiration. Intrinsic mechanisms are present within cells that reduce proliferation of and damage by ROS generated during metabolism. However, of great concern is the overproduction of ROS, which can assault natural defense mechanisms and lead to cellular damage. This is exemplified by the damage that results from the overproduction of ROS within the skin after ultraviolet (UV) radiation exposure. Solution phase and cell culture studies have shown that after absorption of UV radiation, epidermal chromophores (riboflavin, NADH/NADPH, tryptophan, urocanic acid, **refs. *1–6***, and cellular components [mitochondria, **ref. *7***, and melanin, **refs. *7*** and ***8***]) generate ROS, including singlet oxygen, hydrogen peroxide, superoxide anion, and hydroxyl radical. These ROS may, for example, react with lipid membranes and proteins ***(9)***, alter the immune response ***(10)***, and cause lesions within deoxyribonucleic acid (DNA; **ref. *11***) and apoptosis ***(12)***, prompting researchers to argue that UV-induced skin ROS significantly affect photoaging ***(13)***, actinic keratosis ***(14)***, and tumorigenesis ***(15).***

From: *Methods in Molecular Biology, vol. 289:*
Epidermal Cells: Methods and Protocols
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

With advances in laser technology and fluorescence microscopy methods, we now have the tools available to study ROS and subsequent ROS-mediated pathways within the skin. This chapter gives a detailed two-photon fluorescence microscopy protocol that is used to detect ROS levels within all layers of the epidermis and the dermis. Two-photon fluorescence microscopy is an ideal method to detect ROS in skin because it affords submicron spatial resolution and submillimeter depth penetration with negligible photodamage. The components for a two-photon fluorescence microscope (laser, optics, and microscope) are all commercially available. It is a method that allows for ex vivo skin samples, skin equivalents or whole animals to be easily studied. Endoscope attachments allow for human subject applications as well.

Becaue of their short lifetimes and nonfluorescent nature, ROS are detected indirectly using the ROS fluorescent probe dihydrorhodamine (DHR). DHR is nonfluorescent until it reacts with ROS, including H_2O_2, $^1O_2$, $ONOO^-$, when it is converted to fluorescent rhodamine-123 (R123). R123 exhibits a strong and easily detectable fluorescence, peaking at 525 nm. Other ROS probes are available that may have specific cellular targets ***(16)***. Herein, we show how two-photon fluorescence microscopy is used to detect, on the subcellular level, ROS through R123 fluorescence. We also discuss how this method can be used to determine the level of ROS that are generated at a specific skin depth, which allows for tests to be conducted on the effect of externally applied agents upon ROS generation within the skin.

With current advancement in probe technology, two-photon fluorescence imaging can be used to detect ROS at specific locations within cellular components (mitochondria, nuclei, lipid membranes) and to follow ROS-mediated pathways.

2. Materials

2.1. Tissue Samples and Preparation

*2.1.1. Ex Vivo Tissue (*see ***Note 1****)*

1. Human breast, abdominal, or facial skin.
2. Uncoated plastic dishes (BD Falcon plastic dishes 35 × 10 mm, Fisher Scientific; cat. no. 08757100a).
3. 4°C Refrigeration source.
4. Indicator-free RPMI–1640 (Invitrogen; cat. no. 11835030).
5. L-glutamine (Sigma Aldrich; cat. no. G7513).
6. Gentamicin (Sigma Aldrich; cat. no. G1397).
7. Prepare storage media: (1) 500 mL indicator-free RPMI-1640; (2) 2 m*M* L-glutamine (final concentration); and (3) 10 µg/mL gentamicin (5 mg/500 mL).
8. Prepare ex vivo tissue samples: (1) Using a scalpel, remove excess subcutaneous fat from skin and discard; (2) using a scalpel, divide skin into 0.5-cm × 0.5-cm samples; (3) place samples in clean, uncoated plastic storage dishes; and (4) transfer enough storage media into the dish such that each sample is surrounded by a reservoir of media and concurrently the stratum corneum surface remains uncovered by media; (5) store samples in media-filled dish at 4°C for no more than 5 d; and (6) replace media every 24 h.

*2.1.2. Skin Equivalent (*see ***Note 2****)*

1. EpiDerm™ 200 or Epiderm-FT™ 200 tissues and growth media (MatTek Corp., Ashland, MA).
2. Forceps.
3. Incubator 37°C, 5% CO_2.

4. Follow the manufacturer's instructions for preparation and storage of skin equivalents (*see* **Note 2**).

2.2. Fluorophores

1. ROS Probe: dihydrorhodamine-123 (Molecular Probes, Eugene, OR; cat. no. D632).
2. Viability probe: Mitotracker Green FM *special packaging* (Molecular Probes, Eugene, OR; cat. no. M7514).

2.3. Two-Photon Fluorescence Imaging Microscope (Fig. 1)

A titanium:sapphire laser (Tsunami, Spectra-Physics) is the two-photon excitation source ***(17)***. The 785-nm output (3 mW measured before the scanning mirrors) of the laser is coupled through the epifluorescence port of a Zeiss Axiovert microscope. The excitation beam is diverted to the sample by a dichroic filter (Q560LP, Chroma Technologies), and the fluorescence is detected with a Hamamatsu (R3996) photomultiplier. Two BG39 filters (0.5-cm thick each) are placed in the fluorescence emission path to block scattered IR and pass the R123 fluorescence. One BG39 filter (0.5-cm thick) also is placed in the eyepiece to block damaging laser light from reaching the eye. Scanning mirrors position the excitation beam through a ×40 infinity-corrected oil objective (Zeiss F Fluar, 1.3 N.A.). The scanner is controlled by the program SimFCS (Enrico Gratton, Laboratory for Fluorescence Dynamics, University of Illinois, Urbana-Champaign) through a three-axis plug-in card (ISS, Champaign, IL). Areas up to 4000 μm^2 can be imaged. Depth z-slices are obtained by adjusting the objective focus with a motorized driver (ASI Multi-Scan 4). The photocurrent output from the photomultiplier is digitized using a 12-bit plug-in analog-to-digital converter card (ISS, Champaign, IL). Data are acquired using the data acquisition section of SimFCS.

2.4. Irradiation Equipment

1. UVA-UVB (280 nm–450 nm) solar simulator (PMA2100; Solar Light Co.).
2. UVA-UVB irradiance detector (PMA2105 DCS Detector; Solar Light Co.).
3. Lamp power supply (XPS 200 Xe lamp power supply; Solar Light Co.).

2.5. Sample Housing

1. Hanging drop slides (76 × 26 mm, 0.5-mm concave well, 18-mm diameter, Fisher Scientific; cat. no. 12-560A).
2. Cover slips (No. 1.5, 40 × 24 mm, Fisher Scientific; cat. no. 12-518-105E).

3. Methods

3.1. Fluorophore Labeling of Skin

3.1.1. Skin Labeling With ROS Fluorogenic Probe (Dihydrorhodamine)

Two different protocols are used depending on the sample source: ex vivo tissue or living skin equivalents (*see* **Note 4**).

3.1.1.1. Ex Vivo Skin

1. Make a <200 μ*M* dihydrorhodamine solution in 6:1 phosphate-buffered saline (PBS):ethanol using the absorption of DHR at 280 nm and Beer's Law. The PBS:ethanol solution does not inhibit mitochondrial respiration nor esterase or phosphatase activity ***(18)***. *See* **Note 5**.

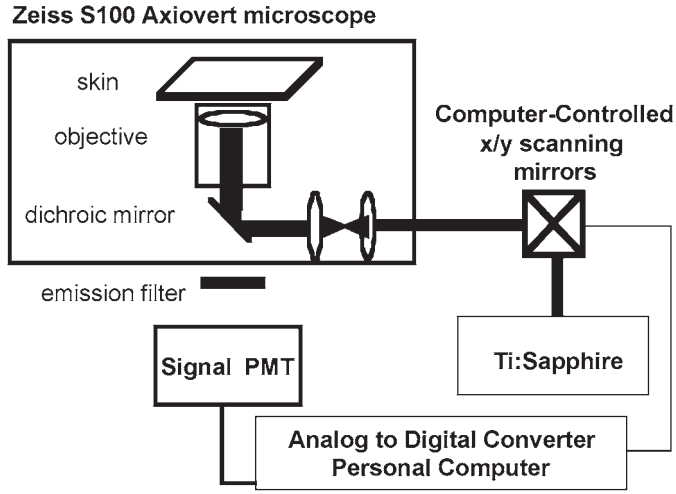


Fig. 1. Two-photon fluorescence microscope instrument.

2. Transfer 200 µL DHR solution into a 1-mL Eppendorf tube.
3. Transfer one 0.5-cm × 0.5-cm ex vivo skin sample to the Eppendorf tube.
4. Submerge ex vivo skin sample completely in DHR solution.
5. Incubate 10 min at 4°C.
6. Remove skin sample.
7. Rinse in PBS, pH 7.2.
8. Remove excess solution from skin using KimWipe.
9. Transfer skin sample to hanging drop slide with stratum corneum surface facing up.
10. Cover skin sample with cover slip.
11. Secure cover slip using four minibinder clips.

3.1.1.2. Skin Equivalents

1. Make a <200 µ*M* dihydrorhodamine solution in 6:1 PBS:ethanol using the absorption of DHR at 280 nm and Beer's Law (*see* **Note 5**).
2. Make a 10 µ*M* DHR solution in PBS from the 200 µ*M* DHR stock solution.
3. After sample equilibration (follow manufacturer's instructions), using sterile techniques (*see* **Note 4**), transfer a 100-µL aliquot of DHR solution to sample surface.
4. Incubate at 37°C, 5% CO_2 for <1 h.
5. Using forceps under sterile conditions, remove each EpiDerm™ from its housing, rinse in pH 7.2 PBS, and place dermis-side down on hanging drop slide.
6. Cover with cover slip and secure using minibinder clips.

3.1.2. Skin Labeling for Viability (Mitochondrial Respiration) Assay

This section describes the protocol followed for labeling the skin with the fluorescence probe Mitotracker Green FM, which determines whether mitochondria are actively respiring. This assay provides important data describing the viability of the tissue sample being used, allowing the researcher to show that the skin sample is metabolically active. The intact membrane of respiring mitochondria facilitates staining by Mitotracker Green FM. Inactive mitochondria do not retain Mitotracker Green FM as

well as their respiring counterparts. Other mitochondria-specific fluorophores are available ***(16)***. In addition, enzymatic activity (esterase, phosphatase) like calcein-AM (Molecular Probes, Eugene, OR) or fluorescein diphosphate (Molecular Probes, Eugene, OR) also can be detected using fluorescence probes ***(16)***. The same protocol as described below can be followed for labeling the skin with such probes.

1. For ex vivo samples, remove the stratum corneum (*see* **Note 6**). (1) Holding the skin sample (ex vivo or EpiDerm™) in place, apply the sticky-side of cellophane tape to the surface of the stratum corneum. (2) Repeat application of the tape to the skin until the stratum corneum is removed. The skin will appear shiny by eye. Confirmation that the stratum corneum is removed can be made by checking the sample under the microscope. (3) Transfer the tissue to a clean dish placing the sample dermis-side down.
2. Prepare Mitotracker incubation solution. (1) Follow the product information sheet provided by Molecular Probes. Make a stock solution by dissolving contents of one vial (50 µg) in anhydrous dimethyl sulfoxide for a final concentration of 1 m*M*. (2) Make an incubating solution of 500 n*M* from the stock solution using PBS pH 7.2 for the dilution. (3) For ex vivo samples, transfer tape-stripped skin to an Eppendorf tube containing 200 µL of the 500 n*M* Mitotracker solution and incubate at 4°C for 10 min. (4) For skin equivalent samples, apply 100 µL of the 500 n*M* Mitotracker solution and incubate at 37°C for 1 h. (5) Remove tissue, rinse in pH 7.2 PBS, and pat dry with KimWipe. (6) Place sample dermis side down on well-slide, cover with cover slip and secure cover slip with minibinder clips. Image sample as described in **Subheadings 3.2., steps 1–12**.

3.2. Two-Photon Fluorescence Imaging Before UV Irradiation

This section describes the protocol used to image skin samples on a Zeiss Axiovert S100 microscope before UVB and/or UVA irradiation. Collection of images before irradiation is important to determine background levels of fluorescence from both autofluorescence and fluorophore probes.

1. Block laser beam with a beam block so it does not enter epifluorescence port of the microscope.
2. Place sample slide on microscope stage with the cover slip facing the objective.
3. Secure slide with clips onto the stage.
4. Place one drop of Zeiss microscope oil onto the 40X oil-objective aperture well.
5. Using the macroadjustment dial, adjust the objective until the oil comes in contact with the cover slip.
6. Look through the eyepiece and using the microadjustment dial, adjust the objective until the skin surface comes into focus.
7. Secure the motorized z-stage, where z = 0 at the surface of the skin.
8. Cover the box with black-cloth such that room-light is blocked from the objective.
9. Turn off room-lights.
10. In the data collection portion of SimFCS, establish the following parameters: 1) Z increment (>1-µm increment steps); 2) X-Y image area (≤200 µm × 200 µm); 3) sampling time (50 µs per pixel); and 4) points collected per pixel ***(1)***.
11. Unblock the laser beam and collect images at each desired z-depth.
12. Remove the black-cloth and the blackened box.
13. Remove the sample slide from the microscope stage.
14. Remove the cover slip from the slide.

3.3. Irradiate Sample

This section describes the protocol used to irradiate skin samples with a solar simulator (Solar Light Co.) after background fluorescence levels have been acquired at different z depths (**Subheading 3.3., steps 1–7**).

1. Turn on the solar simulator.
2. Following the manufacturer's instructions, establish the irradiation dose (100 J m^{-2} = 1 standard erythemal dose; **ref. *19***) and irradiation spectrum (UVB and UVA (280–450 nm) or UVA (320–450 nm).
3. Ensure that the glass cover slip is removed from the sample slide before irradiation (glass attenuates UV light).
4. Place the sample slide in the light path such that the sample stratum corneum surface faces the light. Ensure that the sample area is fully irradiated by the light source.
5. Following the manufacturer's instructions, irradiate the sample for the desired UV dose or time.
6. Remove the sample from the solar simulator.
7. Secure a cover slip on the sample using four mini-binder clips.

3.4. Two-Photon Fluorescence Imaging After UV Irradiation (Figs. 2 and 3)

1. Re-image the sample following **steps 1–12** in **Subheading 3.2.**
2. Image at similar z-depths as were used to acquire the before- UV image data (*see* **Note 7**).

3.5. Determine the Level of ROS

Determination of the concentration of generated ROS is complicated by our limited understanding of the reaction kinetics between dihydrorhodamine (and other ROS fluorophores) and UV-induced ROS. Several ROS are generated following UV irradiation of the skin, and many, but not all, may react with dihydrorhodamine ***(18)***. Thus, a simplified kinetic model has been proposed to calculate the concentration of ROS that are generated following a particular UV dose. The reader is referred to reference ***(18)*** for a complete description. For this work, we focus on a straight-forward method for those who would be interested in studying the effect of a compound upon the level of ROS at a particular depth in the skin.

Of particular interest is the ability of compounds, like antioxidants, to reduce the number of damaging ROS that are generated. To determine the reduction in UV-induced ROS at depth z, Eq. 1 is used.

$$\%-\text{reduction}(z) = 100 - 100\left\{\frac{I(z)_{\text{sample}}}{I(z)_{\text{control}}}\right\} \quad (1)$$

At each epidermal depth z, the fluorescence intensity is calculated by averaging the intensity of each pixel over the entire image. For multiple areas studied on the same skin sample, these intensity data are averaged together ($I(z)_{\text{sample}}$). $I(z)_{\text{control}}$ is calculated identically, where the control images are those acquired on skin incubated with DHR only and indicate the control level of ROS that are generated at the UV irradiance used.

4. Notes

1. Human skin is obtained from local plastic surgeries after patient-requested plastic surgery with University Review Board approval.

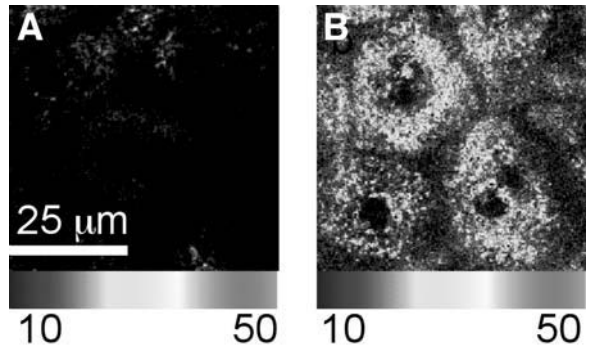


Fig. 2. Two-photon fluorescence intensity images before **(A)** and after **(B)** UV irradiation (600 J m^{-2}) of ex vivo human breast tissue incubated with DHR. The depth from the skin's surface is 15 μm, and the cells are keratinocytes of the stratum granulosum. The intensity scale is below each image and corresponds to a voltage reading of the PMT. Image **(A)** is black to blue in color, indicating little autofluorescence. After UV irradiation, R123 fluorescence increases predominately in the cytoplasm of the cells indicating the generation of ROS. Mitochondrdia, lipid granules, and melanin are all sources of ROS as seen in the image ***(18)***. See color insert following p. 238.

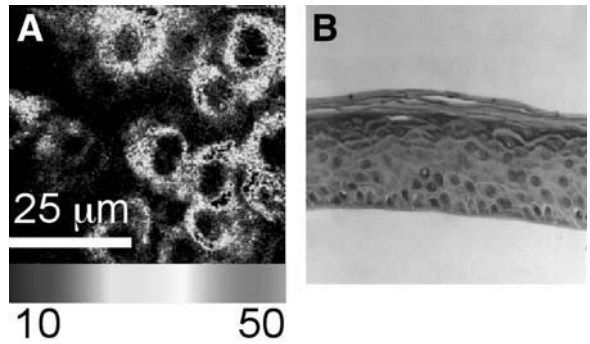


Fig. 3. These images show that similar results as from the ex vivo skin are found with the skin equivalent EpiDerm™ 200. Image **(A)** is the two-photon fluorescence intensity image after UV irradiation (200 J m^{-2}). The depth is 85 μm and the cells are keratinocytes of the stratum basale. The ROS are generated within the cytoplasm of the cells. Image **(B)** shows the histology of the skin sample. See color insert following p. 238.

2. The skin equivalents EpiDerm™ 200 and EpiDerm-FT™ 200 (MatTek Corp., Ashland, MA) are alternatives to ex vivo tissue. The epidermal model, EpiDerm™ 200, is composed of normal human epidermal keratinocytes cultured at the air/liquid interface to produce a highly differentiated three-dimensional organotypic tissue and is cultured in a defined serum-free medium. The epidermis/dermis model, EpiDerm-FT™ 200, also is composed of normal human epidermal keratinocytes; however, it has a well-developed basement membrane at the dermal/epidermal junction. The epidermis is cultured on top of a collagen dermis containing viable normal human dermal fibroblasts, where the NHEKs are cultured at the air/liquid interface.
3. MatTek's product, unlike ex vivo tissue, arrives sterile and thus sterile techniques can be used to preserve sterility of the remaining samples in the snap-well plate until use at a later date. If all of the samples are used in the same day, then sterile techniques are not necessarily required.

4. We have found that the stratum corneum of ex vivo tissue is a greater barrier to penetration of DHR compared to EpiDerm™. Application of DHR only to the surface of ex vivo samples does not always guarantee penetration of enough DHR to allow for detection of ROS in the lower epidermis and the dermis. Thus, submerging ex vivo samples in DHR provides penetration of the fluorophore through the surface and sides of the sample such that DHR labels all layers of the epidermis and dermis. Final concentrations of DHR below the stratum corneum are approx 1 µm with an incubation solution of 100 µm and incubation time of 10 min ***(18)***.
5. Calculation of the concentration is done by Beer's Law: $A = \varepsilon bc$, where A is the sample absorbance at 289 nm, ε is the extinction coefficient (7100 cm^{-1} M^{-1}) at 280 nm, *b* is the cuvet pathlength, and *c* is the calculated concentration.
6. We found that for ex vivo skin, removal of the stratum corneum is critical. Significant penetration of the lipophilic mitochondrial probes is inhibited by the cornified layers. As a result, the fluorescence signal-to-noise ratio is low in the viable epidermal strata inhibiting differentiation between active and inactive mitochondria. The reduced penetration may result from the hydrophobic nature of the mitochondrial probes.
7. Using this protocol, it is impossible to guarantee that the same area will be imaged both before and after UV irradiation of the sample. Typically, the surface (stratum corneum) images yield the greatest background signal (<10%). The viable strata and dermal layers yield <5% background fluorescence when exciting at 785 nm. Thus, imaging different areas before and after UV irradiation is insignificant. A fiber optic could be coupled from the solar simulator with the microscope to allow for UV irradiation of the sample on the microscope. Quartz cover slips would be required to pass the UV light.

References

1 Peak, M. J. and Peak, J. G. (1986) In *The Biological Effects of UVA Radiation* (Gange, F., and Gange, R. W., eds.), Praeger, New York, pp. 42–52.

2. Peak, M. J. and Peak, J. G. (1989) Solar-ultraviolet-induced damage to DNA. *Photodermatology* **6,** 1–15.

3. Cunningham, M. L., Krinsky, N. I., Giovanazzi, S. M., and Peak, M. J. (1985) Superoxide anion is generated from cellular metabolites by solar radiation and its components. *J. Free Radical Biol. Med.* **5,** 381–385.

4 Hanson, K. M., Li, B., and Simon, J. D. (1997) A spectroscopic study of the epidermal chromophore trans-urocanic acid. *J. Am. Chem. Soc.* **119,** 2715–2721.

5 Hanson, K. M. and Simon, J. D. (1998) Epidermal trans-urocanic acid and the UVA-induced photoaging of the skin. Proc. Natl. Acad. Sci. USA ***95,*** *10,576–10,578.*

6 Jurkiewicz, B. A. and Buettner, G. R. (1996) EPR detection of free radicals in UV-irradiated skin: mouse versus human. *Photochem. Photobiol.* **64,** 918–922.

7. Gniadecki, R., Thorn, T., Vicanova, J., Petersen, A., and Wulf, H. C. (2000) Role of mitochondria in ultraviolet-induced oxidative stress. *J. Cell. Biochem.* **80,** 215–222.

8 Nofsinger, J. B., Liu, Y., and Simon, J. D. (2002) Aggregation of eumelanin mitigates photogeneration of reactive oxygen species. *Free Radic. Biol. Med.* **31,** 720–730.

9 Vile, G. F. and Tyrrell, R. M. (1995) UVA radiation-induced oxidative damage to lipids and proteins in vitro and in human skin fibroblasts is dependent on iron and singlet oxygen. *Free Radic. Biol. Med.* **18,** 721–722.

10 Iwai, I., Hatao, M., Nagnauma, M., Kumano, Y., and Ichihashi, M. (1999) UVA-induced immune suppression through an oxidative pathway. *J. Inv. Derm.* **112,** 19–24.

11 Chen, Q., Fischer, A., Reagan, J. D., Yan, L. J., and Ames, B. N. (1995) Oxidative DNA damage and senscence of human diploid fibroblast cells. *Proc. Natl. Acad. Sci. USA* **92,** 4337–4341.

12. Chen, Q. and Ames, B. N. (1994) Senescence-like growht arrest induced by hydrogen peroxide in human diploid fibroblast F65 cells. *Proc. Nat'l. Acad. Sci. USA* **91,** 4130–4134.
13 Brenneisen, P., Sies, H., and Scharffetter-Kochanek, K. (2002) UV-B irradiation and matrix metalloproteinases:from induction via signaling to initial events. *Ann NY Acad. Sci.* **973,** 31–43.
14 Vural, P., Cnbaz, M., and Selcuki, D. (1999) Plasma antioxidant defense in actinic keratosis and basal cell carcinoma. *J. Eur. Acad. Dermatol. Venereol.* **13,** 96–101.
15. Kensler, M. L. and Thrusch, M. A. (1995) Role of oxygen radicals in tumor promotion. *Environ. Mutagen.* **6,** 855–861.
16. Haughland, R. P., ed. (1998) *Handbook of Fluorescent Probes and Research Chemicals*, Molecular Prober, Eugene, OR.
17. So, P. T. C., French, T., Yu, W. M., Berland, K. M., Dong, C. Y., and Gratton, E. (1996) Two-photon fluorescence microscopy: time-resolved and intensity imaging, in *Fluorescence Imaging and Microscopy*, vol. 137 (Herman, B., and Wang, X.F., eds.), John Wiley and Sons, New York, pp. 351–374.
18 Hanson, K. M. and Clegg, R. M. (2002) Observation and quantification of UV-induced ROS generation in ex vivo human skin. *Photochem. Photobiol.* **76,** 57–63.
19. CIE Central Bureau. (1997) Standard Erythema Dose, a Review. l'Eclairage, C. I. d. CIE 125.

V

Transplantation and Gene Therapy

40

In Vivo Transplantation of Engineered Human Skin

Shari Greenberg, Alexander Margulis, and Jonathan A. Garlick

Summary

Tissue engineering approaches have enabled the development of methodologies that allow long-term, in vivo studies in epidermal biology. This has been accomplished through in vivo transplantation of human epidermal cells fabricated as three-dimensional, skin-equivalents in vitro. The methodologies presented in this chapter describe how skin-equivalent (organotypic) cultures are transplanted to nude mice to generate human skin grafts that normalize their tissue architecture, basement membrane structure and barrier function shortly after grafting. By grafting skin equivalents as composite cultures featuring well-differentiated human epidermis and fibroblasts in collagen gel, transplants are "primed" for accelerated take of grafted tissues. The methods outlined can generate stable, human epidermis that mimics the in vivo tissue.

Key Words:

Epidermis; skin equivalents; epidermal transplantation; nude mice; organotypic culture.

1. Introduction

Advances in tissue engineering have paved the way for the development of new approaches to the fabrication of skin and mucosal substitutes. In recent years, two methods for the transplantation of human keratinocytes cultivated in vitro have been established for epidermal grafting for applications in skin biology research. These techniques include the epidermal sheet graft ***(1)*** and the composite, organotypic culture graft ***(2)***. The sheet graft technique involves transplanting a postconfluent sheet of epidermal keratinocytes, grown in submerged culture, directly to a dermal graft site. The second technique is designed to graft a composite tissue grown at an air–liquid interface that includes a dermal component and an epithelium that closely mimics the in vivo tissue. When these two techniques have been compared, the grafting of composite tissues has proven to have numerous advantages over grafting epithelial sheets to nude mice ***(3)***. Because successful transplantation is a function of the degree to which grafted tissues are able to attach to the dermis, survive, differentiate, and proliferate ***(4)***, composite, organotypic cultures demonstrate an engraftment advantage when compared with epithelial sheets. This is not surprising because composite, organotypic cultures have been shown to have many in vivo-like features, such as complete morphologic differentiation, rapid assembly of basement membrane, presence of cells with "stem-like" features, and the presence of dermal fibroblasts that communicate with the epithelium ***(2,5)***. In this light, the construction of such composite cultures allows transplantation of tissues that have been "primed" for accelerated graft take. This

From: *Methods in Molecular Biology, vol. 289:*
Epidermal Cells: Methods and Protocols
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

chapter presents methods for the in vivo transplantation of composite, organotypic cultures composed of human keratinocytes and fibroblasts to nude mice. The technique described is highly reproducible, establishes normalized tissue architecture and barrier function shortly after grafting, and requires no suturing or surgical adaptation of the transplanted tissue. The resultant, stable grafts enable the long-term study of epidermal biology directly in human skin.

2. Materials

1. Four- to six-week-old male nude mice (TAC : N : NHIS-nufDF, Taconic, Germantown, NY). Wait 1 week after mice arrive before grafting.
2. Bandages (Alliance Health Care, Edison, NJ; cat. no. CBD1018096).
3. Vaseline gauze (3" × 9") (Sherwood Medical, Indianapolis, IN; cat. no. 8884-413605).
4. CO_2 chamber: used to transport organotypic cultures to the animal facility.
5. Anesthesia: (1) isofluorane (apply to sterile gauze and place inside a 60-mL syringe tube); (2) ketamine/xylazene/sterile saline, 0.06–0.10 mL/mouse (depending on weight of mouse that is used for deep sedation).
6. 1-mL Tuberculin syringes (Monoject).
7. Sterile surgical kit: includes curved scissors, straight scissors, scalpel, forceps, dental mirror, and stainless-steel biopsy punch (Keyes Cutaneous Punch) with a 1.4-cm diameter (Delasco Surgical Instruments, Council Bluffs, IA; cat. no. KP-14).
8. Teflon cutting board (3 × 5 cm): surface used for cutting with biopsy punch.
9. Sterile gloves, surgical mask, and gown.
10. Laminar flow hood and sterile mouse cage.

3. Methods

3.1. Preparation Before Grafting

1. Sterilize all materials to be used in the grafting procedure.
2. On the day of grafting, the quality of organotypic cultures should be evaluated to determine their suitability for grafting (*see* **Note 1**). Frozen sections are cut and a quick hematoxyllin–eosin stain is performed. Morphologic criteria that would allow grafting are listed in the **Notes** section. If the tissue does not meet these criteria, it is not possible to proceed with grafting (*see* **Note 2**).
3. Prepare fresh anesthetic solution.

3.1.1. Animal Surgery (see ***Note 3***)

1. Transport organotypic cultures to the animal facility in a carbon dioxide chamber. The chamber should have two ports with stopcocks to allow carbon dioxide to replace the air inside.
2. Sedate the mouse by allowing it to crawl into a sterile 60-mL tube to stabilize it. Once inside, administer 0.06–0.10 mL by intraperitoneal injection of the ketamine/xylazene/sterile saline solution by injecting beneath the skin in the inguinal region. The mouse should usually be fully sedated after 5 to 10 min, as verified by testing the foot pad reflex. A negative reflex is seen when gentle pressure on the foot pad does not evoke any muscular response. The mouse should be fully sedated for 45–60 min, which provides ample time to perform this technique.
3. Carefully estimate the area of excision of mouse skin at the graft site (*see* **Note 4**). The superficial edge of the graft should be in line with the fold seen under the anterior limbs. The incision should be several millimeters to the left of the midline, when the animal is viewed from behind, to avoid cutting the large vein found just to the right side of the midline. It is

important that the dimensions of the graft bed be 1 mm smaller than that of the graft. If the excision is too large, the graft margins will remain open and the likelihood of graft take will be decreased.

4. Lift the skin directly in the center of the area of chosen to be removed with narrow forceps and curved scissors are used to excise the mouse skin in one even motion. If there is bleeding or oozing, a sterile gauze can be applied with direct pressure in order to stop the flow before proceeding.
5. The organotypic culture is removed from the insert by using a scalpel to cut the polycarbonate membrane away from the plastic insert **(Fig. 1A)**. This can be performed by turning the insert over and cutting around the insert in an even motion. The culture is then placed onto the Teflon cutting board, and firm, even pressure is placed on the biopsy punch to cut out the culture in the appropriate shape while maintaining it on its polycarbonate membrane. Use moist forceps to remove the culture from its polycarbonate membrane and gently slide it onto the dental mirror which has been premoistened with a drop of media **(Fig. 1B)**. The cultures have sufficient tensile strength to allow them to be manipulated with gentle tugging with the forceps.
6. Move the mirror to the graft site and carefully slide the culture onto the site using the forceps. The culture should be placed so that it's edges overlap slightly over the cut edge of the graft bed. With as little manipulation as possible, gently remove any folds or wrinkles caused by the placement of the graft. At this point, the adjacent mouse skin can be gently stretched until the grafts edges form a smooth junction with the surrounding mouse tissues so that no underlying mouse connective tissue is seen at the margins **(Fig. 1C)**. Do not touch the mouse for 5 min to allow the graft to begin to attach to the mouse connective tissue.
7. Cut sterile vaseline gauze into circular pieces with a curved scissor so that they are 2–3 mm larger than the diameter of the graft. Cover the graft with two thicknesses of gauze and use two overlapping bandages to completely cover the graft site. This is done by gently turning the mouse over and placing the vasoline gauze covering the graft onto the gauze area of an open bandage. The mouse should be turned over by holding only the head and hind quarters to keep the graft from being dislodged (*see* **Note 5**).
8. Once the animal is properly positioned on the gauze, the edges of the bandage are gently yet firmly pulled around the mouse abdomen to ensure that the graft is well adapted to the graft so that it will be kept in place with mild pressure. The sticky ends from a third bandage are then used to seal the areas of closure on the first two bandages on the abdomen to prevent the bandage from being loosened over time. Monitor the mouse until it begins to show signs of reawakening and then transfer it back to the sterile cage.

*3.1.2. Post-Graft Care (*see ***Note 6****)*

1. Check the graft site daily to make sure that the bandages completely cover the graft site and rebandage if any graft margins or gauze are exposed.
2. After 1 wk the Vaseline gauze should be changed and the graft site should be freshly bandaged. Again, allow the mouse to crawl into a 60-mL tube, which will have a piece of sterile gauze soaked in Isofluorane at the opposite end of the tube. Avoid any contact of this anesthetic with the animal, as inhalation of any liquid of this reagent can lead to mortality. The mouse will be sedated within 30 s and will remain this way for one to three min.
3. Bandages should be inspected daily for an additional week and can then be removed.

4. Notes

1. Because the quality of the tissue morphology of organotypic cultures is a major determinant in successful engraftment, it is important to evaluate this parameter before grafting. Because it is not possible to examine cultures by phase-contrast microscopy owing to tissue

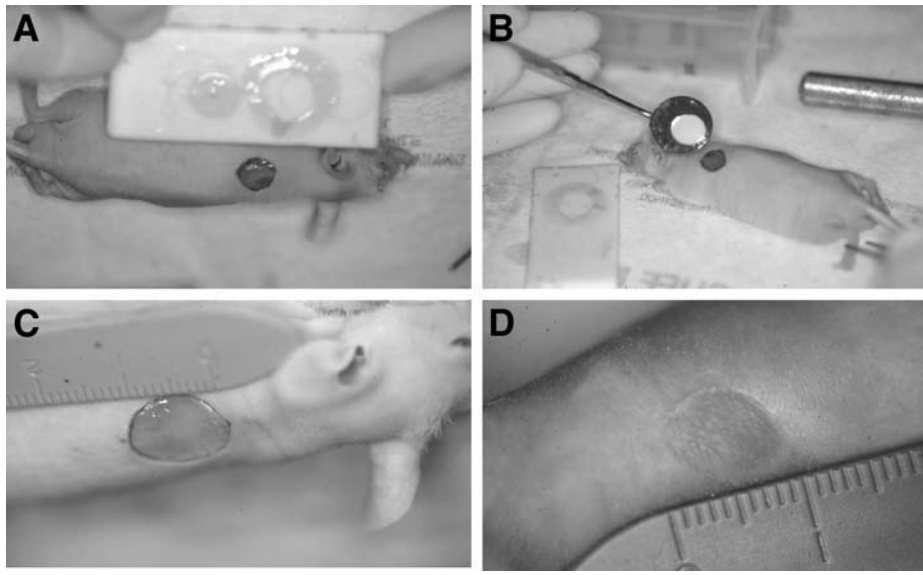


Fig. 1. Transplantation of organotypic cultures to nude mice. **A,** Organotypic cultures are excised from their inserts and placed on a Teflon sheet. A 1.4-cm dermatologic punch is used to prepare a round graft by separating the graft from the insert. **B,** The insert is removed from its polycarbonate membrane by sliding it onto a moist dental mirror. The mirror is then carried to the graft bed and the graft is placed into the site. **C,** A butt joint is formed with adjacent mouse tissue when the graft is placed so that no mouse connective tissue is exposed. **D,** The appearance of a graft 4 wk after transplantation.

thickness, it is necessary to evaluate microscopic features of tissues immediately before grafting. This can be accomplished by growing additional cultures that can be used for examination by frozen section on the day grafts are planned. Morphologic criteria for grafting suitability includes tissues that are greater than five to seven cells in thickness, the presence of a thin stratum corneum and basal cells that are somewhat polarized and cuboidal in shape.

2. An important criteria in the successful transplantation of organotypic cultures is their growth potential at the time of grafting. As a result, it is imperative to optimize conditions to maintain high numbers of dividing cells during the monolayer phase of cell culture to ensure an elevated growth fraction in organotypic cultures. The presence of cells with high replicative potential will greatly increase the success of transplantation.
3. Because all work with nude mice needs to be performed in a sterile field, it is critical to practice sterile technique throughout grafting. It is therefore necessary for grafting to be performed in tandem so that an assistant can prepare all materials that require sterility. It is also helpful to have sentinel mice in the animal facility to ensure the health of the nude mouse colony.
4. The location of the graft bed on the dorsal mouse skin is very important in determining graft take. As described previously, the superior edge of the graft should be in line with the fold of the anterior limbs. This places the graft near a fat pad that is located just superior to this location. In our experience, this fat pad becomes thickened by the time of graft excision, although it remains unclear how it contributes to successful engraftment. In addition, the incision should be centered slightly to the left of the midline when the animal is viewed from behind. This will help avoid a large vein, whose damage might result in bleeding that

is difficult to control. Finally, the size of the incision and the fit of the graft are important in preventing graft rejection. As the graft edge is sensed by the mouse as an open wound, any open space between the graft and the margin of the graft bed will encourage reepithelialization of mouse tissue. To prevent this, it is important that the graft is well-adapted to the margin. This can be accomplished by making sure that the size of the graft bed is slightly smaller than that of the graft.

5. Although organotypic cultures may demonstrate a high degree of morphologic differentiation, they are usually deficient in barrier function at the time of grafting. For this reason, it is important to maintain grafts in a semiocclusive dressing immediately after the grafting period. As described above, this is accomplished by applying vasoline gauze directly to the freshly transplanted tissue and then tightly adapting a bandage to the site. To ensure that the graft is not displaced during the first few days after transplantation, it is important that the bandage be firmly wrapped around the graft. We have found that this also facilitates vascularization of the graft. The bandage should be thought of as a pressure-dressing in addition to providing barrier function.
6. Mice should be inspected daily for 1 wk after grafting to make sure that bandages have neither been displaced or have fallen off. When removing the bandages, use a suture-removal scissor and cut the bandage through the gauze part of the bandage. This makes their removal easier and also allows the bandage to be lifted away from the skin with the scissor so that the skin is not accidentally cut. If grafts are exposed for more than 24 h as a result of a loss of bandages, transepidermal water loss through the graft will result in dessication and loss of transplants. In addition to the establishment of barrier function, vascularization of the transplanted tissue is a second critical event that is required for successful engraftment of organotypic cultures. Typically, endothelial cell ingrowth occurs from the base and the margins of the graft and the degree of vascularization appears to vary from graft to graft. However, it is clear that several days are required for a supportive vascular network to become established. Maintenance of a barrier is essential while this occurs.

References

1 Barrandon, Y., Li, V., and Green, H. (1998) New techniques for the grafting of cultured human epidermal cells onto athymic animals. *J. Invest. Dermatol.* **91,** 315–318.
2 Kolodka, T. M., Garlick, J. A., and Taichman, L. B. (1998) Evidence for keratinocyte stem cells in vitro: long term engraftment and persistence of transgene expression from retrovirus-transduced keratinocytes. *Proc. Natl. Acad. Sci. USA* **95,** 4356–4361.
3 Cooper, M. L., Andree, C., Hansbrough, J. F., Zapata-Sirvent, R. L., and Spielvogel, R. L. (1993) Direct comparison of a cultured composite skin substitute containing human keratinocytes and fibroblasts to an epidermal sheet graft containing human keratinocytes on athymic mice. *J. Invest. Dermatol.* **101,** 811–819.
4 Carver, N., Navsaria, H. A., Fryer, P., Green, C. J., and Leigh, I. M. (1993) Restoration of basement membrane structure in pigs following keratinocyte autografting. *Br. J. Plast. Surg.* **46,** 384–392.
5. Andriani, F., Margulis, A., Lin, N., Griffey, S., and Garlick, J. A. (2003) Analysis of microenvironmental factors contributing to basement membrane assembly and normalized epidermal phenotype. *J. Invest. Dermatol.* **120,** 923–931.

41

Epidermis-Targeted Gene Transfer Using In Vivo Electroporation

Hiroki Maruyama, Jun-Ichi Miyazaki, and Fumitake Gejyo

Summary

The skin is an important target for gene transfer because of its easy accessibility. Recently, we demonstrated that an in vivo electroporation approach could be applied to plasmid deoxyribonucleic acid delivery in rat skin of the abdominal area. Plate-and-fork type electrodes were effective for gene delivery by skin-targeted gene transfer. Eight 12–24 V, 50-m electric pulses were delivered through the electrodes. Skin injection with pCAGGS-*lacZ* showed *lacZ* gene expression in the upper most cell layers (horny, granular, and prickle cell layers) of the epidermis on day 1 and in the subcutaneous muscle layer on day 7 after the pCAGGS-*lacZ* transfer. Slight skin damage as the result of the gene transfer procedure was evident on day 1 but absent by day 7. These results demonstrate that plasmid deoxyribonucleic acid transfer by in vivo electroporation at low voltage is a useful procedure for short-term skin-targeted gene transfer.

Key Words:

Electroporation; gene transfer; skin; plasmid DNA; epidermis; electrodes; CAG promoter.

1. Introduction

Because of its ready accessibility for direct manipulation and clinical monitoring, the skin is an attractive tissue for gene transfer. Direct in vivo plasmid DNA transfer to the skin via injection ***(1,2)*** and topical administration ***(3)*** have been reported. Intradermally injected plasmid DNA rapidly traverses the dermoepidermal junction and is taken up and expressed by keratinocytes in the epidermis ***(4)***. Active transgene expression in the epidermis is lost by 3-d postinjection ***(3)***, a time period that is too short for clinical applications. Moreover, skin takes up and expresses DNA less efficiently than does muscle ***(5)***. Consequently, skin-targeted transfer of plasmid DNA has focused on two principal therapeutic uses: genetic immunization ***(6,7)*** and the expression of biological response modifiers to treat skin disease ***(4)***. Recently, we demonstrated that an in vivo electroporation approach could be applied to plasmid DNA delivery in rat skin of the abdominal area ***(8)***. In this chapter, protocols to transfer plasmid DNA into epidermis using in vivo electroporation will be described in detail.

From: *Methods in Molecular Biology, vol. 289:*
Epidermal Cells: Methods and Protocols
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

2. Materials

2.1. Experimental Animals

1. Rats: We used 9-wk-old male Wistar rats purchased from Charles-River Japan Inc. (Tokyo, Japan). Rats of other ages or strains or mice may be treated similarly.
2. Anesthetic:diethyl ether.
3. Desiccator with lid and porcelaine plate.

2.2. Plasmid DNA

1. Plasmid vector: pCAGGS. The plasmid vector must include an expression unit that is active in skin. We have successfully used the pCAGGS vector ***(9)***. To assess the efficiency of gene transfer, we used pCAGGS-*lacZ* ***(10)***, which were constructed by inserting the *Escherichia coli lacZ* gene into the unique *Eco*RI site between the CAG (cytomegalovirus immediate-early enhancer/chicken β-actin hybrid) promoter and 3′-flanking sequence of the rabbit β-globin gene of pCAGGS. The pCAGGS vector can be provided by Miyazaki Jun-ichi upon request (*see* **Note 1**; e-mail: jimiyaza@nutri.med.osaka-u.ac.jp).
2. Competent cells: The pCAGGS plasmid is based on pUC 13, a high copy number plasmid and is easily grown in *E. coli* DH 5α or other strains.
3. Endofree plasmid Giga kit (Qiagen, Hilden, Germany; cat. no. 12391).
4. Phosphate-buffered saline (PBS), pH 7.4 (Gibco, Invitrogen, Grand Island, NY; cat. no. 10010-031).

2.3. Intradermal DNA Injection and Electroporation

1. Chemical depilatory, Divele (Shiseido Cosmenity, Tokyo, Japan; cat. no. 4901872504701 50470).
2. 27-gauge needle connected to a 1.0-mL capacity syringe (Terumo, Tokyo, Japan; cat. no. SS*01T27139).
3. Plate-and-fork type electrodes, CUY663B (NEPA Gene, Chiba, Japan) consisting of a pair of stainless-steel tweezers, one with a rectangular plate, 10-mm long and 5-mm wide, and the other with a fork consisting of three straight needles at 2.5-mm intervals that are 10-mm long and 0.5 mm in diameter **(Fig. 1A)**.
4. Tube of keratin cream (Fukuda Denshi, Tokyo, Japan; cat. no. OJ-01).
5. Electric pulse generator with square waves, which maintain the constant voltage during the pulse duration, are required in in vivo electroporation. An electric pulse generator, Electro Square Porator T820 (BTX, San Diego, CA) combined with a graphic pulse analyzer, MVC540R (BTX). Another electric pulse generator, CUY21EDIT Square Electroporator (NEPA Gene) equipped with a graphic pulse analyzer **(Fig. 1B)**.

2.4. Assessment of the Efficiency of Gene Transfer

1. Preparations of pCAGGS-*lacZ* using Endofree plasmid Giga kit (Qiagen) at a concentration of 2 μg/μL in PBS.
2. Phosphate buffer (PB) pH 7.4. Mix one volume of 0.1 *M* NaH_2PO_4 (Wako pure chemicals, Osaka, Japan; cat. no. 192-02815) and three volumes of 0.1 *M* Na_2HPO_4 (Wako Pure Chemicals; cat. no. 196-02835) to adjust pH 7.4 of the PB.
3. 4% Paraformaldehyde (Wako Pure Chemicals; cat. no. 160-00515) in PBS.
4. 1 *M* $MgCl_2$ in distilled water, for example, $MgCl_2$ (Wako Pure Chemicals; cat. no. 136-03995) 2.03 g in distilled water to 10 mL. Store at 4°C in the dark.
5. 300 m*M* $K_4Fe(CN)_6$ in distilled water, for example, $K_4Fe(CN)_6 \cdot 3H_2O$ (Wako Pure Chemicals; cat. no. 165-03745) 1.45 g in distilled water to 10 mL. Store at 4°C in the dark.

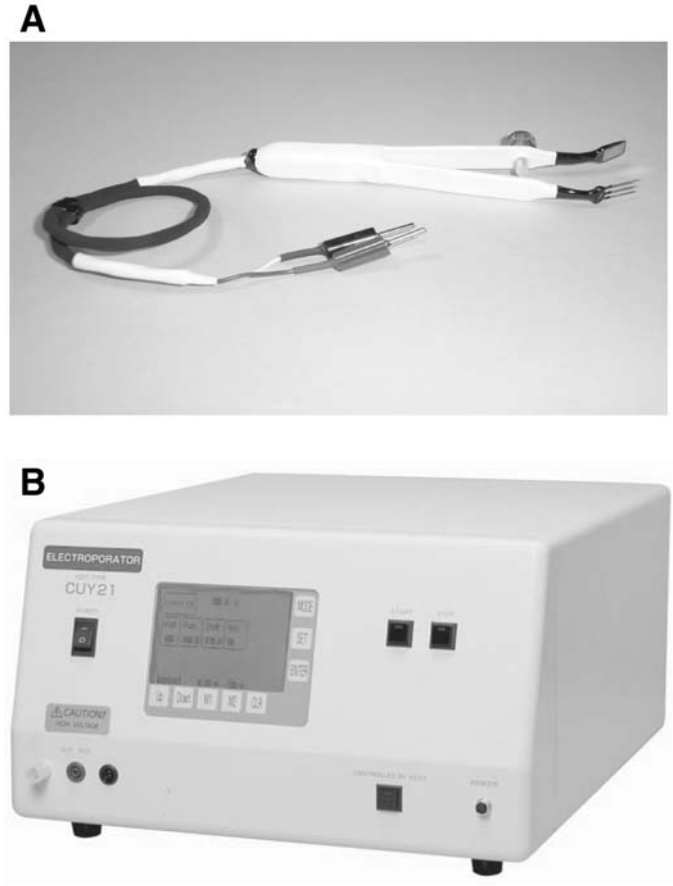


Fig. 1. **(A),** Plate-and-fork type electrodes (CUY663B: NEPA Gene) consist of a pair of stainless-steel tweezers, one with a rectangular plate, 10-mm long and 5-mm wide, and the other with a fork consisting of three straight needles at 2.5-mm intervals, which are 10-mm long and 0.5 mm in diameter. **(B),** Pulse generator (CUY21EDIT Square Electroporator: NEPA Gene).

6. 300 m*M* $K_3Fe(CN)_6$ in distilled water, for example, $K_3Fe(CN)_6$ (Wako Pure Chemicals; cat. no. 161-03725) 0.99 g in distilled water to 10 mL. Store at 4°C in the dark.
7. 10% Igepal CA-630 (Sigma Chemical Co., St. Louis, MO; cat. no. I 3021), which is chemically indistinguishable from Nonidet P-40, in PBS. Store at 4°C in the dark.
8. 40 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal; Takara, Shiga, Japan; cat. no. 9031) in dimethylsulfoxide (Wako Pure Chemicals; cat. no. 046-21981). Store at 4°C in the dark.
9. Tissue-Tek O.C.T. compound (Sakura finetechnical, Tokyo, Japan; cat. no. 06441).
10. Dry ice and acetone.
11. Cryostat, CM 1850 (Leica microsystems Nussloch GmbH, Nussloch, Germany).
12. Slide glasses coated with 3-aminopropyltriethoxysilane (Matsunami glass, Osaka, Japan; cat. no. S-8444).
13. 1.5% Glutaraldehyde (Nacalai tesque, Kyoto, Japan; cat. no. 17003-92) in PB.
14. Nuclear fast red, Kernechtrot stain sol. (Muto pure chemicals, Tokyo, Japan; cat. no. 4087).
15. Mounting medium, malinol (Muto Pure Chemicals; cat. no. 2009).

3. Methods

We describe the method of gene transfer into abdominal skin of rats by in vivo electroporation. It will be necessary to modify this method to use it in skins of other sites or other species.

3.1. Preparation of Plasmid DNA

1. Plasmid DNA is extracted and purified by Endofree plasmid Giga kit (Qiagen; *see* **Note 1**).
2. Dissolve DNA in TE and assess its quantity and quality optical density at 260 and 280 nm. Store the DNA at –20°C.
3. Immediately before injection, dilute the DNA to its final concentration, 2 µg/µL in PBS.

3.2. Intradermal DNA Injection and Electroporation in Vivo

1. Several days before the gene transfer, remove the hair on the abdominal target areas with a chemical depilatory, such as Divele (*see* **Note 2**).
2. Anesthetize rats by diethyl ether in the desiccator with lid and porcelaine plate.
3. Insert the fork electrode into the skin parallel to the surface.
4. Intradermally inject the DNA (100 µg/each site) with a 27-gauge needle connected to a 1.0-mL capacity syringe into the central area of the skin above the electrode (*see* **Note 3**).
5. Squeeze keratin cream out of the cylinder tube, which is approx 5-mm long and 5 mm in diameter. Coat the rectangular plate electrode with the keratin cream to reduce the electrical resistance of the skin. Then, sandwich the skin between the rectangular plate and the fork electrodes (*see* **Note 4**). Both the rectangular plate electrode and the skin are covered with the keratin cream. The extra cream does not disturb the following electroporation procedure.
6. Confirm that the correct insertion into or pinch on the skin was achieved by measuring the electrical resistance between the two electrodes, which is generally below 300 Ω.
7. Deliver electric pulses using the electric pulse generator and monitor with a graphic pulse analyzer as described previously ***(8)***. Four 50-ms pulses followed by another four pulses of the opposite polarity should be administered to each injection site at a rate of 1 pulse/s.

3.3. Assessment of the Efficiency of Gene Transfer

Before introducing the gene of interest by electroporation, it is important to test the effectiveness of experimental procedures by a reporter gene. To this end, we use a plasmid carrying a reporter gene, pCAGGS-*lacZ*, that expresses β-galactosidase.

3.3.1. LacZ Gene Transfer

1. Inject the skin of anesthetized rats with 100 µg of pCAGGS-*lacZ* at a concentration of 2 µg/µL in PBS and deliver electric pulses at 12 V or 18 V as described in **Subheading 3.2.**
2. The following day or day 7 after the injection, sacrifice the rats by diethyl ether in a desiccator with a lid and porcelaine plate.

3.3.2. β-Galactosidase Staining

X-gal staining should be performed according to the methods as described previously ***(8,11)***.

1. Harvest the skins for X-gal staining, embed them in Tissue-Tek O.C.T. compound, and freeze them in a mixture of dry ice and acetone.
2. Cut serial sections (5-µm thick) with a cryostat and place them on glass slides coated with 3-amino-propyltriethoxysilane.

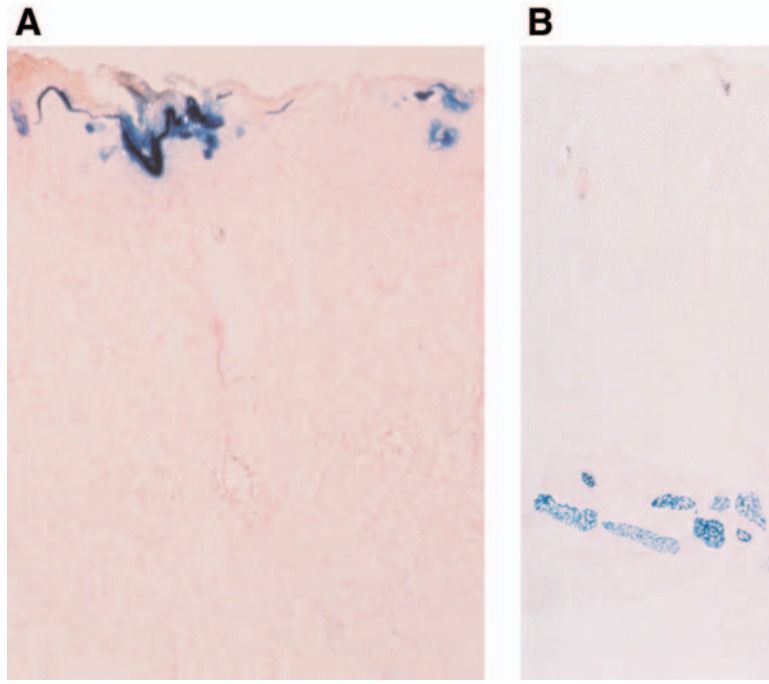


Fig. 2. β-Galactosidase expression on d 1 **(A)** and d 7 **(B)** after the pCAGGS-*lacZ* transfer with electroporation at 18 V. β-Galactosidase was expressed in the upper most cell layers (horny, granular, and prickle cell layers) of the epidermis on d 1 **(A)**, and in the subcutaneous muscle layer on d 7 **(B)**. Magnification: **(A)**, ×250; **(B)**, ×70.

3. Fix the sections in 1.5% glutaraldehyde in PB, pH 7.4, at room temperature for 10 min, and then wash them three times in cold PBS (5 min/wash; *see* **Note 5**).
4. Incubate in X-gal staining solution containing 1 mg/mL X-gal, 2 m*M* $MgCl_2$, 5 m*M* $K_4Fe(CN)_6$, 5 m*M* $K_3Fe(CN)_6$, and 0.5% Igepal CA-630 in PBS, at 37°C for 3 h. X-gal staining solution:
   a. 60 mL
   b. 1 *M* $MgCl_2$ $MgCl_2$, 120 µL
   c. 300 m*M* $K_4Fe(CN)_6$, 1002 µL
   d. 300 m*M* $K_3Fe(CN)_6$, 1002 µL
   e. 10% Igepal CA-630 in PBS, 3 mL
   f. 40 mg/mL X-gal in dimethylsulfoxide, 1.5 mL
   g. Distilled water to 60 mL.
5. Counterstain them with nuclear fast red at room temperature for 4 min, dry the sections for 1 h, and clear three times in xylene. Affix the cover slips with mounting one drop of medium.
6. Observe the sections with a light microscope. β-Galactosidase should be expressed in the uppermost cell layers (horny, granular, and prickle cell layers) of the epidermis on d 1 **(Fig. 2A)** and in the subcutaneous muscle layer on d 7 **(Fig. 2B)**.

4. Notes

1. The endotoxin-free plasmid DNA should be prepared. Contaminating endotoxin may cause local immunologic reactions, which may lead to the early loss of gene expression or affect the experimental results. Endofree plasmid kits (Qiagen; cat. no. 12362, 12381, 12391) are recommended.

2. To avoid the possibility of skin damage caused by the chemical depilatory, we recommend that you remove the hair on the target areas several days before the gene transfer.
3. Before the actual experiments, we recommend that you confirm that you are able to reproducibly inject the skin of anesthetized rats using some kind of dye, for example, Trypan blue.
4. Plate-and-fork type electrodes (CUY663B: NEPA Gene) can cover a relatively small area (50 mm^2), and the injection volume of DNA solution should not exceed 50 µL.
5. It is important for X-gal staining to avoid background staining attributable to the endogenous β-galactosidase. In all but a few specialized eukaryotic cells, lysosomal enzymes that are active only under acid conditions catalyze hydrolysis of β-galactosidic linkages and inactive at the neutral pH. However, *E. coli* β-galactosidase is active at the neutral pH ***(12)***. Therefore, we use PBS, pH 7.4, to assay for *lacZ*.

Acknowledgments

The authors are grateful to Y. Hayakawa of the NEPA Gene Co. Ltd., and Y. Imada of the Unique Medical Imada Co. Ltd., for technical assistance.

References

1 Hengge, U. R, Chan E. F., Foster, R. A., Walker, P. S., and Vogel, J. C. (1995) Cytokine gene expression in epidermis with biological effects following injection of naked DNA. *Nat. Genet.* **10,** 161–166.
2 Hengge, U. R., Walker, P. S., and Vogel, J. C. (1996) Expression of naked DNA in human, pig, and mouse skin. *J. Clin. Invest.* **97,** 2911–2916.
3 Li, L. and Hoffmann, R. M. (1995) The feasibility of targeted selective gene therapy of the hair follicle. *Nat. Med.* **1,** 705–706.
4. Vogel, J. C. (1999) A direct in vivo approach for skin gene therapy. *P. Assoc. Am. Phys.* **111,** 190–197.
5 Wolff, J. A., Malone, R. W., Williams, P., Chong, W., Acsadi, G., Jani, A., et al. (1990) Direct gene transfer into mouse muscle in vivo. *Science* **247,** 1465–1468.
6 Raz, E., Carson, D. A., Parker, S. E., Parr, T. B., Abai, A. M., Aichinger, G., et al. (1994) Intradermal gene immunization: the possible role of DNA uptake in the induction of cellular immunity to viruses. *Proc. Natl. Acad. Sci. USA* **91,** 9519–9523.
7 Fan, H., Lin, Q., Morrissey, G. R., and Khavari, P. A. (1998) Immunization via hair follicles by topical application of naked DNA to normal skin. *Nat. Biotechnol.* **17,** 870–872.
8 Maruyama, H., Ataka, K., Higuchi, N., Sakamoto, F., Gejyo, F., and Miyazaki, J. (2001) Skin-targeted gene transfer using in-vivo electroporation. *Gene Ther.* **8,** 1808–1812.
9 Niwa, H., Yamamura, K., and Miyazaki, J. (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108,** 193–199.
10 Aihara, H. and Miyazaki, J. (1998) Gene transfer into muscle by electroporation in vivo. *Nat. Biotechnol.* **16,** 867–870.
11 Maruyama, H., Higuchi, N., Nishikawa, U., Hirahara, H., Iino, N., Kameda, S., et al. (2002) Kidney-targeted naked DNA transfer by retrograde renal vein injection in rats. *Hum. Gene Ther.* **13,** 455–468.
12. Sambrook, J. and Russell, D. W. (2001) β-galactosidase, in *Molecular Cloning: A Laboratory Manual*, *3rd ed., vol. 3* (Argentine, J., ed.), Cold Spring Harbor Laboratory Press, New York, pp. 17.97–17.99.

42

Gene and Stem Cell Therapy of the Hair Follicle

Robert M. Hoffman

Summary

The hair follicle is a highly complex appendage of the skin containing a multiplicity of cell types. The follicle undergoes constant cycling through the life of the organism including growth and resorption with growth dependent on specific stem cells. The targeting of the follicle by genes and stem cells to change its properties, in particular, the nature of the hair shaft is discussed. Hair follicle delivery systems are described such as liposomes and viral vectors for gene therapy. The nature of the hair follicle stem cells is discussed, in particular, its pluripotency.

Key Words:

Hair follicles; liposomes; adenovirus; genes; stem cells.

1. Introduction

The hair follicle is a complex skin appendage consisting of six concentric cylinders with several distinct cell types that produce highly specialized proteins **(Fig. 1)**. Follicle function is regulated at least in part by the adjacent mesenchymal dermal papilla **(Fig. 1)**. The hair follicle continuously cycles through three major stages: anagen is the hair growth phase, catagen the involution phase, and telogen the resting phase. All three are regulated by specific molecular mechanisms, and thus the follicle offers many potential therapeutic targets ***(1)***.

1.1. Feasibility of Targeted Selective Gene Therapy of the Hair Follicle (2)

Specific therapy of the hair follicle depends on selective targeting of the appendage. We have developed a histoculture method of intact hair-growing skin on sponge-gel matrices (**refs. *3–8***; US Patents 5,849,579 and 6,399,380; Europe Patent 573,606; Japan Patent 2,950,519). We have previously found in histocultured skin that liposomes can selectively target hair follicles and deliver both small and large molecules (**refs. *5–8***; US Patents 5,641,508, 5,753,263, 5,914,126, 5,965,157, 6,224,901, 6,261,776, 6,733,776; Canada Patent 2,159,626; Europe Patent 692,972; Japan Patent 2,950,520). That liposomes can selectively target the hair follicle for delivery has been confirmed in other laboratories ***(9)***. Twenty-six years ago, we introduced the technique of entrapping deoxyribonucleic acid (DNA) in liposomes for use in gene therapy ***(10)***. We used

From: *Methods in Molecular Biology, vol. 289: Epidermal Cells: Methods and Protocols*
Edited by: K. Turksen © Humana Press Inc., Totowa, NJ

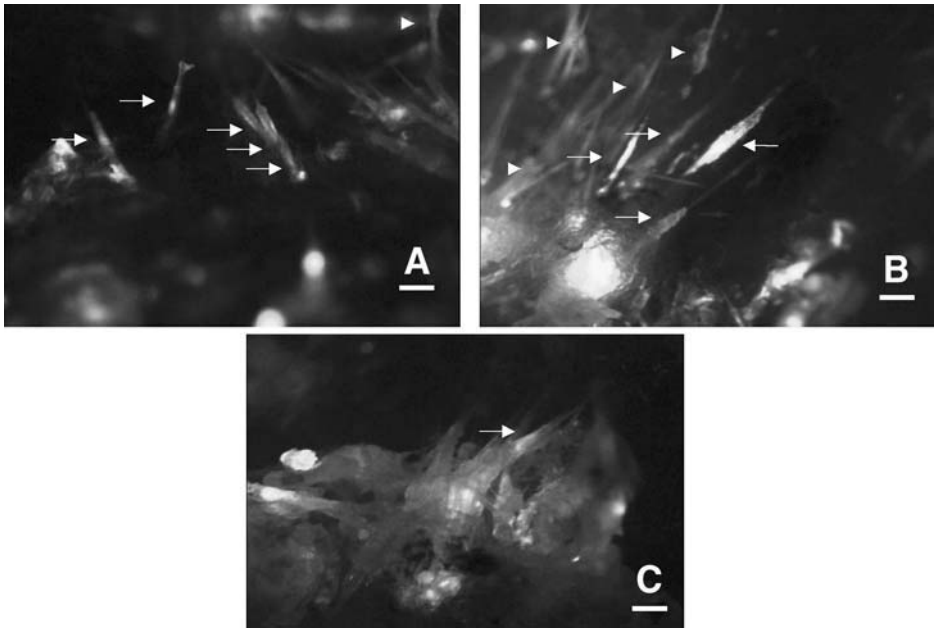


Fig. 1. GFP visualization in hair shafts of adenoviral–GFP-transduced grafted skin. **(A),** Eight days after grafting. Magnification ×32. Bar: 3 mm. (**B** and **C**)**,** Twelve days after grafting. **(B),** Magnification ×80. Bar: 1 mm. **(C),** Magnification ×63. Bar: 1.5 mm. **(A–C),** The view is from the surface. Arrows indicate GFP-positive areas. Arrowheads indicate GFP-negative (autofluorescent) areas. See color insert following p. 238.

DNA liposomes to selectively target the *lacZ* reporter gene to the hair follicles in mice after topical application of the gene entrapped in liposomes. These results demonstrated that highly selective, safe gene therapy for the hair process is feasible.

After topical application of liposome-*lacZ*, expression of the *lacZ* gene was indicated by blue staining of the X-gal substrate in the hair-forming hair matrix cells in the hair follicle bulbs and in the bulge area below the opening of the sebaceous gland ***(2)***. The bulge area is thought to contain the follicle stem cells ***(11,12)***. The transfection frequency was high because many follicles were stained. No other cells were transfected with *lacZ* outside the follicle in the dermis or epidermis. Extensive *lacZ* expression in the hair matrix cells was shown. Topical application of the naked *lacZ* gene did not result in gene transfer, and no *lacZ* staining was seen in follicles in animals not treated with lipsome-*lacZ*.

These results demonstrated that genes can be selectively targeted to the most important cells of the hair follicle by liposomes, which was the most selective targeting of a gene observed thus far in vivo ***(2)***. This selectivity of gene targeting by topical liposome application suggested the feasibility of targeting hair matrix cells and possibly follicle stem cells to potentially restore hair color by delivery and expression of the tyrosinase gene ***(13,14)*** and with genes to restore hair growth. The highly selective nature of the topical application of liposome gene targeting lends itself to the development of practical and safe procedures.

1.2. Efficient Delivery of Transgenes to Human Hair Follicle Cells in Anagen (15)

Human scalp hair follicles are large and are generally in the anagen stage, whereas mouse follicles are small and are predominantly in the telogen stage ***(15,16)***. Domashenko et al. ***(17)*** showed efficient transfection of human follicle cells after topical application of liposome-entrapped *lacZ* in mice as well as in a human scalp xenograft model. These authors found that liposome composition, timing of liposome application to the onset of a new hair cycle, and pretreatment with depilation and retinoic acid were important for transfection efficiency. They found that liposome application during anagen onset resulted in selective transfection of human hair follicle matrix cells. Depilation of the hair and application of retinoic acid to the grafts before liposome application markedly increases the transfection efficiency.

pFx-1, composed of a 1 : 1 mixture of two cationic lipids, was the best preparation found by Domashenko et al. ***(17)***. These authors detected expression of β-galactosidase (β-gal) in the hair follicles of mice transfected only on the first, second, or third day after depilation. During the first 3 d after depilation, hair follicles are in the earliest phases of the anagen stage ***(18)***. The majority of β-gal-expressing cells were matrix keratinocytes located at the leading edge of the epithelial downgrowth at anagen onset ***(17)***. No activity was evident in the epidermis or dermis. β-gal activity was detected for up to 4 d after the last transfection. These authors ***(17)*** noted that at anagen onset, progenitor cells within the hair follicle are proliferating and they are accessible, probably because the follicle lacks an inner root sheath, which normally prevents ingress of material from the environment later in anagen.

To address whether human hair follicles can be transfected with DNA using topically applied liposomes, Domashenko et al. ***(17)*** used a human scalp xenograft model in immunodeficient mice ***(19,20)***. The majority of follicles were in anagen, producing hair shafts. The authors pretreated xenografts with depilation and/or retinoic acid to increase follicles in the beginning of anagen and then applied pFx-1 liposome-containing β-gal. Transfected cells were found only in follicles at anagen onset. Cells positive for β-gal were located at the leading edge of the epithelial downgrowth, in the matrix keratinocytes. Transfected cells were predominantly keratinocytes. No other cells within the hair follicle, sebaceous gland, or epidermis expressed β-gal. The dermis and subcutaneous fat were also negative for β-gal. These results showed that the early anagen follicle in human scalp can selectively express a transgene after topical introduction with liposomes (*see* **Note 1**).

1.3. Specific Genes Affecting Hair Growth (1)

Alexeev et al. used a chimeric RNA–DNA oligonucleotide to correct the albino point mutation in the mouse tyrosinase gene ***(21)***. Chimeric oligonucleotides correct point mutations for homologous recombination and mismatch DNA repair. In this case, the correction restored tyrosinase activity and concomitant melanin synthesis in a heritable manner, and this resulted in the production of pigmented hair shafts in albino mice. The localized gene correction was maintained for at least 3 mo, suggesting that melanocyte precursors may have been converted. The chimeric oligonucleitude was delivered in liposomes as well as by intradermal injection in mice. When the fluorescently labeled chimeric oligonucleotide was topically applied in a liposome prepara-

tion that can penetrate the stratum cornium, fluorescence was detected mainly in the hair follicles and epidermis. Intradermal injection resulted in more efficient, but less specific delivery; fluorescence was detected in the dermis as well as the hair follicles. The low efficiency observed may be a problem for cosmetically successful restoration of hair pigment, since a minimum number of melanocytes located deep in the hair bulb must be converted to color a single hair shaft. Because partial restoration will not give desired results, in vivo genetic pigment restoration will be a difficult task given the present efficiencies.

Gene therapy targeting of hair growth, however, may not be as demanding, because even partial restoration could potentially give satisfactory results. In this line, a recent study by Sato et al. ***(22)*** showed that the sonic hedgehog *(shh)* gene, delivered with an adenovirus vector, stimulated anagen development and hair shaft production in the C57BL/6 mouse. The human hairless gene, which is responsible for alopecia universalis, has also been cloned ***(23)***, providing another in what is probably a multitude of potentially therapeutic genes.

1.4. Ex Vivo Approaches to Increasing the Efficacy of Gene Modification of the Hair Follicle

Ex vivo approaches allow close to 100% gene transfer ***(24,25)*** and can be applied to the hair follicle. This takes on added importance in light of the recent discovery that hair follicle dermal sheath cells taken from the scalp of an adult human male could form new dermal papilli and hair follicles that produce hair shafts when transplanted in the skin of an unrelated female ***(25)***. This type of transplantation takes advantage of the so-called immune privilege of hair follicle cells that prevents their rejection, and the possible totipotency or inductive properties of the follicle dermal sheath cells ***(25)***. Follicle formation by transplantation of follicle dermal sheath cells and by other hair transplantation procedures now used in the clinic or under development give rise to many possibilities of genetically altering hair follicles ex vivo for subsequent reimplantation or even transplantation to individuals other than the donor.

We have recently described a highly efficient genetic modification technique for hair follicles. It enables high transgene expression in growing hair shafts in genetically modified mouse skin grafted onto nude mice ***(26)***. Mouse anagen skin fragments, maintained in histoculture, were genetically modified at high efficiency with adenoviral-green fluorescent protein (GFP). The histocultured skin fragments were treated with collagenase, which made hair follicles accessible to the adenoviral–GFP gene, allowing high-efficiency transduction. These skin fragments were subsequently grafted onto nude mice, where GFP was readily visualized in as many as 75% of hair follicles ***(26)***. GFP fluorescence was visualized in the root sheath cells and matrix cells of the hair bulb. Most follicles produced GFP-fluorescent growing hair shafts. GFP expression was predominantly in hair follicles and occurred to a much lesser extent in the upper epidermis and dermal fibroblasts. This novel technique has produced, for the first time, efficient genetic modification of the hair shaft.

The transduced GFP gene continued expression in hair follicles that were maintained in implanted skin. Reverse transcription polymerase chain reaction (RT-PCR) analysis detected GFP-specific mRNA in vivo after grafting the GFP-containing skin into nude mice.

The ex vivo delivery system targeted both the epidermis and dermis in the floating collagenase-treated skin histocultures. This can explain, at least in part, the high-efficiency of gene delivery achieved. The high efficiency was also the result of collagenase treatment, which exposed the follicles more completely to GFP adenovirus. This ex vivo approach is the first to demonstrate expression of a delivered transgene in large numbers of growing hair shafts, which were seen after re-implantation in vivo (*see* **Note 1**).

1.5. The Hair Follicle Genetically Modified to Produce Therapeutic Proteins (1)

The hair follicle has a high capacity for manufacturing proteins, a characteristic that could be exploited by in vivo and ex vivo gene therapy to produce molecules other than those involved directly in hair shaft production and pigmentation. In a recent study, topical application of both naked and liposome-entrapped plasmid vectors for the hepatitis surface antigen (HbsAg) resulted in antigen-specific immune responses *(27)*. This topical vaccine gene therapy depended on the presence of normal hair follicles, suggesting that targeting and manufacture of the gene product occurs in the follicles. One can envisage many applications for such hair follicle factories.

1.6. Visualization and Isolation of Hair Follicle Stem Cells (28)

The intermediate filament protein, nestin uniquely marks progenitor cells of the central nervous system (CNS). Such CNS stem cells were selectively labeled by placing GFP under the control of the nestin regulatory sequences. During early anagen or growth phase of the hair follicle, nestin-expressing cells, marked by GFP fluorescence in nestin-GFP transgenic mice, appear in the permanent upper hair follicle immediately below the sebaceous glands in the follicle bulge. This is where stem cells for the hair follicle outer root sheath are thought to be located. The relatively small, oval shaped, nestin-expressing cells in the bulge area surround the hair shaft and are interconnected by short dendrites. The precise locations of the nestin-expressing cells in the hair follicle vary with the hair-cycle (**Fig. 2**). During telogen or resting phase and in early anagen, the GFP-positive cells are mainly in the bulge area. However, in mid- and late anagen, the GFP-expressing cells are located in the upper outer root sheath as well as in the bulge area but not in the hair matrix bulb. These observations show that the nestin-expressing cells form the outer-root sheath. Results of the immunohistochemical staining showed that nestin, GFP, keratin 5/8, and keratin 15 colocalize in the hair follicle bulge cells, outer root sheath cells, and basal cells of the sebaceous glands. These data indicate that nestin-expressing cells, marked by GFP, in the hair follicle bulge are indeed progenitors of the follicle outer root sheath. The expression of the unique protein, nestin, in both neural stem cells and hair follicle stem cells suggests a possible relation (**ref.** ***28***; *see* **Note 2**). GFP expression enables specific isolation of the hair follicle stem cells, which can then be targeted for gene therapy.

2. Materials

2.1. Hair Follicle Gene Therapy

1. *LacZ* gene (pM-MuLV-SV-*lacZ*; obtained from Joshua R. Sanes of Washington University, St. Louis, MO).

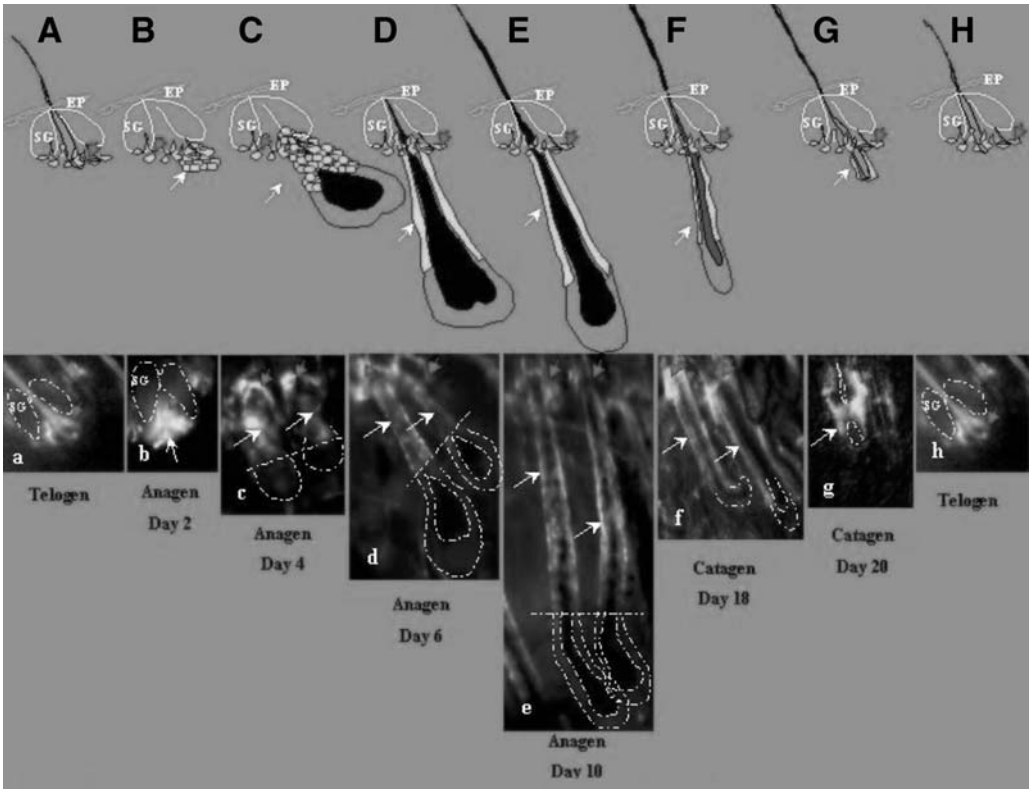


Fig. 2. Hair follicle stem cells in hair growth cycle *(28)*. Top, illustration showing position of nestin–GFP stem cells at each stage of hair follicle cycle. Bottom, Nestin–GFP-expressing hair follicle stem cells (red arrow) located in the hair follicle bulge area in telogen phase **(A)**; d 2 after anagen induction by depilation. Note the new hair follicle cells (white arrow) formed directly from the bulge nestin-GFP-expressing stem cells **(B)**; d 4 **(C)**, d 6 **(D)**, and d 10 **(E)** after anagen induction by depilation. Note the nestin–GFP-expressing outer root sheath cells (white arrows) in the upper two-thirds of the hair follicle d 18 **(F)** and d 20 **(G)** after depilation. Note that the hair follicles are in the catagen phase and are undergoing regression and degeneration, including the nestin–GFP-expressing cells in the outer root sheath. The bulge area nestin–GFP-expressing stem cells remain. Hair follicles are cycling to another telogen phase **(H)**. See color insert following p. 238.

2. Promega Wizard™ Megaprep DNA purification system (Promega Corporation, Madison, WI; cat. no. A7300).
3. Phosphatidylcholine:cholesterol:phosphatidylethanolamine (Avanti Polar Lipids, Alabaster, AL).
4. BALB/c mice (Harlan Sprague Dawley, San Diego, CA; cat. no. BALB/c).
5. X-gal staining solution (Promega Corporation, Madison, WI; cat. no. V3941).
6. GFP-containing adenovirus vector, pQBI-AdCMV5GFP (available from Qbiogene Inc., Montreal, Quebec, Canada; cat. no. AES0525).
7. C57BL/10 (8 wk old; available from Charles River Laboratories, Wilmington, MA; cat. no. C57BL/10).
8. C57BL/6-Tyr<c-2J>/+ mice (8 wk old; available from Charles River Laboratories, Wilmington, MA; cat. no. C57BL/6-Tyr<c-2J>/+).

9. CD-1 nude mice (7 wk old; available from Charles River Laboratories, Wilmington, MA; cat. no. CD-1 nude).
10. Ketamine (50 mg; available from Burns Veterinary Supply, Vancouver, WA; cat. no. Ketaset).
11. RPMI 1640 containing 10% fetal bovine serum (FBS) (available from Pittsburgh, PA; cat. no. 10-040-CV).
12. Type I collagenase (available from Sigma-Aldrich, St. Louis, MO; cat. no. C0130).
13. Nikon fluorescent microscope (available from Nikon, Tokyo, Japan).
14. Leica fluorescence stereo microscope model LZ12 (available from Leica Inc., Deerfield, IL; cat. no. LZ12).
15. Long-pass filter GG475 (available from Chroma Technology, Brattleboro, VT; cat. no. GG475).
16. C5810 3-chip cooled color CCD camera (available from Hamamatsu Photonics Systems, Bridgewater, NJ; cat. no. C5810).
17. Leica CM1850 Cryostat (available from Hacker Instruments, Inc., Fairfield, NJ; cat. no. CM1850).
18. Glass slides (available from Fisher Scientific, Pittsburgh, PA; cat. no. 12-550-12).
19. Tri Reagent (available from Sigma-Aldrich, St. Louis, MO; cat. no. T9424).
20. AMV reverse transcriptase (available from Stratagene, San Diego, CA; cat. no. 600081).

2.2. Visualization of GFP-Expressing Hair Follicle

1. EGFP transgenic mice carrying enhanced green fluorescent protein (EGFP) under the control of the nestin second-intron enhancer (obtained from Grigori N. Enikolopov, Ph.D., Cold Spring Harbor Laboratory, enik@cshl.org).
2. Leica CM1850 Cryostat (available from Leica Inc., Deerfield, IL; cat. no. CM1850).
3. MRC-600 confocal imaging system (available from Bio-Rad, Hercules, CA; MRC-600).
4. DAKO ARK animal research kit (nestin and keratins; available from DAKO, Carpinteria, CA; cat. no. K3954 [15 mL] or K3955 [110 mL]).
5. DAKO EnVision Doublestain System (available from DAKO, Carpinteria, CA; cat. no. K1395).
6. Nestin MAb (Rat 401; available from DSHB at the University of Iowa, Iowa City, IA, cat. no. RAT401).
7. Keratin 5/8 MAb (MAB3228; available from Chemicon International, Temecula, CA; cat. no. MAB3228).
8. Keratin 15 MAb (CBL 272; available from Chemicon International, Temecula, CA; cat. no. CBL272).

2.3. Solutions

X-gal staining solution: contains 1 mg/mL X-gal, 5 m*M* potassium ferricyanide, 5 m*M* potassium ferrocyanide, and 2 m*M* $MgCl_2$ in phosphate-buffered saline (PBS).

3. Methods

3.1. Hair Follicle Gene Therapy Methods

3.1.1. Purification of LacZ DNA (2)

1. A recombinant retrovirus containing the *lacZ* gene (pM-MuLV-SV-*lacZ*) was obtained from Joshua R. Sanes (Washington University, St. Louis, MO).
2. The plasmid *lacZ* was transformed to HB101 *Escherichia coli* competent cells (Promega Corporation) using standard procedures.

3. The plasmid *lacZ* DNA was purified using the Promega Wizard™ Megaprep DNA purification system.

3.1.2. Preparation of LacZ-Liposomes (2)

1. A total of 20 mg of lipid in a ratio of 5:3:2 of phosphatidylcholine:cholesterol: phosphatidylethanolamine were rotary evaporated for 1 h with a vacuum drier from a chloroform solution, to form a thin film on the wall of a 5-mL round-bottomed flask.
2. The dried thin film lipid was suspended in 0.6-mL Tris-ethylene diamine tetraacetic acid (TE) buffer containing approx 1 mg of *lacZ* DNA on a vortex mixer.
3. The *lacZ* was entrapped in liposomes by sonication in a compact water bath sonicator for 20 min followed by freezing (at –70°C) and thawing (at room temperature) three times.

3.1.3. Topical Application of LacZ Liposomes (2)

1. Preshaved 5- to 6-wk-old BALB/c mice were used.
2. The skin area for application of liposome-*lacZ* was prehydrated with PBS for 10–30 min.
3. Liposome-*lacZ* formulation (50 µL) was placed directly on the skin with reapplication after 1 h. Untreated mice and mice treated with naked *lacZ* DNA were used as controls.
4. The skin was carefully cleaned by 70% isopropyl alcohol before harvest for X-gal staining 3 d after application of the liposome–*lacZ* formulation.

3.1.4. Detection of LacZ DNA Expression by X-gal Staining (2)

1. The harvested skin samples were immediately put into a modified Eagle's medium wash containing a combination of antibiotics at 4°C for 1 h.
2. The skin was fixed in 2% (vol/vol) formaldehyde-0.2% (vol/vol) glutaraldehyde in PBS for 30 min at 4°C.
3. Tissues were then rinsed with PBS three times and incubated in the X-gal staining solution at 37°C for 18 h.
4. Skin tissues were processed for paraffin sectioning by standard histological procedures and photographed under light microscopy after counter-staining with 0.1% nuclear fast red.

3.1.5. Adenovirus GFP Vector (26)

The GFP-containing adenovirus vector, pQBI-AdCMV5GFP was purchased from Qbiogene Inc. (Montreal, Quebec, Canada).

3.1.6. Mice (26)

1. C57BL/10 and C57BL/6-Tyr<c-2J>/+ mice (8 wk old) were used as skin donors.
2. CD-1 nude mice (7 wk old) were used as skin graft recipients.

3.1.7. Induction of Anagen (26)

1. Telogen mice (judged by their pink skin color) were anesthetized (50 mg ketamine [Burns Veterinary Supply, Vancouver, WA] per kg body weight).
2. The dorsal area (3 × 5 cm) of the mice was depilated with hair removal wax to induce a synchronized anagen phase.

3.1.8. Skin Histoculture and Collagenase Treatment (26)

1. Animals were sacrificed by cervical dislocation on the sixth day after depilation.
2. The back skin was dissected at the level of the subcuitis.
3. Subcutaneous tissue was removed and the skin was rinsed in calcium- and magnesium-free phosphate buffered saline (CMF-PBS, pH 7.4).

4. The skin samples were cut into small pieces (1 mm × 1 mm ~ 2 cm × 2 cm).
5. A fraction of the specimens was directly used as untreated controls cultured in RPMI 1640 containing 10% FBS.
6. The remainder of the specimens was incubated in a 2 mg/mL type I collagenase (Sigma-Aldrich, St. Louis, MO) solution in medium from 45 min to 3 h 45 min at 37°C and rinsed in CMF-PBS.

3.1.9. GFP Transduction of Hair Follicles in Histocultured Skin **(26)**

1. The collagenase-treated skin histocultures were infected with pQBI-AdCMV5GFP at 2.4×10^6 to 5×10^9 plaque-forming units (pfu) per milliliter of culture medium.
2. The skin histocultures were incubated in fresh RPMI 1640 (10% FBS) at 37°C for 1–6 h.

3.1.10. Grafting of GFP-Transduced Skin Histocultures **(26)**

1. To visualize the expression of the transgene in vivo, histocultured skin was grafted to nude mice or C57BL/10 mice after viral GFP transduction. Histocultured specimens were grafted within 24 h of harvest. Grafting surgery was performed in a laminar-flow hood using sterile procedures.
2. Mice were anesthetized with ketamine.
3. 1- × 1-cm pieces of skin were grafted to a bed of similar size that was prepared by removing recipient mouse skin down to the fascia. Skin grafts were fixed in place with 6-O non-absorbable monofilament sutures.

3.1.11. Fluorescence Microscopy of GFP-Transduced Hair Follicles and Shafts **(26)**

1. A Nikon (Tokyo, Japan) fluorescent microscope and a Leica fluorescence stereo microscope model LZ12 (Leica Inc., Deerfield, IL) equipped with a mercury 50-W lamp power supply were used.
2. Emitted fluorescence was collected through a long-pass filter GG475 (Chroma Technology, Brattleboro, VT) on a Hamamatsu C5810 3-chip cooled color CCD camera (Hamamatsu Photonics Systems, Bridgewater, NJ).

3.1.12. Quantification of GFP-Transduced Hair Follicles **(26)**

1. The number of hair follicles and GFP-positive hair follicles was determined under bright-field microscopy and fluorescence microscopy. The calculations were based on the average number of hairs from five randomly chosen microscopic fields covering an area of 0.581 mm^2 (one field of ×200 magnification).
2. At least 500 hairs per group were counted to generate the percentage of GFP-positive hair follicles.
3. Hair follicles in which GFP was visualized anywhere in the hair bulb or shaft were scored as GFP positive.

3.1.13. Isolation of Hair Follicles and Dermal Papillae From GFP-Transduced Histocultured and Grafted Skin **(26)**

1. After viral GFP transduction, hair follicles were isolated from histocultured skin and grafted skin at several time points to determine the location of GFP expression.
2. Pieces of histocultured skin or skin grafts were incubated in a 2 mg/mL type I collagenase solution in culture medium for 2 h at 37°C and rinsed in culture medium to release hair follicles.

3.1.14. Preparation of Skin Sample for Histological and Cytological Studies (26)

1. After viral GFP transduction, skin pieces were taken at several time points for histological study to determine the location of GFP expression.
2. Pieces of histocultured skin or skin grafts were stored at –80°C.
3. Frozen specimens were sectioned on a Leica CM1850 cryostat (Hacker Instruments, Inc., Fairfield, NJ) and collected onto glass slides (Fisher Scientific, Pittsburgh, PA).

3.1.15. Isolation of RNA From Histocultured Skin and RT-PCR (26)

1. Skin samples (100 mg) were homogenized in 1 mL of Tri Reagent (Sigma-Aldrich, St. Louis, MO) to extract RNA.
2. For RT-PCR, approx 10 µg of RNA was reversely transcribed to first cDNA chains.
3. Reverse transcription was conducted in 20 µL first-strand buffer, 500 µ*M* of each dNTP, and 20 U of AMV reverse transcriptase (Strategene, San Diego, CA). The primer for the first strand was pGFP antisense.
4. Incubation was at 42°C for 50 min.
5. The products of the reverse transcription were then amplified by the PCR. Mouse β-actin mRNA was used as the standard. The sequence of the GFP upstream primer was 5′-ATG GCT AGC AAA GGA GAA GAA CT-3′. The downstream primer was 5′-TCA GTT GTA CAG TTC ATC ACT G-3′.
6. The PCR conditions for both GFP and β-actin were as follows: first denaturation at 97°C for 30 s; annealing at 55°C for 30 s; and extension at 72°C for 45 s; then a final extension at 72°C for 10 min.

3.2. Visualization of GFP-Expressing Hair Follicles (28)

3.2.1. Nestin-GFP Transgenic Mice (28)

Nestin is an intermediate filament gene that is a marker for central nervous system (CNS) progenitor cells and neuroepithelial stem cells. EGFP transgenic mice carrying EGFP under the control of the nestin second-intron enhancer are used. Hair follicle stem cells also strongly express nestin as evidenced by nestin-regulated EGFP expression.

3.2.2. Induction of Anagen (28)

1. Nestin-regulated GFP transgenic mice, 6-8 wk old, in the telogen phase of hair growth, were depilated by a hot mixture of rosin and beeswax.
2. Samples (5×5 mm^2) were excised from the dorsal skin right before depilation (telogen) and at days 1, 2, 3, 4, 5 (early anagen); days 8 and 10 (middle anagen); days 14 and 15 (late anagen); and days 19 and 20 (catagen) after depilation.
3. The skin samples were divided into two parts, one for fluorescence microscopy and the other for frozen sections.
4. Briefly, the skin samples were embedded in tissue freezing embedding medium and frozen at –80°C overnight.
5. Sections (8-µm thick) were cut with a Leica CM1850 cryostat.
6. The frozen sections were air-dried and counterstained with propidium iodide for fluorescence microscopy.

3.2.3. Fluorescence and Confocal Microscopy (28)

After subcutaneous tissue was dissected, the nestin–GFP skin samples were directly observed with dermis up and epidermis down under a Nikon-fluorescent microscope

equipped with GFP optics. An MRC-600 confocal imaging system (Bio-Rad), mounted on a Nikon Optiphot using a ×10 PlanApo objective, was also used.

3.2.4. Immunohistochemical Staining **(28)**

1. Colocalization of nestin, keratin (5, 8, and 15) and GFP in the paraffin-embedded C57Bl6 mouse and nestin-GFP transgenic mouse skin sections was detected with DAKO ARK animal research kit (nestin and keratins) and DAKO EnVision Doublestain System, following the manufacturer's instruction manual.
2. The activity of endogenous peroxidase in the skin samples was quenched with incubation in a peroxidase blocking solution for 5 min.
3. Slides were then incubated with the prepared biotinylated primary antibody (GFP MAb 1 : 100, nestin MAb 1 : 80, keratin 5/8 MAb 1 : 250, and keratin 15 MAb 1 : 100) for 15 min, followed by incubation with strepavidin-peroxidase for 15 min.
4. The staining was completed by incubation with substrate–chromogen DAB (3,3′-diaminobenzidine) or nuclear fast red for 5 min. Brown (DAB) or cherry-red (nuclear fast red) staining was used for antigen staining. Nestin MAb (Rat 401) was purchased from DSHB at the University of Iowa, Iowa City, IA. Keratin 5/8 MAb (MAB3228) and keratin 15 MAb (CBL 272) were purchased from Chemicon International, Temecula, CA.

4. Notes

1. The location and structure of the hair follicle offers unique therapeutic opportunities. As described previously, we and others have shown that the follicle can be selectively targeted with functional genes. Two experimental strategies have been used: topical application of liposome-entrapped DNA and ex vivo targeting with adenoviral gene vectors. These highly specific methods for targeting the hair follicle offer the possibility of screening for genes that can correct defects in the hair follicle such as alopecia or loss of pigment, as well as take advantage of the synthetic capacity of the hair follicle to manufacture therapeutic proteins for systemic diseases ***(2,26)***.
2. With the use of the nestin-GFP transgenic mice, we were able to visualize the hair follicle stem cells in real time. This capability gives rise to the possibility of selectively targeting the hair follicle stem cells for gene therapy, as well as the possibility of using the stem cells themselves therapeutically for the growth of new hair follicles ***(28)***.

References

1 Hoffman, R. M. (2000) The hair follicle as a gene therapy target. *Nat. Biotechnol.* **18,** 20–21.
2 Li, L. and Hoffman, R. M. (1995) The feasibility of targeted selective gene therapy of the hair follicle. *Nat. Med.* **1,** 705–706.
3 Li, L., Margolis, L. B., and Hoffman, R. M. (1991) Skin toxicity determined in vitro by three-dimensional, native-state histoculture. *Proc. Natl. Acad. Sci. USA* **88,** 1908–1912.
4 Li, L., Margolis, L. B., Paus, R., and Hoffman, R. M. (1992) Hair shaft elongation, follicle growth, and spontaneous regression in long-term gelatin sponge-supported histoculture of human scalp skin. *Proc. Natl. Acad. Sci. USA* **89,** 8764–8768.
5 Li, L., Margolis, L. B., Lishko, V. K., and Hoffman, R. M. (1992) Product-delivering liposomes specifically target hair follicles in histocultured intact skin. *In Vitro Cell Dev. Biol.* **28A,** 679–681.
6. Li, L., Lishko, V. K., and Hoffman, R. M. (1993) Liposomes can specifically target entrapped melanin to hair follicles in histocultured skin. *In Vitro Cell Dev. Biol.* **29A,** 192–194.
7. Li, L., Lishko, V. K., and Hoffman, R. M. (1993) Liposome targeting of high molecular weight DNA to the hair follicles of histocultured skin: a model for gene therapy of the hair growth processes. *In Vitro Cell Dev. Biol.* **29A,** 258–260.

8. Li, L., Lishko, V., and Hoffman, R. M. (1994) High efficiency liposome-mediated transfection of the tyrosinase gene to cultured cells: a model for the gene therapy of hair color restoration. *In Vitro Cell Dev. Biol.* **30A,** 135–138.
9 Yarosh, D., Buana, C., Cox, P., Alas, L., Kibitel, J., Kripke, M. (1994) Localization of liposomes containing a DNA repair enzyme in murine skin. *J. Invest. Dermatol.* **103,** 461–468.
10 Hoffman, R. M., Margolis, L. B., and Bergelson, L. D. (1978) Binding and entrapment of high molecular weight DNA by lecithin liposomes. *FEBS Lett.* **93,** 365–368.
11 Cotsarelis, G., Sun, T. T., and Lavker, R. M. (1990) Label-retaining cells reside in the bulge area of pilosebaceous unit: Implication for follicular stem cells, hair cycle and skin carcinogenesis. *Cell* **61,** 1329–1337.
12 Huelsken, J., Vogel, R., Erdmann, B., Cotsarelis, G., and Birchmeier, W. (2001) β-Catenin controls hair follicle morphogenesis and stem cell differentiation in the skin. *Cell* **105,** 533–545.
13. Shibahara, S., Tomita, Y., Tagami, H., Muller, R. M., and Cohen, T. (1988) Molecular basis for the heterogeneity of human tyrosinase. *J. Exp. Med.* **156,** 403–414.
14 Tanaka, S., Yamamoto, H., Takeuchi, S., and Takeuchi, T. (1990) Melanization in albino mice transformed by introducing cloned mouse tyrosinase gene. *Development* **108,** 223–227.
15 Cotsarelis, G. (1997) The hair follicle: dying for attention. *Am. J. Pathol.* **151,** 1505–1509.
16 Paus, R. and Cotsarelis, G. (1999) The biology of hair follicles. Review. *N. Engl. J. Med.* **341,** 491–497.
17 Domashenko, A., Gupta, S., and Cotsarelis, G. (2000) Efficient delivery of transgenes to human hair follicle progenitor cells using topical lipoplex. *Nat. Biotechnol.* **18,** 420–423.
18 Chase, H. (1954) Growth of the hair. *Physiol. Rev.* **34,** 113–126.
19 Gilhar, A., Ullmann, Y., Berkutzki, T., Assy, B., and Kalish, R. S. (1998) Autoimmune hair loss (alopecia areata) transferred by T lymphocytes to human scalp explants on SCID mice. *J. Clin. Invest.* **101,** 62–67.
20 Wrone-Smith, T. and Nickoloff, B. J. (1996) Dermal injection of immunocytes induces psoriasis. *J. Clin. Invest.* **98,** 1878–1887.
21 Alexeev, V., Igoucheva, O., Domashenko, A., Cotsarelis, G., and Yoon, K. (2000) Localized in vivo genotypic and phenotypic correction of the albino mutation in skin by RNA-DNA oligonucleotide. *Nat. Biotechnol.* **18,** 43–47.
22 Sato, N., Leopold, P. L., and Crystal, R. G. (1999) Induction of the hair growth phase in postnatal mice by localized transient expression of Sonic hedgehog. *J. Clin. Invest.* **104,** 855–864.
23 Ahmad, W., Faiyaz ul Haque, M., Brancolini, V., Tsou, H. C., ul Haque, S., Lam, H., et al. (1998) Alopecia universalis associated with a mutation in the human hairless gene. *Science* **279,** 720–724.
24 Choate, K. A., Medalie, D. A., Morgan, J. R., and Khavari, P. A. (1996) Corrective gene transfer in the human skin disorder lamellar ichthyosis. *Nat. Med.* **2,** 1263–1267.
25 Reynolds, A. J., Lawrence, C., Cserhalmi-Friedman, P. B., Christiano, A. M., and Jahoda, C. A. (1999) Trans-gender induction of hair follicles. *Nature* **402,** 33–34.
26. Saito, N., Zhao, M., Li, L., Baranov, E., Yang, M., Ohta, Y., et al. (2002) High efficiency genetic modification of hair follicles and growing hair shafts. *Proc. Natl. Acad. Sci. USA* **99,** 13,120–13,124.
27 Fan, H., Lin, Q., Morrissey, G. R., and Khavari, P. A. (1999) Immunization via hair follicles by topical application of naked DNA to normal skin. *Nat. Biotechnol.* **17,** 870–872.
28. Li, L., Mignone, J., Yang, M., Matic, M., Penman, S., Enikolopov, G., et al. (2003) Nestin expression in hair follicle sheath progenitor cells. *Proc. Natl. Acad. Sci. USA* **100,** 9958–9961.

Index

G