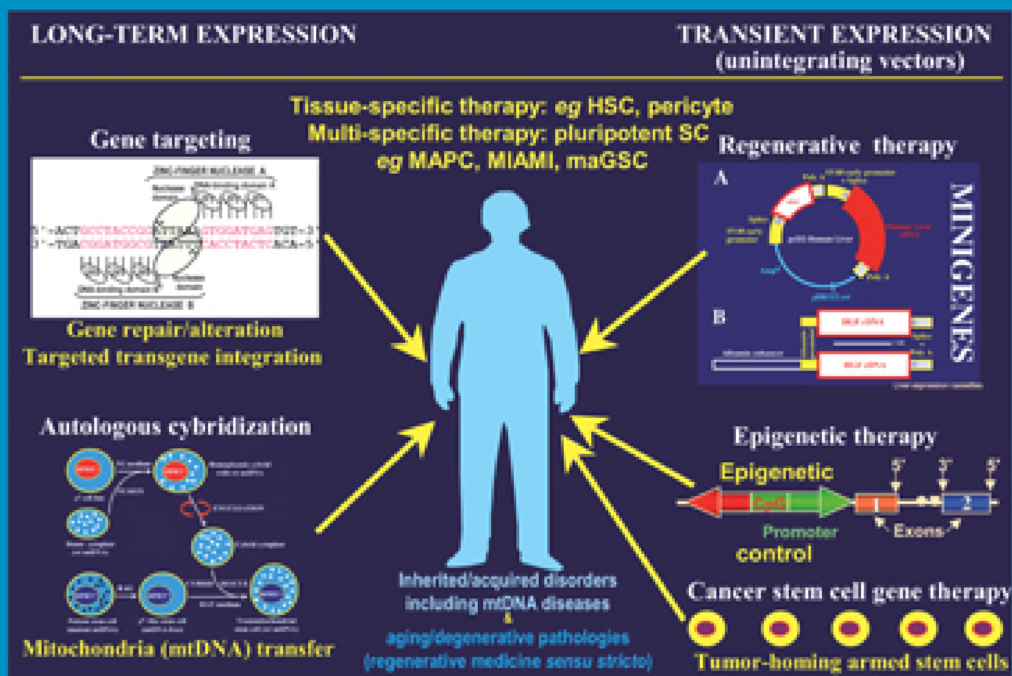
干细胞之家www.stemcell8.cn ←点击进入

Progress in Gene Therapy - Vol. 3

AUTOLOGOUS AND CANCER STEM CELL GENE THERAPY

Editors
Roger BERTOLOTTI
Keiya OZAWA



World Scientific
www.worldscientific.com
Singapore · New Jersey · London
Hong Kong · Taipei · Banglore

干细胞之家www.stemcell8.cn ←点击进入

Progress in Gene Therapy — Vol. 3

AUTOLOGOUS AND CANCER STEM CELL GENE THERAPY

干细胞之家www.stemcell8.cn ←点击进入

This page intentionally left blank

干细胞之家www.stemcell8.cn ←点击进入

Progress in Gene Therapy — Vol. 3

AUTOLOGOUS AND CANCER STEM CELL GENE THERAPY

Editors

Roger BERTOLOTTI
University of Nice–Sophia Antipolis, France

Keiya OZAWA
Jichi Medical University, Japan

NEW JERSEY • LONDON • SINGAPORE • BEIJING • SHANGHAI • HONG KONG • TAIPEI • CHENNAI

干细胞之家www.stemcell8.cn ←点击进入

Published by

World Scientific Publishing Co. Pte. Ltd.
5 Toh Tuck Link, Singapore 596224
USA office: 27 Warren Street, Suite 401-402, Hackensack, NJ 07601
UK office: 57 Shelton Street, Covent Garden, London WC2H 9HE

Library of Congress Cataloging-in-Publication Data
Autologous and cancer stem cell gene therapy / edited by Roger Bertolotti, Keiya Ozawa.
p. ; cm. -- (Progress in gene therapy ; v. 3)
Includes bibliographical references and index.
ISBN-13: 978-981-277-586-3 (hardcover : alk. paper)
ISBN-10: 981-277-586-2 (hardcover : alk. paper)
1. Stem cells--Therapeutic use. 2. Stem cells--Transplantation. 3. Gene therapy.
4. Cancer--Gene therapy. 5. Autotransplantation. I. Bertolotti, R. (Roger) II. Ozawa, Keiya, 1953- III. Series.
[DNLM: 1. Gene Therapy. 2. Stem Cells. 3. Regenerative Medicine. 4. Stem Cell Transplantation. 5. Transplantation, Autologous. 6. Tumor Stem Cells. QZ 325 A939 2007]
QH588.S83A98 2007
615.8'95--dc22

2007043710

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library.

Copyright © 2008 by World Scientific Publishing Co. Pte. Ltd.

All rights reserved. This book, or parts thereof, may not be reproduced in any form or by any means, electronic or mechanical, including photocopying, recording or any information storage and retrieval system now known or to be invented, without written permission from the Publisher.

For photocopying of material in this volume, please pay a copying fee through the Copyright Clearance Center, Inc., 222 Rosewood Drive, Danvers, MA 01923, USA. In this case permission to photocopy is not required from the publisher.

Typeset by Stallion Press
Email: enquiries@stallionpress.com

Printed in Singapore.

干细胞之家www.stemcell8.cn ←点击进入

Preface

Adult/Postnatal stem cells provide for life-long cell replacement in tissues and organs. They have an inherent ability for self-renewal and differentiation into multiple cell types that are characteristic of their tissue of origin, and have been identified in an increasing number of tissues/organs even in brain and myocardium in which cell turnover was reputedly absent. In addition, many stem cells have inherent homing abilities that are instrumental in therapeutic applications. Stem cells are also the driving force of cancer, where genetic/epigenetic alterations culminate in uncontrolled self-renewal and tumorigenesis either in tissue stem cells or in some of their progenitor/differentiated derivatives. As a rare subset of the tumor, cancer stem cells are the only drive of tumor initiation/propagation and, upon transplantation, have been shown to recapitulate the hierarchical clonogenic differentiating/differentiated organization of the original tumor cell population. Stem cells are thus the key targets of 1) long-term gene therapy and broad/synergistic transient regenerative/epigenetic gene therapy for both inherited diseases and acquired/aging disorders on their autologous side, and 2) effective anti-cancer therapy on their dark side.

Autologous stem cells have been instrumental in the first unequivocal successes for gene therapy (2000–2004), whereby *ex vivo* retrovirally corrected hematopoietic stem cells have been returned to the patients. Such a stem cell gene therapy achievement that relies on random integration of therapeutic transgenes into host chromosomes is presented together with emerging experimental approaches aimed at eliminating random integration oncogenic hazards through site-specific integration or gene targeting. Breakthrough endonuclease-boosted gene targeting for gene correction (inherited diseases) or targeted integration of therapeutic transgene (other pathologies), culminating in an efficiency compatible with clinical correction of a disease gene, is one of the highlights of the book. Other highlights include the pioneering transplantation of adult pluripotent stem cells as a substitute for tissue-specific stem cells, thereby pinpointing the breakthrough potential of such autologous cells able to contribute to all three

干细胞之家www.stemcell8.cn ←点击进入

germ layers both for multi-systemic diseases and for the development of a universal stem cell gene therapy platform. The autologous side is thus discussed in terms of magnifying stem cell therapeutic homing/regenerative capabilities through transient regenerative gene therapy, and in terms of tackling most pathologies (including mitochondrial DNA diseases and aging disorders) through stem cell repopulation dynamics into appropriate niches (long-term engraftment) and tissues (cell turnover). Regarding the dark side, focus is on both the increasing number of identified tissue-specific cancer stem cells as the ultimate targets for recurrence-free cancer therapy and on the development of armed stem cells as tumor-homing vectors for targeted anti-cancer stem cell gene therapy.

Roger Bertolotti
Keiya Ozawa

干细胞之家www.stemcell8.cn ←点击进入

Contents

Preface v
Roger Bertolotti & Keiya Ozawa

List of Contributors xi

Introduction

Toward a Universal Platform for Autologous Stem Cell Gene Therapy xxi
Roger Bertolotti

Long-term Stem Cell Gene Therapy: From Current Clinical Random–Integration Achievements to Stem Cell Gene Targeting/Cybridization

I. From pioneering clinical retroviral gene therapy to experimental site-specific integrative gene therapy

1 Stem Cell Gene Therapy for ADA-Deficiency without Myelopreparative Conditioning 1
Makoto Otsu and Tadashi Ariga

2 Targeted Insertion of Transgene into a Specific Site on Chromosome 19 by Using Adeno-Associated Virus Integration Machinery 19
Masashi Urabe, Yoko Obara, Takayuki Ito, Hiroaki Mizukami, Akihiro Kume and Keiya Ozawa

3 Herpes Simplex Virus Type 1 / Adeno-Associated Virus Hybrids as Site-Specific Integrating Vectors 47
Cornel Fraefel, Daniel L. Glauser, Thomas Heister and Mathias Ackermann

干细胞之家www.stemcell8.cn ←点击进入

II. Stem cell gene targeting/cybridization: toward endonuclease-boosted gene repair/alteration, custom site-specific integrative gene therapy and transmitochondrial therapy/rejuvenation

4 Endonuclease-Boosted Gene Targeting and Cybridization for Long-Term Stem Cell Gene Therapy 81
Roger Bertolotti

5 Cell-Free Protein-Evolution Systems for Engineering of Novel Sequence-Specific DNA-Binding and -Modifying Activities 115
Armin Sepp, Farid Ghadessy and Yen Choo

6 Advances in Engineering Homing Endonucleases for Gene Targeting: Ten Years After Structures 135
Barry L. Stoddard, Andrew M. Scharenberg and Raymond J. Monnat, Jr

7 Gene Targeting Mediated by Helper-dependent Adenoviral Vectors 169
Kohnosuke Mitani

Adult Pluripotent Stem Cells: Emerging Stem Cell/Gene Therapy Breakthrough

8 Multipotent Progenitor Cells: A New Era in Stem Cell-Mediated Gene Therapy? 187
Marta Serafini and Catherine M. Verfaillie

Cancer Stem Cell Gene Therapy

I. Cancer stem cells as breakthrough targets of cancer gene therapy

9 Leukemia Stem Cells, A Pioneering Paradigmatic Model 203
Daniel J. Pearce and Dominique Bonnet

10 Mammary Stem Cells 261
Mark Shackleton, Marie-Liesse Asselin-Labat, François Vaillant, Geoffrey J. Lindeman and Jane E. Visvader

干细胞之家www.stemcell8.cn ←点击进入

11 Human Brain Tumor Stem Cells 299
Sara G. M. Piccirillo and Angelo L. Vescovi

12 CD44 as a Functional Cancer Stem Cell Marker and a Potential Therapeutic Target 317
Lubna Patrawala and Dean G. Tang

II. Armed stem cells as tumor-homing vectors for cancer gene therapy

13 Neural Stem Cell-mediated Therapy of Primary and Metastatic Solid Tumors 335
Joseph Najbauer, Mary K. Danks, Nils-Ole Schmidt, Seung U. Kim and Karen S. Aboody

14 Antitumor Activity of Adenovirally Transduced $CD34^+$ Cells Expressing Membrane-bound TRAIL 373
Carmelo Carlo-Stella, Cristiana Lavazza, Antonino Carbone and Alessandro M. Gianni

15 Mesenchymal Stem Cells as Vehicles for Delivering Therapeutics and Oncolytic Viruses 393
Larisa Pereboeva and Justin Roth

Index 445

干细胞之家www.stemcell8.cn ←点击进入

This page intentionally left blank

干细胞之家www.stemcell8.cn ←点击进入

List of Contributors

Karen S. Aboody
Division of Hematology/Hematopoietic
Cell Transplantation, City of Hope
National Medical Center and
Beckman Research Institute
Duarte, CA, USA
and
Division of Neurosciences
City of Hope National
Medical Center and Beckman Research Institute
Duarte, CA, USA

Mathias Ackermann
Institute of Virology
University of Zurich
Switzerland

Tadashi Ariga
Department of Pediatrics
Hokkaido University Graduate School of Medicine
Sapporo, 060-8638
Hokkaido, Japan

Marie-Liesse Asselin-Labat
The Walter and Eliza Hall Institute of Medical Research
Parkville
Victoria 3050, Australia

干细胞之家www.stemcell8.cn ←点击进入

Roger Bertolotti
CNRS, Gene Therapy and Regulation Research
Faculty of Medicine
University of Nice Sophia Antipolis
06107 Nice, France

Dominique Bonnet
Hematopoietic Stem Cell Laboratory
Cancer Research UK
London Research Institute
London, WC2A 3PX, UK

Antonino Carbone
Department of Pathology
Fondazione IRCCS Istituto Nazionale Tumori
Milano, Italy

Carmelo Carlo-Stella
"Cristina Gandini" Medical Oncology Unit
Fondazione IRCCS Istituto Nazionale Tumori
Milano, Italy
and
Medical Oncology
University of Milano
Milano, Italy

Yen Choo
Plasticell Ltd
Imperial Bioincubator
Bessemer Building (RSM)
Prince Consort Road
South Kensington
London SW7 2BP, UK

干细胞之家www.stemcell8.cn ←点击进入

Mary K. Danks
Department of Molecular Pharmacology
St. Jude Children's Research Hospital
Memphis, TN, USA

Cornel Fraefel
Institute of Virology
University of Zurich
Switzerland

Farid Ghadessy
Institute of Molecular and Cell Biology
61 Biopolis Drive
Singapore 138673

Alessandro M. Gianni
"Cristina Gandini" Medical Oncology Unit
Fondazione IRCCS Istituto Nazionale Tumori
Milano, Italy
and
Medical Oncology
University of Milano
Milano, Italy

Daniel L. Glauser
Institute of Virology
University of Zurich
Switzerland

Thomas Heister
Institute of Virology
University of Zurich
Switzerland

干细胞之家www.stemcell8.cn ←点击进入

Takayuki Ito
Division of Genetic Therapeutics
Center for Molecular Medicine
Jichi Medical University
Tochigi 329-0498, Japan

Seung U. Kim
Division of Neurology
UBC Hospital
University of British Columbia
Vancouver, Canada
and
Institute for Regenerative Medicine
Gachon University
Inchon, Korea

Akihiro Kume
Division of Genetic Therapeutics
Center for Molecular Medicine
Jichi Medical University
Tochigi 329-0498, Japan

Cristiana Lavazza
"Cristina Gandini" Medical Oncology Unit
Fondazione IRCCS Istituto Nazionale Tumori
Milano, Italy
and
Medical Oncology
University of Milano
Milano, Italy

Geoffrey J. Lindeman
The Walter and Eliza Hall Institute of Medical Research
Parkville
Victoria 3050, Australia

干细胞之家www.stemcell8.cn ←点击进入

Kohnosuke Mitani
Gene Therapy Division
Research Center for Genomic Medicine
Saitama Medical University
Hidaka, Saitama 350-1241, Japan

Hiroaki Mizukami
Division of Genetic Therapeutics
Center for Molecular Medicine
Jichi Medical University
Tochigi 329-0498, Japan

Raymond J Monnat, Jr
Departments of Pathology and Genome Sciences
University of Washington
Seattle WA 98195, USA

Joseph Najbauer
Division of Hematology/Hematopoietic
Cell Transplantation, City of Hope
National Medical Center and
Beckman Research Institute
Duarte, CA, USA

Yoko Obara
Division of Genetic Therapeutics
Center for Molecular Medicine
Jichi Medical University
Tochigi 329-0498, Japan

Makoto Otsu
Laboratory of Stem Cell Therapy
Center for Experimental Medicine
Institute of Medical Science
University of Tokyo
108-8639 Tokyo, Japan

干细胞之家www.stemcell8.cn ←点击进入

Keiya Ozawa
Division of Genetic Therapeutics
Center for Molecular Medicine
Jichi Medical University
Tochigi 329-0498, Japan

Lubna Patrawala
Department of Carcinogenesis
The University of Texas MD Anderson Cancer Center
Science Park–Research Division
Smithville, TX 78957, USA

Daniel J. Pearce
Hematopoietic Stem Cell Laboratory
Cancer Research UK
London Research Institute
London, WC2A 3PX, UK

Larisa Pereboeva
Division of Human Gene Therapy
Departments of Medicine, Obstetrics and Gynecology,
Pathology, and Surgery and the Gene Therapy Center
University of Alabama at Birmingham
Birmingham, AL 35294-2172, USA
and
Division of Molecular and Cellular Pathology
Department of Pathology
University of Alabama at Birmingham
Birmingham, AL 35294, USA

Sara G. M. Piccirillo
Department of Biotechnology and Biosciences
University of Milan-Bicocca
Milan, Italy

干细胞之家www.stemcell8.cn ←点击进入

Justin Roth
Division of Molecular and Cellular Pathology
Department of Pathology
University of Alabama at Birmingham
Birmingham, AL 35294, USA

Andrew M Scharenberg
Department of Pediatrics
University of Washington
Seattle WA 98195, USA
and
Children's Hospital and Regional
Medical Center
Seattle WA 98109, USA

Nils-Ole Schmidt
Department of Neurosurgery
University Medical Center Hamburg-Eppendorf
Hamburg, Germany

Armin Sepp
43 Bullen Close
Cambridge CB1 8YU, UK

Marta Serafini
Centro Ricerca M. Tettamanti
Clinica Pediatrica Università Milano-Bicocca
Ospedale San Gerardo
Monza, Italy

干细胞之家www.stemcell8.cn ←点击进入

Mark Shackleton
Life Sciences Institute
University of Michigan
Ann Arbor, MI, USA
and
The Walter and Eliza Hall Institute of Medical Research
Parkville
Victoria 3050, Australia

Barry L Stoddard
Division of Basic Sciences
Fred Hutchinson Cancer Research Center
Seattle WA 981089, USA

Dean G. Tang
Department of Carcinogenesis
The University of Texas MD Anderson Cancer Center
Science Park — Research Division
Smithville, TX 78957, USA
and
Program in Environmental & Molecular Carcinogenesis
Graduate School of Biomedical Sciences (GSBS)
Houston, TX 77030, USA

Masashi Urabe
Division of Genetic Therapeutics
Center for Molecular Medicine
Jichi Medical University
Tochigi 329-0498, Japan

François Vaillant
The Walter and Eliza Hall Institute of Medical Research
Parkville
Victoria 3050, Australia

干细胞之家www.stemcell8.cn ←点击进入

Catherine M. Verfaillie
Stamcelinstituut Leuven
Katholieke Universiteit Leuven
Leuven, Belgium

Angelo L. Vescovi
Department of Biotechnology and Biosciences
University of Milan-Bicocca, Milan, Italy
and
StemGen Spa, Milan, Italy

Jane E. Visvader
The Walter and Eliza Hall Institute of Medical Research
Parkville
Victoria 3050, Australia

干细胞之家www.stemcell8.cn ←点击进入

This page intentionally left blank

干细胞之家www.stemcell8.cn ←点击进入

Introduction

Toward a Universal Platform for Autologous Stem Cell Gene Therapy

Roger Bertolotti*

Adult/Postnatal stem cells provide for life-long cell replacement in tissues and organs. They have an inherent ability for self-renewal and differentiation into multiple cell types that are characteristic of their tissue of origin, and have been identified in an increasing number of tissues/organs even in brain and myocardium in which cell turnover was reputedly absent. They are thus the obvious targets of both long-term and transient-regenerative gene therapy, whereby they either provide for life-long transgenic cell turnover (e.g., gene-corrected hematopoietic stem cells in SCID patients) or are mobilized/recruited in a regenerative process (e.g., vasculogenesis in patients incurring critical limb ischemia). In both cases, their inherent homing ability is essential to their therapeutic repopulation dynamics into appropriate niches (long-term engraftment) and tissues (cell turnover), culminating in synergistic combinations aimed at magnifying their homing/regenerative/differentiative potential. Importantly enough, the homing ability of relevant stem/progenitor cells is a unique opportunity to substitute stem cell-mediated delivery for transient topical gene therapy both for regenerative medicine (e.g., aforementioned vasculogenesis) and for cancer therapy. Indeed, as "never-healing wounds", tumors mobilize neovascularizing and connective-mesenchymal stem cells that are indispensable to their growth, thereby prompting stem cell-mediated tumor-targeted gene therapy protocols for maximized efficiency and minimized off-target side effects. Unexpectedly, neural stem/progenitor cells have been found to have such a tumor-homing propensity that they are currently used to track disseminated metastatic cells. Therapeutic homing is thus the driving force of our autologous stem cell gene therapy platform that comprises 1) a long-term gene therapy branch that relies on endonuclease-boosted gene targeting and cybridization, and 2) a transient regenerative/epigenetic/cancer gene therapy branch. Endonuclease-boosted gene targeting and *ex vivo* cybridization are true genomic homeostatic tools for gene repair/alteration (nuclear inherited diseases) and whole wild-type mitochondrial genome (mtDNA) transfer (mtDNA diseases including aging disorders),

*Corresponding author. Roger.Bertolotti@unice.fr
CNRS, Gene Therapy and Regulation Research, Faculty of Medicine, University of Nice Sophia Antipolis, 06107 Nice, France

干细胞之家www.stemcell8.cn ←点击进入

respectively, thereby eliminating random-integration hazards (dysregulations and adverse oncogenic events) that hamper current clinical gene therapy trials. The same holds true for emerging custom site-specific integrative gene therapy mediated by endonuclease-boosted gene targeting (mtDNA-independent acquired and degenerative/aging disorders). Transient regenerative gene therapy is a critical arm of our platform, since it is aimed both at driving regenerative medicine *sensu stricto* (wound healing and aging/degenerative pathologies) and at synergizing long-term gene therapy, thereby magnifying the repopulating/regenerative ability of transfected/transduced stem cells and cybrids (inherited/acquired disorders including mtDNA diseases). The transient epigenetic and cancer gene therapy arms are discussed in terms of 1) potential long-term inactivation/activation of endogenous genes through transient expression of promoter-specific siRNA and 2) breakthrough targeting of cancer stem cells for recurrence-free cancer therapy, respectively. Importantly enough, emerging adult/postnatal pluripotent stem cells that have an extensive/clonogenic *ex vivo* growth potential and are able to differentiate into cells of the three germ lineages (pluripotency) are presented as the ultimate autologous drive of our universal stem cell gene therapy platform, culminating in strategies aimed at selective *ex vivo* amplification of engineered stem cells and cybrids and at tackling both tissue-specific and multisystemic pathologies/regenerative needs.

Keywords: Autologous pluripotent stem cells; cybrid stem cells; cancer stem cells; tissue- and tumor-homing; endonuclease-boosted gene targeting; gene repair/alteration; targeted transgene integration; long-term and transient gene therapy.

1. Therapeutic Homing as the Very Drive of Both Long-term and Transient-Regenerative Stem Cell Gene Therapy

Adult/Postnatal stem cells have both self-renewing and differentiative capabilities, thereby providing for life-long cell replacement in tissues and organs. In addition, many stem cells have inherent homing abilities that are instrumental in therapeutic applications (see below). They are thus the obvious target of both transient-regenerative and long-term gene therapy (see Bertolotti, 2001, 2003a and 2003b).

As illustrated by the first unequivocal successes for gene therapy, the selective engraftment and repopulating abilities of *ex vivo* gene-corrected hematopoietic stem cells (HSCs) have been instrumental in the clinical outcome of patients with X-linked severe combined immunodeficiency (X-SCID) and adenosine deaminase (ADA)-deficient SCID (Cavazzana-Calvo *et al.*, 2000; Aiuti *et al.*, 2002; Gaspar *et al.*, 2004). In these trials, efficient *ex vivo* retroviral transduction of bone marrow (BM) HSCs has been synergized to the BM niche-homing ability of these transduced stem cells and to the selective growth advantage of their lymphoid progenitor derivatives over mutant SCID cognates. The self-renewing and differentiative capabilities of engrafted gene-corrected stem cells thus secure long-term transgenic

干细胞之家www.stemcell8.cn ←点击进入

lymphoid cell turnover (Hacein-Bey-Abina *et al.*, 2002; see Bertolotti, 2003a and 2003b) and, like in conventional BM transplantations, are expected to result in a life-long cure for these inherited diseases, thereby establishing the very paradigm of life-long therapeutic gene expression mediated by targeted autologous stem cells.

Stem cells are also the targets of transient gene therapy protocols, where they are mobilized and recruited in a regenerative process such as the formation of new blood vessels (see Bertolotti, 2001, 2002 and 2003c). Indeed, pioneering vascular endothelial growth factor (VEGF) gene therapy trials with topical transgene expression drove Isner and co-workers to the identification of circulating endothelial progenitor cells (EPCs or angioblasts) in peripheral blood (Asahara *et al.*, 1997) and to the demonstration that neovascularization in adult ischemic tissue is not restricted to angiogenesis (sprouting of endothelial cells [ECs] from pre-existing vessels; Folkman, 1971) but also involves vasculogenesis (Asahara *et al.*, 1997), where mobilization of BM EPCs (Takahashi *et al.*, 1999; Asahara *et al.*, 1999a) is increased by VEGF gene therapy (Asahara *et al.*, 1999b; Kalka *et al.*, 2000a and 2000c), culminating in the incorporation of EPCs into foci of neovascularization and in their differentiation into mature ECs (Asahara *et al.*, 1997 and 1999b; Kalka *et al.*, 2000a and 2000c). Importantly enough, mobilization of BM EPCs is correlated to the transient expression of the therapeutic VEGF transgene and subsequent transient increase in plasma VEGF (Kalka *et al.*, 2000a and 2000c), indicating that one of the main targets of VEGF gene therapy is indeed BM stem/progenitor cells, i.e., EPC precursors such as putative adult hemangioblasts (precursors of both HSCs and angioblasts; see Reyes *et al.*, 2002), multipotent lineage-negative c-kit-positive (lin^- $c\text{-}kit^+$) stem cells (Orlic *et al.*, 2001) and/or pluripotent mesenchymal stem cells (MSCs)/pre-MSCs (Reyes *et al.*, 2002; Jiang *et al.*, 2002; Anjos-Afonso and Bonnet, 2007).

As shown by Isner and co-workers, a key feature of circulating EPCs is their propensity to home into ischemic tissues (Asahara *et al.*, 1997 and 1999b; Kalka *et al.*, 2000a and 2000c). Such a regenerative homing of EPCs prompted the development of 1) stem cell-mediated gene delivery to ischemic/neovascularizating tissues (Asahara *et al.*, 1997; Iwaguro *et al.*, 2002) and 2) autologous vasculogenic stem cell therapy. The first approach is a breakthrough for both regenerative medicine and cancer gene therapy (see below). The second approach was initiated on experimental models for both critical limb ischemia (Kalka *et al.*, 2000b) and myocardial ischemia (Kawamoto *et al.*, 2001; Kocher *et al.*, 2001), and then readily moved from bench to bedside because stem cell transplantation was already

干细胞之家www.stemcell8.cn ←点击进入

a well-established medical practice (BM transplantation) (see: Matsubara, 2003; Brehm *et al.*, 2003; Assmus *et al.*, 2003; Bertolotti, 2003c).

Such a stimulation of the homing ability of EPCs to ischemic foci by transient topical gene therapy prompted us to devise a synergistic approach for long-term stem cell gene therapy, in which *ex vivo* protocols are combined with transient topical gene therapy in order to maximize the homing, regenerative and differentiative capabilities of the autologous therapeutic stem cells when they are returned to the patients (Bertolotti, 2001, 2003a and 2003b). The same synergistic combination with transient topical gene therapy applies to straight stem cell regenerative therapy too, as initially proposed for cardiovascular stem cell gene therapy (Bertolotti, 2002 and 2003c). Importantly enough, the intensive investigations that are aimed at unraveling the molecular mechanisms driving stem cell homing in a variety of niches and tissues/organs will be instrumental in the development of highly specific transient gene therapy protocols (see Bertolotti, 2003a and 2003b).

2. Stem/Progenitor Cells as Tissue- or Tumor-homing Vectors for Targeted Transient Regenerative/ Cancer Gene Therapy

2.1. *Autologous Stem/Progenitor Cells as Tissue-Homing Vectors for Targeted Transient Regenerative Gene Therapy*

The homing ability of EPCs has been pioneered by Isner and co-workers as a substitute for topical VEGF gene therapy in order to overcome autologous stem cell scarcity in aged patients through an increase of the *in vivo* revascularizative action of adult EPCs. Indeed, in this experimental trial on nude mice, *ex vivo* expansion and subsequent adenoviral transduction with a VEGF transgene strongly increased both the *in vitro* proliferative index and the *in vivo* revascularizative action of adult human EPCs. VEGF transduction was such a powerful booster that it reduced the effective therapeutic dose of EPCs to one thirtieth of its original value (Iwaguro *et al.*, 2002; Asahara *et al.*, 2002). The relative contribution of VEGF-induced angiogenesis, VEGF-induced mobilization/vasculogenesis of endogenous EPCs and transplanted-EPC–mediated vasculogenesis remains to be evaluated. Importantly enough, in this case homing does not apply to stem cells that are bound to engraft in their specific niche for life-long transgene expression (see HSCs in above SCID trials), but to progenitors cells that are bound to the tissue in which they incorporate through differentiation and in which

干细胞之家www.stemcell8.cn ←点击进入

they will incur basic cell turnover (see Iwaguro *et al.*, 2002). Under these conditions, transient expression of the transgene is granted because adenoviral transgenes do not integrate into host chromosomal DNA and are thus lost by dilution through the last bursts of EPC divisions that precede final differentiation.

Such a transient stem cell gene therapy has obviously many applications in regenerative medicine, and is part of the transient regenerative gene therapy arm of our universal platform (Bertolotti, 2007; see below). It has been pioneered with EPCs, but also with MSCs and with neural stem cells (NSCs) that might work as progenitors and/or real stem cells (see below).

2.2. *Autologous and Immortalized Stem/Progenitor Cells as Tumor-homing Vectors for Targeted Cancer Gene Therapy*

Like train servicing a combat unit, angiogenesis/vasculogenesis is essential both to most regenerative processes and to tumor growth/development. As expected, mobilization of BM EPCs to the tumor bed has been shown to be essential to tumor angiogenesis/vasculogenesis and growth (Lyden *et al.*, 2001). In fact, the formation of tumor stroma closely resembles wound healing and is so tightly associated to cancer pathological growth that tumors are seen as "never-healing wounds" (Dvorak, 1986). Increased neovascularization and turnover/proliferation of connective stromal cells in tumors appear thus to rely on both EPCs and MSC regenerative potential, thereby paving the way to EPC-mediated (Ferrari *et al.*, 2003) and MSC-mediated targeted cancer therapy (Studeny *et al.*, 2002). The propensity of both EPCs and MSCs to home into the tumor bed is the very drive of an emerging EPC- and MSC-based strategy for targeted delivery of therapeutics and oncolytic viruses to primary and metastatic tumors (see: Ferrari *et al.*, 2003; Moore *et al.*, 2004; Hall *et al.*, 2007; Pereboeva and Roth, this volume).

Importantly enough, neural stem/progenitor cells (NSCs) have been found to display extensive tropism for pathology (Aboody *et al.*, 2000) and, in experimental models, appear to be fairly efficient for targeted cancer gene therapy both for intracranial gliomas and for a variety of primary and metastatic tumors (see Yip *et al.*, 2006; Aboody *et al.*, 2006; Shah, 2007; Neujbauer *et al.*, this volume). On the other hand, $CD34^{+}$ HSCs have been successfully used to target leukemias in another experimental system (Carlo-Stella *et al.*, 2006 and this volume).

For cancer therapy, we are dealing with a transient gene therapy protocol even though armed stem cells can be transduced with an integrating vector. Therefore, the main point is to have an efficient homing into the tumor

干细胞之家www.stemcell8.cn ←点击进入

beds or nodules in order to topically deliver a transient transgene product, thereby maximizing the therapeutic effect and minimizing off-target side effects. Such a strategy is well fitted to autologous stem cells that are easy to obtain for *ex vivo* expansion and transfection/transduction (MSCs, EPCs and possibly circulating $CD34^+$ HSCs), but cannot apply to autologous NSCs and many other tissue-stem cells. For these reasons and for the sake of standardization and easiness, off-the-shelf cell lines or preconditioned heterologous stem/progenitor cells are currently favored over autologous stem/progenitor cells (see: Hall *et al.*, 2007; Neujbauer *et al.*, this volume). This is why cancer stem cell gene therapy has not been formally included in our autologous stem cell gene therapy platform (see below).

2.3. *Cancer Stem Cells as Breakthrough Targets for Stem Cell-mediated Gene Therapy and Other Cancer Therapies*

Cancer stem cell gene therapy has a double meaning. It covers either the aforedescribed approach in which armed stem cells are used for tumor-targeted delivery of a transgene or an oncolytic virus (see above), or emerging gene therapy approaches aimed at targeting cancer stem cells. However, we favor a third option conceptualizing an emerging breakthrough approach based on armed tumor-homing stem cells to specifically target cancer stem cells, thereby opening a promising avenue for recurrence-free cancer therapy.

Cancer stem cells are indeed the driving force of cancer (or at least of many of them), where genetic/epigenetic alterations have culminated in uncontrolled self-renewal and tumorigenesis either in tissue stem cells or in some of their progenitor/differentiated derivatives (see: Pearce and Bonnet, this volume; Piccirillo and Vescovi, this volume; Shackleton *et al.*, this volume; Patrawala and Tang, this volume). As a rare subset of the tumor, cancer stem cells are the only drive of tumor initiation/propagation and, upon transplantation, have been shown to recapitulate the hierarchical clonogenic differentiating/differentiated organization of the original tumor cell population (Bonnet and Dick, 1997; Reya *et al.*, 2001). Cancer stem cells have been identified in leukemia (Lapidot *et al.*, 1994; Bonnet and Dick, 1997) and in an increasing number of solid tumors such as breast cancer (Al-Hajj *et al.*, 2003), brain cancer (Singh *et al.*, 2003 and 2004; Galli *et al.*, 2004), prostate cancer (Collins *et al.*, 2005; Patrawala *et al.*, 2007), colon cancer (O'Brien *et al.*, 2007; Ricci-Vitiani *et al.*, 2007) and pancreatic cancer (Li *et al.*, 2007).

Cancer stem cells have the exclusive ability to reinitiate tumors (tumor-initiating cells) and are thus the very targets of a tantalizing

干细胞之家www.stemcell8.cn ←点击进入

progression-free, recurrence-free therapy. Intensive investigations are thus aimed both at their identification in an exhaustive number of tumor types (Clarke *et al.*, 2006) and at developing cancer stem cell-specific therapeutic regimens (see: Piccirillo and Vescovi, this volume; Patrawala and Tang, this volume; Bao *et al.*, 2006; Piccirillo *et al.*, 2006; Jin *et al.*, 2006; Krause *et al.*, 2006). In this respect, we believe that cancer stem cell gene therapy protocols capable of efficiently driving a stem cell-mediated destruction of specific cancer stem cells while sparing their normal tissue-stem cell counterparts will soon be available.

3. Therapeutic Homing and Autologous Stem Cells as the Main Drive of Our Stem Cell Gene Therapy Platform

As schematized in Fig. 1, autologous stem cells are the universal drive of our stem cell gene therapy platform (Bertolotti, 2007). They are the targets of both long-term and transient gene therapy protocols that culminate in synergistic combinations (Bertolotti, 2001, 2003a and 2003b). As discussed above, therapeutic homing drives both long-term engraftment of gene-corrected autologous stem cells into their original niche when they are returned to the patient (e.g., $CD34^+$ HSCs into the BM for life-long supply of transgenic lymphoid cells in the aforedescribed SCID patients) and tissue-specific integration of stem/progenitor cells that have been mobilized by transient gene therapy (e.g., EPCs in transient topical VEGF gene therapy in the aforementioned critical limb ischemic patients). It also drives the tissue-specific migration of transgenic stem cells aimed at transient topical regenerative gene therapy (e.g., aforedescribed EPC-mediated VEGF gene therapy for experimental critical limb ischemia) or the tumor-targeted armed stem cell delivery of transgene products for maximized efficiency of transient cancer gene therapy (e.g., Aboody *et al.*, 2000; Studeny *et al.*, 2002; Shah *et al.*, 2005; Carlo-Stella *et al.*, 2006; Komarova *et al.*, 2006). As discussed above, cancer stem cell gene therapy has not been formally included in our autologous stem cell gene therapy platform because, for the sake of standardization and easiness/availability, off-the-shelf cell lines or preconditioned heterologous stem/progenitor cells are currently favored over autologous stem/progenitor cells (see: Hall *et al.*, 2007; Neujbauer *et al.*, this volume).

As schematized in Fig. 1, the long-term gene therapy branch of our platform also has a transmitochondrial arm aimed at tackling mitochondrial DNA (mtDNA) diseases (Bertolotti, 2005). In this case, autologous

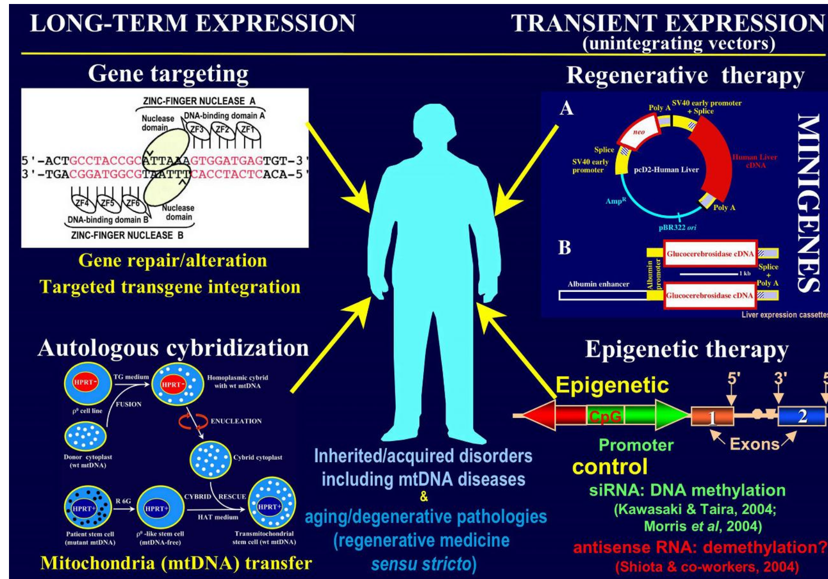


Fig. 1. Stem cell gene therapy: a universal platform (Poster Exhibition of June 2, 2005, ASGT 8th Annual Meeting, Saint-Louis: Bertolotti, 2005a). A schematic overview of our universal platform in which autologous stem cells are the targets of both long-term (*left*: endonuclease-boosted gene targeting and cybridization; Bertolotti, 2004a and 2005) and transient (*right*: regenerative medicine and epigenetic therapy*; Bertolotti, 2000c, 2001 and 2003b) gene therapy protocols culminating in synergistic combinations (Bertolotti, 2001 and 2003a). Cancer stem cell gene therapy has not been included because off-the-shelf stem cell lines or preconditioned heterologous stem/progenitor cells are currently favored over autologous stem/progenitor cells for tumor-targeted stem cell gene therapy (see text). Autologous adult/neonatal stem cells include emerging multipotent/pluripotent stem cells with an extensive *ex vivo* growth potential (e.g., MAPCs, MIAMI cells, hBMSCs and maGSCs; see text), umbilical cord derivatives and potential ES-like cells that might arise from intensive cell reprogramming investigations (see Bertolotti, 2001). Such a universal platform is aimed at eliminating hazardous random integration of therapeutic DNA, at reversing inherited diseases by re-establishing wild-type genomic homeostasis, and at tackling most pathologies through stem cell repopulation dynamics into appropriate niches (long-term engraftment) and tissues (cell turnover). *Although the experimental work of Kawasaki and Taira is under fraud suspicion investigations (Fuyuno and Cyranoski, 2006), siRNA-mediated transcriptional gene silencing in human cells has been also described by Morris *et al*. (2004) and more recent data (see text); on the other hand, siRNA-mediated gene activation has now been shown to occur too (Li *et al*., 2006; Janowski *et al*., 2007). (Based on Fig. 1 of: Bertolotti R., *Gene Therapy and Regulation*, **3**: 1–14 (2007), with permission of WSPC.)

干细胞之家www.stemcell8.cn ←点击进入

transmitochondrial stem cells obtained through *ex vivo* cybridization (see below) are returned to the patient like a conventional long-term gene therapy trial (e.g., aforedescribed SCID trials). However, in this case, one of the first potential targets might be the heart instead of the BM, and efficient engraftment of relevant cybrid stem cells might need the synergistic magnification of their homing/repopulating ability by the transient regenerative gene therapy arm of our platform (Bertololotti, 2005 and this volume).

Importantly enough, transient regenerative gene therapy is a critical arm of our platform since it is aimed both at driving regenerative medicine *sensu stricto* (healing disorders and aging/degenerative pathologies) and at synergizing long-term gene therapy, thereby magnifying the repopulating/regenerative ability of autologous native *ex vivo* expanded stem cells, transfected/transduced stem cells and cybrids.

4. Autologous Pluripotent Stem Cells: Toward a Universal Stem Cell Gene Therapy Platform

Using the therapeutic homing ability of adult/postnatal stem cells, we have thus designed an autologous stem cell gene therapy platform aimed at tackling most diseases (Bertolotti, 2005 and 2007). As schematized in Fig. 1, the long-term gene therapy branch comprises a gene targeting arm aimed at tackling 1) nuclear inherited diseases through gene repair/alteration and 2) mtDNA-independent acquired/degenerative/aging disorders through custom site-specific integration of therapeutic transgenes (see below). The long-term gene therapy branch also has a cybridization arm in which autologous transmitochondrial stem cells are used to treat all mtDNA diseases (Bertolotti, 2005). On the other hand, as emphasized above, the transient gene therapy branch comprises a regenerative arm for regenerative medicine *sensu stricto* and for the synergistic magnification of the homing/regenerative/differentiative potential of *ex vivo* engineered stem cells from the long-term gene therapy arm (see above), and an epigenetic arm for long-term inactivation/activation of endogenous genes through transient expression of promoter-specific siRNAs and antisense RNA (see below). In addition, the transient gene therapy arm of our stem cell gene therapy platform includes a potential cancer gene therapy arm for stem cell-mediated, tumor-targeted delivery of transgenic products or oncolytic viruses (see above). Some of the main features of our platform are detailed below.

干细胞之家www.stemcell8.cn ←点击进入

4.1. *Gene Targeting and Cybridization as Genomic Homeostatic Tools*

4.1.1. *Endonuclease-boosted gene targeting*

Pioneering gene therapy clinical trials relied on retrovirus-mediated transduction of transgenes encoding the wild-type product of the disease genes (Blaese *et al.*, 1995; Kohn *et al.*, 1995; Bordignon *et al.*, 1995). Such an approach culminated in the early 2000s with the first unequivocal successes for gene therapy (Cavazzana-Calvo *et al.*, 2000; Aiuti *et al.*, 2002; Gaspar *et al.*, 2004). Unfortunately, this therapeutic breakthrough is plagued by adverse oncogenic events resulting from the fact that retroviral vectors integrate at random into host chromosomal DNA (Hacein-Bey-Abina *et al.*, 2003b; Baum, 2007). Random integration of transgenic DNA into host chromosomal DNA is an insertional mutagenesis event that can hit a cancer-prone gene (e.g., oncogene, tumor suppressor or DNA-repair gene), thereby promoting a true long-term carcinogenesis hazard as experienced by patients from the first unequivocal successful gene therapy trial (Cavazzana-Calvo *et al.*, 2000; Hacein-Bey-Abina *et al.*, 2003b). In this trial, in which four out of eight successfully treated X-SCID patients incurred retroviral vector-mediated random-insertional T cell lymphomas, the first lymphoma case occurred almost three years after successful therapeutic gene transfer (Hacein-Bey-Abina *et al.*, 2003a) and the fourth case a few months ago (Baum, 2007).

Although a theoretically low-probability risk, random insertional oncogenesis is a true safety hazard of retroviral vectors. In addition, random integration into host chromosomal DNA does not provide for optimal transgene expression/regulation, a major concern when, unlike with SCID patients, we deal with a tightly regulated function (see Bertolotti, 1998 and 2000a). Site-specific integrating vectors are thus under intensive investigations, and are now emerging for both viral and nonviral gene therapy protocols (see: Olivares and Calos, 2003; Allen and Samulski, 2003; Wilkinson *et al.*, 2005; Urabe *et al.* and Fraefel *et al.*, this volume). However, these site-specific integrating vectors lack chromosomal target flexibility and might thus incur differentiation background trouble if one wishes to target a broad spectrum of tissue stem cells. Unlike these approaches, integration of exogenous DNA mediated by gene targeting is driven by homologous recombination with chromosomal DNA (see Capecchi, 1989) and has thus no target restriction, since the integration site is not specified by the DNA-binding domain of a protein but by the very DNA sequence of the targeting vector that is homologous to target chromosomal DNA. Gene targeting mediates flexible DNA

干细胞之家www.stemcell8.cn ←点击进入

exchanges between chromosomal DNA and transfecting/transducing DNA, thereby providing the means to modify at will the sequence of target chromosomal DNA (Capecchi, 1989). Gene targeting stands thus not only as the ultimate process for site-specific (i.e., targeted) transgene integration, but also for gene repair/alteration (Bertolotti, 1996 and 1999). Therefore, clinical gene targeting is discussed both in terms of gene repair/alteration for inherited diseases/anti-viral therapy and in terms of custom site-specific integrative gene therapy for acquired/degenerative and aging disorders (Bertolotti, 1999, 2000a and 2004a).

Conventional gene targeting is overwhelmed by random integration and is inefficient unless a double-strand break (DSB) hits target chromosomal DNA (see Bertolotti, this volume). Emerging DSB-boosted gene targeting relies on chimeric zinc-finger endonucleases (ZFNs) and customized homing endonucleases that create site-specific DSBs (see Bertolotti, 2007). Optimization of zinc-finger DNA-binding domains culminates now in targeting efficiencies compatible with clinical single-base correction: homologous recombination mediates strand exchanges between chromosomal and transfecting therapeutic DNA under negligible random integration of both therapeutic DNA and ZFN vectors (Urnov *et al*, 2005). Hot-off-the-press data show that the same holds true for targeted integration of up to ≈ 8 kb transgenes (Moehle *et al.*, 2007), thereby opening a promising custom site-specific integrative gene therapy era in which therapeutic transgenes can be integrated at selected hazard-free locations for optimized regulated expression.

Endonuclease-boosted gene targeting is thus a breakthrough for clinical gene therapy (see Bertolotti, 2004a and this volume). However, customization of both ZFNs and homing endonucleases for clinical applications is still a tedious and time-consuming process, thereby limiting their availability (see Bertolotti, 2007 and this volume). In addition, either with ZFNs or emerging custom homing endonucleases, efficiency is excellent when the distance between the target bases and the DSB is short (less than ~50 bp), but quickly drops when the distance increases (Elliott *et al.*, 1998; Miller *et al.*, 2005; Porteus, 2006). Such a limitation is a major hurdle for the clinical management of most multi-base mutations. In addition, it pinpoints the personalized facet of current endonuclease-boosted gene repair since most sites of a disease gene have to be targeted with specific sets of designed nucleases. Therefore, current strategic choices focus on polyvalent ZFNs/homing endonucleases that target mutation hotspots and nearby mutation sites. In order to reach full clinical potentialities, vectorization of therapeutic DNA

干细胞之家www.stemcell8.cn ←点击进入

might need optimization (see Bertolotti, 2004a and 2004b). An alternate approach is related to our autologous cybridization arm (see below).

4.1.2. *Cybridization: transmitochondrial stem cells for mtDNA diseases*

Due to the current lack of effective techniques for intramitochondrial genetic engineering, gene targeting is not amenable to mtDNA and wild-type mtDNA sequence cannot be restored in mtDNA mutants. Therefore, we have devised a strategy in which autologous transmitochondrial stem cells are generated *ex vivo* and then returned to the patient as in standard long-term stem cell gene therapy (Bertolotti, 2005). Current approaches rely on nuclear transgenes for allotopic expression of mtDNA-encoded proteins (e.g., Owen *et al.*, 2000; Manfredi *et al.*, 2002; Guy *et al.*, 2002) or for mutant mtDNA cleavage/methylation (e.g., Srivastava and Moraes, 2001; Tanaka *et al.*, 2002; Minczuk *et al.*, 2006), culminating in the mtDNA-free mitochondria concept in which all mtDNA-encoded proteins are converted into allotopic gene products (Zullo, 2001). Unlike these approaches, stem cell cybrid gene therapy can accommodate all types of mtDNA mutations and is not hampered by random-integration hazards and transgene dysregulations associated with current long-term nuclear gene therapy (Bertolotti, 2005).

In this approach, patients' stem cells are cured from their mtDNA content by a transient growth period in the presence of rhodamine 6G and then repopulated by wild-type mitochondria through fusion with relevant enucleated cells (cytoplasts), thereby generating cybrids which are true transmitochondrial stem cells (Bertolotti, 2005). Together with their differentiating/differentiated progeny, cybrid stem cells are thus expected to convert their gain in energy metabolism efficiency into a selective *in vivo* growth advantage over resident mtDNA mutant cognate cells. Like the nuclear γC transgene in the seminal X-linked SCID trial (Cavazzana-Calvo *et al.*, 2000), wild-type mtDNA could thus drive effective therapeutic repopulation dynamics of autologous engineered stem cells and differentiated derivatives into appropriate niches and relevant tissues/organs of patients, respectively. Importantly enough, magnification of this repopulating/regenerative capability will be under the control of the transient regenerative gene therapy arm of our stem cell gene therapy platform (see above).

4.1.3. *Genomic homeostasis and hazard-free custom site-specific integrative gene therapy*

The aforedescribed approaches are thus ideal to tackle inherited diseases, since the mutant nuclear genome can be repaired by gene targeting and

干细胞之家www.stemcell8.cn ←点击进入

the wild-type mitochondrial genome (mtDNA) substituted for its mutant counterpart by cybridization, thereby restoring a perfect wild-type genomic homeostasis in both cases (Bertolotti, 1996, 2005 and 2007). Such an approach eliminates dysregulations and oncogenic hazards that hamper random integration of conventional or allotopic transgenes into host chromosomal DNA (Bertolotti, 1998 and 2005). Importantly enough, for other long-term stem cell gene therapy applications (mtDNA-independent acquired and degenerative/aging disorders), the hot-off-the-press gene targeting breakthrough achievement that culminated in high-efficiency integration of an ≈8 kb transgene (Moehle *et al.*, 2007) opens a promising custom site-specific integrative gene therapy era in which therapeutic transgenes can be integrated at selected hazard-free locations for optimized regulated expression.

4.2. *Epigenetic Therapy: Potential Long-Term Gene Inactivation/Activation Through Transient siRNA Gene Therapy*

The epigenetic arm of our transient gene therapy branch is aimed at long-term inactivation/activation of endogenous genes through transient expression of promoter-specific siRNAs (Morris *et al.*, 2004; Li *et al.*, 2006; Janowski *et al.*, 2007). Transcriptional gene silencing mediated by siRNAs is tightly sequence-specific (Morris *et al.*, 2004; Castanotto *et al.*, 2005; Suzuki *et al.*, 2005; Ting *et al.*, 2005; Weinberg *et al.*, 2006; Han *et al.*, 2007), thereby necessitating potential patient personalization and, possibly, allele adjustment in heterozygote patients. On the other hand, siRNA (Li *et al.*, 2006; Janowski *et al.*, 2007) and noncoding antisense RNAs (Imamura *et al.*, 2004) are also amenable to converse epigenetic endogenous gene activation. Whether long-term gene silencing (Suzuki *et al.*, 2005) or activation (Janowski *et al.*, 2007) can be achieved under current conditions is not yet clear, and may be associated with efficient promoter methylation (Suzuki *et al.*, 2005).

4.3. *Emerging Pluripotent Stem Cells as Potential Universal Drives for Tissue-Specific and Multisystemic Gene Therapy*

Emerging adult/postnatal multi/pluripotent stem cells such as MAPCs (multipotent adult progenitor cells; Jiang *et al.*, 2002), MIAMI cells (marrow-isolated adult multilineage inducible cells; D'Ippolito *et al.*, 2004), hBMSCs (human bone marrow multipotent stem cells; Yoon *et al.*, 2005) or maGSCs (multipotent adult germ stem cells; Guan *et al.*, 2006) have an extensive/clonogenic *ex vivo* growth potential, and are therefore amenable to drastic

干细胞之家www.stemcell8.cn ←点击进入

selective growth conditions. They are thus instrumental in our transmitochondrial stem cell production platform for the selective cybrid rescue step (Bertolotti, 2005 and this volume). Such an extended *in vitro* growth potential also opens a promising avenue for emerging selective gene targeting, whereby relevant engineered autologous stem cells are selected/sorted and amplified *ex vivo* in order to compensate for nonclinical gene targeting frequencies incurred when the mutation track is too long or too far from the endonuclease target site (Bertolotti, 2006 and 2007; see Bertolotti, this volume).

Importantly enough, the breakthrough ability of the aforementioned adult/postnatal stem cells to differentiate into cells of the three germ lineages (pluripotency) is an important parameter in the development of our universal autologous stem cell gene therapy platform, since it opens exciting avenues to tackle all tissue-specific pathologies and tissue regeneration needs with a single stem cell population (Bertolotti, 2007; see Serafini and Verfaillie, this volume). In this respect, the pioneering transplantation of MAPCs as a substitute for tissue-specific stem cells (HSCs) with full functional reconstitution of the hematopoietic system stands as a promising proof of concept (Serafini *et al.*, 2007). However, one of the most exciting features of autologous pluripotent cells is that they should be amenable to multisystemic diseases such as progeria or most of the mtDNA diseases (see Bertolotti, this volume). Importantly enough, in order to take full advantage of the extraordinary potential of these cells culminating in multisystemic applications, magnification/optimization of their homing, regenerative, and differentiative abilities will exhaustively depend on the transient regenerative arm of our universal platform. Therefore, thanks to synergistic combinations between the different arms of our autologous stem cell gene therapy platform, we believe that both tissue-specific and multisystemic pathologies/regenerative needs (including mtDNA diseases and aging disorders) can be tackled through pluripotent stem cell repopulation dynamics into appropriate niches (long-term engraftment) and tissues (cell turnover) (Bertolotti, 2005 and 2007).

References

Aboody KS, Brown A, Rainov NG, *et al.* (2000) Neural stem cells display extensive tropism for pathology in adult brain: evidence from intracranial gliomas. *Proc Natl Acad Sci USA* **97**: 12846–51.

Aboody KS, Bush RA, Garcia E, *et al.* (2006) Development of a tumor-selective approach to treat metastatic cancer. *PLoS ONE* **1**: e23.

干细胞之家www.stemcell8.cn ←点击进入

Aiuti A, Slavin S, Aker M, *et al.* (2002) Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. *Science* **296**: 2410–13.

Al-Hajj M, Wicha MS, Benito-Hernandez A, *et al.* (2003) Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci USA* **100**: 3983–88.

Allen NA, Samulski RJ. (2003) The pros and cons of using the mechanism of AAV site-specific recombination in gene delivery. *Gene Ther Regul* **2**: 121–38.

Anjos-Afonso F, Bonnet D. (2007) Non-hematopoietic/endothelial SSEA-1+ cells defines the most primitive progenitors in the adult murine bone marrow mesenchymal compartment. *Blood* **109**: 1298–306.

Asahara T, Murohara T, Sullivan A, *et al.* (1997) Isolation of putative progenitor endothelial cells for angiogenesis. *Science* **275**: 964–67.

Asahara T, Masuda H, Takahashi T, *et al.* (1999a) Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ Res* **85**: 221–28.

Asahara T, Takahashi T, Masuda H, *et al.* (1999b) VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. *EMBO J* **18**: 3964–72.

Asahara T, Kawamoto A, Kalka C, Masuda H. (2002) Therapeutic potential of bone marrow-derived endothelial progenitor cells for cardiovascular ischemic diseases. *Gene Ther Regul* **1**: 361–74.

Assmus B, Schächinger V, Dimmeler S, Zeiher AM. (2003) Transplantation of adult progenitor cells in ischemic heart disease: *TOPCARE-AMI* trial and other current clinical trials. In: *Progress in Gene Therapy: Pioneering Stem Cell/Gene Therapy Trials*, Bertolotti R, Ozawa K, Hammond HK (eds.), VSP, Utrecht, NL, Vol. 2, pp. 233–52.

Bao S, Wu Q, McLendon RE, *et al.* (2006) Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature* **444**: 756–60.

Baum C. (2007) Fourth case of leukaemia in the first SCID-X1 gene therapy trial, and the diversity of gene therapy. *http://www.esgct.org/upload/4th_CaseofLeukemial.pdf*

Bertolotti R. (1996) Recombinase-mediated gene therapy: strategies based on Lesch-Nyhan mutants for gene repair/inactivation using human RAD51 nucleoprotein filaments. *Biogenic Amines* **12**: 487–98.

Bertolotti R. (1998) Gene therapy 1998: transient or stable minigene expression and gene repair/inactivation. *Biogenic Amines* **14**: 389–406.

Bertolotti R. (1999) Recombinase-DNA nucleoprotein filaments as vectors for gene repair/inactivation and targeted integration of minigenes. *Biogenic Amines* **15**: 169–95.

Bertolotti R. (2000a) RNA and gene repair/alteration: from inherited diseases to acquired disorders and tantalizing applications for non-disease conditions. *Gene Ther Regul* **1**: 115–22.

Bertolotti R. (2000b) Gene therapy: *dsDNA*-cored presynaptic filaments as vectors for gene repair and targeted integration of transgenes. In: *Progress in Gene Therapy — Basic and Clinical Frontiers*, Bertolotti R, Parvez H, Nagatsu T (eds.), VSP, Utrecht, NL, pp. 513–49.

干细胞之家www.stemcell8.cn ←点击进入

Bertolotti R. (2001) Adult and embryonic-like stem cells: toward a major gene therapy breakthrough relying on autologous multipotent stem cells. *Gene Ther Regul* **1**: 207–12.

Bertolotti R. (2002) From therapeutic angiogenesis to myocardium regeneration. *Gene Ther Regul* **1**: 287–95.

Bertolotti R. (2003a) Stem cell gene therapy: breakthrough culminating in combination of *ex vivo* protocols with transient topical gene therapy. *Gene Ther Regul* **2**: 91–102.

Bertolotti R. (2003b) Stem cell gene therapy: a breakthrough combination magnified by therapeutic stem cell homing. In: *Progress in Gene Therapy: Pioneering Stem Cell/Gene Therapy Trials*, Bertolotti R, Ozawa K, Hammond HK (eds.), VSP, Utrecht, NL, Vol. 2, pp. 1–31.

Bertolotti R. (2003c) From therapeutic angio/vasculogenesis toward myocardium regeneration. In: *Progress in Gene Therapy: Pioneering Stem Cell/Gene Therapy Trials*, Bertolotti R, Ozawa K, Hammond HK (eds.), VSP, Utrecht, NL, Vol. 2, pp. 253–73.

Bertolotti R. (2004a) Zinc finger nuclease-boosted gene targeting and synergistic transient regenerative gene therapy for long-term stem cell gene therapy. *Biogenic Amines* **18**: 503–38.

Bertolotti R. (2004b) Zinc finger nuclease-boosted gene targeting: toward clinical gene repair/alteration and custom site-specific integrative gene therapy. *Gene Ther Regul* **2**: 177–89.

Bertolotti R. (2005) Transmitochondrial stem cells: from mouse models of mtDNA diseases to autologous cybrid stem cell gene therapy. *Gene Ther Regul* **2**: 275–82.

Bertolotti R. (2005a) Zinc finger nuclease-boosted gene targeting and synergistic transient regenerative stem cell gene therapy: toward clinical gene repair/alteration and custom site-specific integrative gene therapy. *Mol Ther* **11**: S148–49.

Bertolotti R. (2006) Stem cell gene therapy: toward a universal platform relying on custom endonuclease-boosted gene targeting, cybridization, transient regenerative/epigenetic gene therapy and synergistic combinations. *Mol Ther* **13**: S392.

Bertolotti R. (2007) Autologous stem cell gene therapy: toward a universal platform for personalized therapy. *Gene Ther Regul* **3**: 1–14.

Blaese RM, Culver K, Miller AD, *et al.* (1995) T lymphocyte-directed gene therapy for ADA-SCID: initial trial results after 4 years. *Science* **270**: 475–80.

Bonnet D, Dick JE. (1997) Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med* **3**: 730–37.

Borchiellini P, Angulo JF, Bertolotti R. (1997) Genes encoding mammalian recombinases: cloning approach with anti-RecA antibodies. *Biogenic Amines* **13**: 195–215.

Bordignon C, Notarangelo LD, Nobili N, *et al.* (1995) Gene therapy in peripheral blood lymphocytes and bone marrow for ADA-immunodeficient patients. *Science* **270**: 470–75.

干细胞之家www.stemcell8.cn ←点击进入

Brehm M, Zeus T, Strauer BE. (2003) Autologous mononuclear bone marrow cell transplantation for myocardial infarction: first pilot study and other pioneering trials. In: *Progress in Gene Therapy: Pioneering Stem Cell/Gene Therapy Trials*, Bertolotti R, Ozawa K, Hammond HK (eds.), VSP, Utrecht, NL, Vol. 2, pp. 203–31.

Capecchi M. (1989) The new mouse genetics: altering the genome by gene targeting. *Trends Genet* **5**: 70–76.

Carlo-Stella C, Lavazza C, Di Nicola M, *et al.* (2006) Antitumor activity of human CD34(+) cells expressing membrane-bound tumor necrosis factor-related apoptosis-inducing ligand. *Hum Gene Ther* **17**: 1225–40.

Castanotto D, Tommasi S, Li M, *et al.* (2005) Short hairpin RNA-directed cytosine (CpG) methylation of the RASSF1A gene promoter in HeLa cells. *Mol Ther* **12**: 179–83.

Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, *et al.* (2000) Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science* **288**: 669–72.

Clarke MF, Dick JE, Dirks PB, *et al.* (2006) Cancer stem cells — perspectives on current status and future directions: AACR Workshop on Cancer Stem Cells. *Cancer Res* **66**: 9339–44.

Collins AT, Berry PA, Hyde C, *et al.* (2005) Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Res* **65**: 10946–51.

D'Ippolito G, Diabira S, Howard GA, *et al.* (2004) Marrow-isolated adult multilineage inducible (MIAMI) cells, a unique population of postnatal young and old human cells with extensive expansion and differentiation potential. *J Cell Sci* **117**: 2971–81.

Dvorak HF. (1986) Tumors: wounds that do not heal: similarities between tumor stroma generation and wound healing. *N Engl J Med* **315**: 1650–59.

Elliott B, Richardson C, Winderbaum J, *et al.* (1998) Gene conversion tracts from double-strand break repair in mammalian cells. *Mol Cell Biol* **18**: 93–101.

Ferrari N, Glod J, Lee J, *et al.* (2003) Bone marrow-derived, endothelial progenitor-like cells as angiogenesis-selective gene-targeting vectors. *Gene Ther* **10**: 647–56.

Folkman J. (1971) Tumor angiogenesis: therapeutic implications. *N Engl J Med* **285**: 1182–86.

Fuyuno I, Cyranoski D. (2006) Doubts over biochemist's data expose holes in Japanese fraud laws. *Nature* **439**: 514.

Galli R, Binda E, Orfanelli U, *et al.* (2004) Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. *Cancer Res* **64**: 7011–21.

Gaspar HB, Parsley KL, Howe S, *et al.* (2004) Gene therapy of X-linked severe combined immunodeficiency by use of a pseudotyped gammaretroviral vector. *Lancet* **364**: 2181–87.

干细胞之家www.stemcell8.cn ←点击进入

Guan K, Nayernia K, Maier LS, *et al*. (2006) Pluripotency of spermatogonial stem cells from adult mouse testis. *Nature* **440**: 1199–203.

Guy J, Qi X, Pallotti F, *et al*. (2002) Rescue of a mitochondrial deficiency causing Leber hereditary optic neuropathy. *Ann Neurol* **52**: 534–42.

Hacein-Bey-Abina S, Le Deist F, Carlier F, *et al*. (2002) Sustained correction of X-linked severe combined immunodeficiency by *ex vivo* gene therapy. *N Engl J Med* **346**: 1185–93.

Hacein-Bey-Abina S, von Kalle C, Schmidt M, *et al*. (2003a) A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *N Engl J Med* **348**: 255–56.

Hacein-Bey-Abina S, Von Kalle C, Schmidt M, *et al*. (2003b) LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* **302**: 415–19.

Hall B, Dembinski J, Sasser AK, *et al*. (2007) Mesenchymal stem cells in cancer: tumor-associated fibroblasts and cell-based delivery vehicles. *Int J Hematol* **86**: 8–16.

Han J, Kim D, Morris KV. (2007) Promoter-associated RNA is required for RNA-directed transcriptional gene silencing in human cells. *Proc Natl Acad Sci USA* **104**: 12422–27.

Imamura T, Yamamoto S, Ohgane J, *et al*. (2004) Non-coding RNA directed DNA demethylation of Sphk1 CpG island. *Biochem Biophys Res Commun* **322**: 593–600.

Iwaguro H, Yamaguchi J, Kalka C, *et al*. (2002) Endothelial progenitor cell vascular endothelial growth factor gene transfer for vascular regeneration. *Circulation* **105**: 732–38.

Janowski BA, Younger ST, Hardy DB, *et al*. (2007) Activating gene expression in mammalian cells with promoter-targeted duplex RNAs. *Nat Chem Biol* **3**: 166–73.

Jiang Y, Jahagirdar BN, Reinhardt RL, *et al*. (2002) Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* **418**: 41–49.

Jin L, Hope KJ, Zhai Q, *et al*. (2006) Targeting of CD44 eradicates human acute myeloid leukemic stem cells. *Nat Med* **12**: 1167–74.

Kalka C, Masuda H, Takahashi T, *et al*. (2000a) Vascular endothelial growth factor(165) gene transfer augments circulating endothelial progenitor cells in human subjects. *Circ Res* **86**: 1198–202.

Kalka C, Masuda H, Takahashi T, *et al*. (2000b) Transplantation of *ex vivo* expanded endothelial progenitor cells for therapeutic neovascularization. *Proc Natl Acad Sci USA* **97**: 3422–27.

Kalka C, Tehrani H, Laudenberg B, *et al*. (2000c) VEGF gene transfer mobilizes endothelial progenitor cells in patients with inoperable coronary disease. *Ann Thorac Surg* **70**: 829–34.

Kawamoto A, Gwon H-C, Iwaguro H, *et al*. (2001) Therapeutic potential of *ex vivo* expanded endothelial progenitor cells for myocardial ischemia. *Circulation* **103**: 634–37.

干细胞之家www.stemcell8.cn ←点击进入

Kawasaki H, Taira K. (2004) Induction of DNA methylation and gene silencing by short interfering RNAs in human cells. *Nature* **431**: 211–17.

Kocher AA, Schuster MD, Szabolcs MJ, *et al*. (2001) Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. *Nat Med* **7**: 430–36.

Kohn DB, Weinberg KI, Nolta JA, *et al*. (1995) Engraftment of gene-modified cells from umbilical cord blood in neonates with adenosine deaminase deficiency. *Nat Med* **1**: 1017–26.

Komarova S, Kawakami Y, Stoff-Khalili MA, *et al*. (2006) Mesenchymal progenitor cells as cellular vehicles for delivery of oncolytic adenoviruses. *Mol Cancer Ther* **5**: 755–66.

Krause DS, Lazarides K, von Andrian UH, Van Etten RA. (2006) Requirement for CD44 in homing and engraftment of BCR-ABL-expressing leukemic stem cells. *Nat Med* **12**: 1175–80.

Lapidot T, Sirard C, Vormoor J, *et al*. (1994) A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature* **367**: 645–48.

Li C, Heidt DG, Dalerba P, *et al*. (2007) Identification of pancreatic cancer stem cells. *Cancer Res* **67**: 1030–37.

Li LC, Okino ST, Zhao H, *et al*. (2006) Small dsRNAs induce transcriptional activation in human cells. *Proc Natl Acad Sci USA* **103**: 17337–42.

Lyden D, Hattori K, Dias S, *et al*. (2001) Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. *Nat Med* **7**: 1194–201.

Manfredi G, Fu J, Ojaimi J, *et al*. (2002) Rescue of a deficiency in ATP synthesis by transfer of MTATP6, a mitochondrial DNA-encoded gene, to the nucleus. *Nat Genet* **30**: 394–99.

Matsubara H. (2003) Therapeutic angiogenesis for cardiac and peripheral vascular diseases by autologous bone marrow cell transplantation. In: *Progress in Gene Therapy, Vol. 2: Pioneering Stem Cell/Gene Therapy Trials*, Bertolotti R, Ozawa K, Hammond HK (eds.), VSP, Utrecht, NL, Vol. 2, pp. 181–201.

Miller JC, Urnov FD, Lee Y-L, *et al*. (2005) Development of zinc finger nucleases for therapeutic gene correction of sickle cell anemia. *Mol Ther* **11**: S35.

Minczuk M, Papworth MA, Kolasinska P, *et al*. (2006) Sequence-specific modification of mitochondrial DNA using a chimeric zinc finger methylase. *Proc Natl Acad Sci USA* **103**: 19689–94.

Moehle EA, Rock JM, Lee YL, *et al*. (2007) Targeted gene addition into a specified location in the human genome using designed zinc finger nucleases. *Proc Natl Acad Sci USA* **104**: 3055–60.

Moore XL, Lu J, Sun L, *et al*. (2004) Endothelial progenitor cells' "homing" specificity to brain tumors. *Gene Ther* **11**: 811–18.

Morris KV, Chan SW, Jacobsen SE, Looney DJ. (2004) Small interfering RNA-induced transcriptional gene silencing in human cells. *Science* **305**: 1289–92.

干细胞之家www.stemcell8.cn ←点击进入

O'Brien CA, Pollett A, Gallinger S, Dick JE. (2007) A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature* **445**: 106–10.

Olivares EC, Calos MP. (2003) Phage ϕC31 integrase-mediated site-specific integration for gene therapy. *Gene Ther Regul* **2**: 103–20.

Orlic D, Kajstura J, Chimenti S, *et al*. (2001) Bone marrow cells regenerate infarcted myocardium. *Nature* **410**: 701–705.

Owen R IV, Lewin AP, Peel A, *et al*. (2000) Recombinant adeno-associated virus vector-based gene transfer for defects in oxidative metabolism. *Hum Gene Ther* **11**: 2067–78.

Patrawala L, Calhoun-Davis T, Schneider-Broussard R, Tang DG. (2007) Hierarchical organization of prostate cancer cells in xenograft tumors: the $CD44^{+}\alpha 2\beta 1^{+}$ cell population is enriched in tumor-initiating cells. *Cancer Res* **67**: 6796–805.

Piccirillo SGM, Reynolds BA, Zanetti N, *et al*. (2006) Bone morphogenetic proteins inhibit the tumorigenic potential of human brain tumour-initiating cells. *Nature* **444**: 761–65.

Porteus MH. (2006) Mammalian gene targeting with designed zinc finger nucleases. *Mol Ther* **13**: 438–46.

Reya T, Morrison SJ, Clarke MF, Weissman IL. (2001) Stem cells, cancer, and cancer stem cells. *Nature* **414**: 105–11.

Reyes M, Dudek A, Jahagirdar B, *et al*. (2002) Origin of endothelial progenitors in human postnatal bone marrow. *J Clin Invest* **109**: 337–46.

Ricci-Vitiani L, Lombardi DG, Pilozzi E, *et al*. (2007) Identification and expansion of human colon-cancer-initiating cells. *Nature* **445**: 111–15.

Serafini M, Dylla SJ, Oki M, *et al*. (2007) Hematopoietic reconstitution by multipotent adult progenitor cells: precursors to long-term hematopoietic stem cells. *J Exp Med* **204**: 129–39.

Shah K. (2007) Neural stem cells and armed derivatives: fate and therapeutic potential in the brain. *Gene Ther Regul* **3**: 91–109.

Shah K, Bureau E, Kim DE, *et al*. (2005) Glioma therapy and real-time imaging of neural precursor cell migration and tumor regression. *Ann Neurol* **57**: 34–41.

Singh SK, Clarke ID, Terasaki M, *et al*. (2003) Identification of a cancer stem cell in human brain tumors. *Cancer Res* **63**: 5821–28.

Singh SK, Hawkins C, Clarke ID, *et al*. (2004) Identification of human brain tumour initiating cells. *Nature* **432**: 396–401.

Srivastava S, Moraes CT. (2001) Manipulating mitochondrial DNA heteroplasmy by a mitochondrially targeted restriction endonuclease. *Hum Mol Genet* **10**: 3093–99.

Studeny M, Marini FC, Champlin RE, *et al*. (2002) Bone marrow-derived mesenchymal stem cells as vehicles for interferon-beta delivery into tumors. *Cancer Res* **62**: 3603–608.

Suzuki K, Shijuuku T, Fukamachi T, *et al*. (2005) Prolonged transcriptional silencing and CpG methylation induced by siRNAs targeted to the HIV-1 promoter region. *J RNAi Gene Silencing* **1**: 66–78.

干细胞之家www.stemcell8.cn ←点击进入

Takahashi T, Kalka C, Masuda H, *et al*. (1999) Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. *Nat Med* **5**: 434–38.

Tanaka M, Borgeld HJ, Zhang J, *et al*. (2002) Gene therapy for mitochondrial disease by delivering restriction endonuclease SmaI into mitochondria. *J Biomed Sci* **9**: 534–41.

Ting AH, Schuebel KE, Herman JG, Baylin SB. (2005) Short double-stranded RNA induces transcriptional gene silencing in human cancer cells in the absence of DNA methylation. *Nat Genet* **37**: 906–10.

Urnov FD, Miller JC, Lee YL, *et al.* (2005) Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature* **435**: 646–51.

Weinberg MS, Villeneuve LM, Ehsani A, *et al.* (2006) The antisense strand of small interfering RNAs directs histone methylation and transcriptional gene silencing in human cells. *RNA* **12**: 256–62.

Wilkinson TA, Tan W, Chow SA. (2005) Safe delivery of therapeutic genes into specific chromosomal sites using engineered retroviral integrase. *Gene Ther Regul* **2**: 369–85.

Yip S, Sabetrasekh R, Sidman RL, Snyder EY. (2006) Neural stem cells as novel cancer therapeutic vehicles. *Eur J Cancer* **42**: 1298–308.

Yoon YS, Wecker A, Heyd L, *et al*. (2005) Clonally expanded novel multipotent stem cells from human bone marrow regenerate myocardium after myocardial infarction. *J Clin Invest* **115**: 326–38.

Zullo SJ. (2001) Gene therapy of mitochondrial DNA mutations: a brief, biased history of allotopic expression in mammalian cells. *Semin Neurol* **21**: 327–35.

干细胞之家www.stemcell8.cn ←点击进入

This page intentionally left blank

干细胞之家www.stemcell8.cn ←点击进入

Chapter 1

Stem Cell Gene Therapy for ADA-Deficiency without Myelopreparative Conditioning

Makoto Otsu*,† and Tadashi Ariga*,‡

Severe combined immunodeficiency (SCID) due to adenosine deaminase (ADA)-deficiency has been one of the best target diseases for clinical gene therapy. Stem cell gene therapy (SCGT) mediated by *ex vivo* transduction of hematopoietic stem cells with an *ADA* transgene can be curative for ADA-SCID patients, having a potential to enable life-long immune reconstitution. We have treated two ADA-SCID patients by retroviral-mediated gene transfer into bone marrow CD34$^+$ cells. Of note, we did not give any myelopreparative conditioning to our patients before cell infusion, while the concurrent successful SCGT trials utilized chemotherapy with the aim of enhancing engraftment of infused gene-corrected cells. At ~3 years post SCGT, clinical benefits are evident for both patients even in the absence of myelopreparative conditioning. Gene-corrected CD34$^+$ cells have engrafted in both patients and still keep producing mature hematopoietic cells expressing ADA levels sufficient to allow systemic detoxification. Our SCGT trial has also been successful in providing protective immunity to our two patients. The extent and kinetics of immune reconstitution, however, are significantly different from those achieved in the SCGT trials with preconditioning. These results indicate that preconditioning-free SCGT for ADA-deficiency may be further improved since it does not yet match the clinical outcomes of concurrent preconditioning-based SCGT trials. Unlike the pioneering SCGT trial for X-SCID in which success has been plagued by some random-integration oncogenic cases, all ADA-SCID SCGT trials, including ours, are thus far free from adverse events. However, myelopreparative conditioning might have a negative effect on the long-term repopulating potential of the HSC pool. Therefore, our protocol through worldwide cooperation will eventually culminate in the

*Corresponding authors. motsu@ims.u-tokyo.ac.jp; Tada-ari@med.hokudai. ac.jp
†Laboratory of Stem Cell Therapy, Center for Experimental Medicine, Institute of Medical Science, University of Tokyo, 108-8639 Tokyo, Japan
‡Department of Pediatrics, Hokkaido University Graduate School of Medicine, Sapporo, 060-8638 Hokkaido, Japan

干细胞之家www.stemcell8.cn ←点击进入

establishment of a long-term risk-free SCGT protocol for ADA-deficiency, thereby maximizing the effectiveness of current successful myelopreparative-conditioning SCGT strategies.

Keywords: Adenosine deaminase; severe combine immunodeficiency; gene therapy; clinical trials; hematopoietic stem cells; myelopreparative conditioning.

1. Introduction

Severe combined immunodeficiency, SCID, is a heterogeneous group of disorders with which affected patients have profound defects in their immune functions (Buckley, 2004; Fischer *et al.*, 2005). Among the currently recognized SCID diseases, adenosine deaminase(ADA)-deficiency represents ~15% of all cases (Buckley *et al.*, 1997; Stephan *et al.*, 1993). ADA-deficiency has been one of the best target diseases for clinical gene therapy since the pioneering trial conducted in 1990, which was originally aimed at genetic correction of patients' T cells (Blaese *et al.*, 1995). Extensive efforts have been made to improve efficacy and safety of genetic correction for ADA-deficiency either by targeting patients' T cells or hematopoietic stem cells (HSCs) (Onodera *et al.*, 1998a; Hoogerbrugge *et al.*, 1996; Kohn *et al.*, 1995; Bordignon *et al.*, 1995). These efforts have culminated in the recent success achieved in the stem cell gene therapy trial conducted by Aiuti *et al.*, which has for the first time introduced nonmyeloablative conditioning for stem cell gene therapy (SCGT) (Aiuti *et al.*, 2002a). In this trial, the use of a low-dose of busulfan as a cytoreductive reagent is believed to have played a key role for the beneficial immune reconstitution achieved in patients by helping efficient engraftment and expansion of gene-corrected HSCs. Another successful trial reported by Gasper *et al.* that used melphalan as a preconditioning drug has further supported the idea that creating some "space" in patient's marrow can lead to a better outcome in SCGT for ADA-deficiency (Gaspar *et al.*, 2006). Although possibly in rare instances, however, there seem to be some cases in which the use of chemotherapeutic reagents may not be appropriate because of unpredictable hypersensitivity of patients' hematopoietic cells to the drugs (Engel *et al.*, 2007). Moreover, since the long-term outcomes of treated patients still remain to be determined, it may be desirable to keep seeking for the ultimate treatment procedures that can provide ADA-deficient SCID patients with life-long immune reconstitution while simultaneously eliminating any treatment-related risks.

Of note, we have had our own clinical trial of SCGT in which two ADA-deficient SCID patients received autologous gene-corrected HSCs without any myelopreparative conditioning. Both patients clearly obtained clinical

干细胞之家www.stemcell8.cn ←点击进入

benefits and have no need for ADA enzyme replacement for more than 3 years with partial, but still protective reconstitution of their immunity. In this chapter, we will summarize our clinical trial and try to make a comparison between different concurrent trials. We believe that the results of our trial can add important pieces of information to the field of SCGT and that the collective efforts will eventually lead to the establishment of an ideal treatment for ADA-deficiency.

2. Severe Combined Immunodeficiency due to ADA-deficiency

ADA is the purine salvage enzyme ubiquitously expressed in mammalian cells. Genetic defects in the *ADA* gene that is located on chromosome 20 lead to accumulation of cellular toxic metabolites, which can cause death of lymphocytes and consequently profound deficiency in immune systems (Markert, 1994; Hirschhorn, 1993; Giblett *et al.*, 1972). Systemic involvement due to metabolic toxicities of various non-lymphoid organs, including liver, kidney and gut is also a feature in ADA-deficient SCID patients, making this disorder distinctive from other major SCID diseases such as X-linked SCID. Because ADA-deficiency is virtually a "metabolic" disease, the curative treatment requires correction of not only immune deficiency, but also generalized toxicities.

Allogeneic bone marrow transplantation (BMT) or HSC transplantation (HSCT) has been successful as a curative treatment for ADA-deficiency when HLA-identical sibling donors are available (Myers *et al.*, 2002). Because the lack of such ideal donors is not uncommon for most patients, BMT from HLA-haploidentical parental donors have been performed with limited success (Haddad *et al.*, 1998; Buckley *et al.*, 1999). HLA-matched unrelated donor procedures are also shown to result in frequent transplantation failure, making ADA-deficiency one of hard-to-manage SCID phenotypes (Booth *et al.*, 2007).

The presence of another life-saving option is one remarkable feature of ADA-deficiency; polyethylene glycol-modified ADA (PEG-ADA) was developed as an enzyme replacement reagent (Hershfield, 1995). PEG-ADA has provided over 150 patients worldwide with life-saving effects, but recent reports have highlighted still protective, but somehow limited immune reconstitution observed in treated patients with long follow-up (Chan *et al.*, 2005; Malacarne *et al.*, 2005). Despite the above-mentioned limitation, it is still true that PEG-ADA stands as one of the major life-saving treatment options for ADA-SCID capable of stabilizing patients prior to other curative treatments such as HSCT and SCGT (Booth *et al.*, 2007).

干细胞之家www.stemcell8.cn ←点击进入

3. Gene Therapy Trials for ADA-deficiency

3.1. *T Cell-Directed Gene Transfer as a Treatment for ADA-deficiency*

In the early clinical trials including ours (Onodera *et al.*, 1998a), peripheral T lymphocytes were used as target cells for gene transduction. These trials have demonstrated that retroviral-mediated gene transfer could be safely completed and lead to long-term gene marking/expression in patients' T cells (Blaese *et al.*, 1995; Bordignon *et al.*, 1995; Onodera *et al.*, 1998a). Although limited ADA-transgene expression in the T cell compartment seems sufficient to provide protective immunity with the concomitant use of PEG-ADA, it likely fails to supply ADA in sufficient amounts to detoxify systemic organs (Aiuti *et al.*, 2002b).

3.2. *Early Stem Cell Gene Therapy Trials*

To achieve life-long immune reconstitution by gene therapy, targeting HSCs is thought to be the ideal measure because of their ability to self-renew and differentiate into multiple hematopoietic lineages. Several pioneering trials aimed at genetic correction of HSCs via retroviral-mediated gene transfer have been conducted (Bordignon *et al.*, 1995; Kohn *et al.*, 1995; Hoogerbrugge *et al.*, 1996). These trials resulted in long-term engraftment of gene-marked cells, thereby establishing that hematopoietic progenitor/stem cells with repopulating abilities can be targeted by retroviral-mediated gene transfer (Schmidt *et al.*, 2003). Treated patients, however, did not gain clear clinical benefits from transduced cell populations. The reasons for poor immune recovery in these trials are now thought to be largely attributable to insufficient gene transduction into HSCs and to impairment due to continuous use of PEG-ADA of selective growth/survival advantages normally expected for gene-corrected cells.

3.3. *Second Generation of Stem Cell Gene Therapy Trials for ADA-deficiency*

Based on the cumulative experience of early clinical trials and the advancement in gene transfer techniques into HSCs, a team in Italy started the new generation of SCGT trials for ADA-deficiency (Aiuti *et al.*, 2002a). In sharp contrast to the early trials, the treatment provided most patients with systemic detoxification and good immune reconstitution, thereby eliminating the need for PEG-ADA administration. This trial has introduced two major

干细胞之家www.stemcell8.cn ←点击进入

amendments in its protocol, i.e., to treat patients who are not on PEG-ADA and to use a low-dose of busulfan as a preconditioning reagent. These two modifications have most likely played important roles in the improvement of the clinical outcomes. Usefulness of mild preconditioning for SCGT has been further confirmed by a report from Gaspar *et al.*, which demonstrated meaningful immune reconstitution in an ADA-SCID patient treated following the Italian SCGT protocol but using Melphalan instead of busulfan (Gaspar *et al.*, 2006). Together with two other clinical trials, one conducted in the USA (Engel *et al.*, 2007) and the other by us in Japan (see below), SCGT is now establishing its status as a curative treatment option for ADA-deficiency. We believe that cumulative experience from different trials will help establish the best treatment protocol for SCGT for ADA-deficiency.

4. Stem Cell Gene Therapy for ADA-deficiency without Myelopreparative Conditioning

In 1995, we started the clinical trial that was aimed at full genetic correction of patient T cells by retroviral-mediated gene transfer. Although the patient clearly benefited from the treatment, we could not withdraw PEG-ADA replacement because gene-marked T cells alone seemed incapable of producing sufficient ADA for systemic detoxification (Kawamura *et al.*, 1998; Onodera *et al.*, 1998a; Kawamura *et al.*, 1999). While seeking for a better treatment option for this patient, we happened to have another ADA-deficient SCID patient who did not have a HLA-identical sibling donor (Ariga *et al.*, 2001). PEG-ADA had helped her growth and raised her immune functions to a level compatible with a transition from hospital to day care, but she had to stay home most of the time in order to avoid severe infections. We therefore decided to conduct the clinical trial of stem cell-directed gene therapy with the aim of giving patients sustained immune reconstitution by eliminating the necessity for supportive treatment including PEG-ADA.

The above-mentioned two ADA-deficient SCID patients (pt 1, a 4-year-old girl and pt 2, a 13-year-old boy) have been enrolled in the trial. PEG-ADA was stopped ~5 weeks prior to treatment. Bone marrow (BM) cells were collected and then subjected to $CD34^+$ cell-purification procedures. We then transduced $CD34^+$ cells (~1×10^6 cells/kg) with the retroviral vector GCsapM-ADA (Onodera *et al.*, 1998b) pseudotyped with gibbon ape leukemia virus (GALV) envelope. We used for cell culture, a serum-free medium supplemented with a combination of cytokines: SCF, TPO, Flt3-L, IL-6, and soluble IL-6R (sIL-6R). After 3 rounds of transduction, we obtained ~1×10^6 cells/kg of $CD34^+$ cells that expressed high levels of ADA

干细胞之家www.stemcell8.cn ←点击进入

with ~50% of cells transduced. Gene-corrected autologous BM cells were intravenously injected into pt 1 on December 2003 and into pt 2 on February 2004 without any preconditioning. No acute adverse reaction was noted.

For the first several weeks in the absence of PEG-ADA replacement, some symptoms related to ADA-deficiency, including mild anorexia became evident in both patients. Consistent with the clinical ADA-deficient state, the levels of accumulated toxic metabolites measured in erythrocytes reached the levels of the untreated ADA-deficient SCID patients. Liver toxicity was also noted (Fig. 1). Starting from as early as 1 week post SCGT, however, these ADA-deficiency-related abnormalities gradually disappeared. Of note, liver enzyme values completely normalized for both patients (Fig. 1), and toxic metabolite values continuously remain low for up to ~3 years post treatment, the levels being similar to those achievable in successful HSCT cases. These results indicate that SCGT without myelopreparative preconditioning can lead to systemic detoxification in ADA-deficient SCID patients.

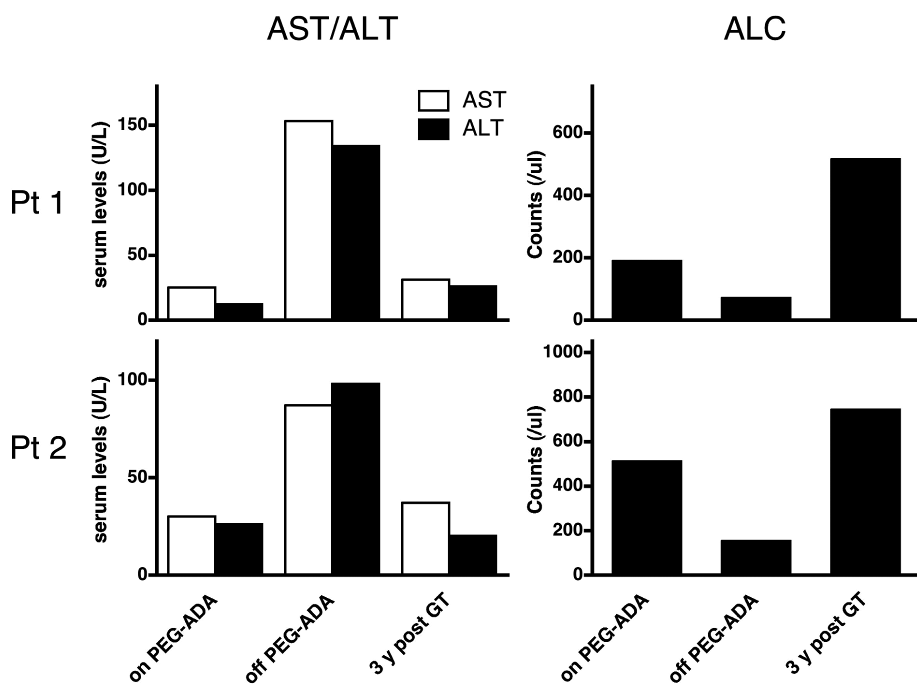


Fig. 1. Effectiveness of stem cell gene therapy for ADA-deficiency without myelopreparative preconditioning. *Left:* Serum levels of liver enzymes. Serum aspartate aminotransferase (AST, empty columns) and alanine aminotranferase (ALT, solid columns) activities are shown before cessation of PEG-ADA (on PEG-ADA), at peak levels (38 and 41 days after cessation of PEG-ADA for pt 1 and pt 2, respectively), and ~3 years after gene therapy (3 y post GT). *Right:* Absolute lymphocyte counts (ALC). Time points are the same as for serum levels of liver enzyme activities.

干细胞之家www.stemcell8.cn ←点击进入

Reconstitution of hematopoiesis and immune functions was also achieved, although with much slower kinetics than those observed in other trials that used mild preconditioning (Aiuti *et al.*, 2002a; Gaspar *et al.*, 2006). Lymphocyte counts showed a clear increase from ~4–6 months post treatment, then reached levels higher than those observed in their pre-SCGT status (300−400/μl for pt 1 and 500–700/μl for pt 2; Fig. 1). Immunophenotyping of these lymphocytes revealed that T cells represented a major subset of polyclonal nature as evidenced by TCR-spectratyping analysis. Accordingly, proliferative responses of T cells to mitogenic stimuli were remarkably improved in both patients. In contrast, increase of B and NK cells did not occur until ~10 months after treatment for pt 1. Once developed, however, these lymphocytes showed steady improvement of their counts. This delayed reconstitution is in sharp contrast to that achieved in the Italian trial that showed quick recovery of B and NK cells starting as early as 2 months post treatment (Aiuti *et al.*, 2002a). For pt 2, NK cell counts increased, although to a lesser extent, while B cell counts remained low throughout the observation period. Because antibody production after SCGT is currently considered insufficient, both patients are still on immunoglobulin replacement therapy. Overall, SCGT has improved both patients' immunity to levels compatible with protection from life-threatening infections without the need for PEG-ADA administration.

Assessment of gene-marking levels revealed that virtually ~100% of T cells possessed the transgene in both patients. Similar analysis was available for B and NK cells only for pt 1, and showed ~60% and ~80% marking levels for B cells and NK cells, respectively. It is noteworthy that we could confirm the presence of transgene in ~10% of colony-forming cells derived from BM at ~3 years post SCGT. Similar marking levels determined in BM $CD34^+$ cells by quantitative PCR assay further support the idea that significant portions of hematopoietic stem/progenitor cells have been successfully transduced and stably engrafted in patients' marrow without cytoreductive preconditioning.

We have carried out sequential analysis of retroviral integration sites in peripheral blood mononuclear cell samples, and have demonstrated the oligoclonal nature of transduced cells. During the ~3 year-observation period, we have not observed in either patients any severe adverse events, including leukemic expansion of abnormal clones. At the time of writing, pt 1 is an elementary school pupil while pt 2 is a high school student. Although we have not achieved complete withdrawal of supportive treatment, e.g., prophylactic antibiotics, it may be of great significance that both patients have been off PEG-ADA for as long as three years without serious infectious episodes. Further follow-up is necessary to evaluate the long-term

干细胞之家www.stemcell8.cn ←点击进入

safety and efficacy of SCGT for ADA-deficiency without myelopreparative conditioning.

5. Factors that Likely Affect the Safety and Efficacy of Stem Cell Gene Therapy for ADA-deficiency

To establish the ultimate protocol with the maximal efficacy and minimal risk, it is valuable to consider what factors likely affect patient outcomes after SCGT. Although there should be more than those listed below, we will focus on the five factors that are considered in particular to have influenced the outcome of our patients.

5.1. *Age of Patients*

Our patients were relatively old (4-year-old and 13-year-old) at the beginning of SCGT. From the clinical experience obtained from either BMT (Myers *et al.*, 2002; Buckley *et al.*, 1999) or SCGT (Hacein-Bey-Abina *et al.*, 2002; Cavazzana-Calvo *et al.*, 2000) for SCID diseases, younger age of patients seems critical for treatment effectiveness. This is probably due to several reasons that include higher quality of "younger" HSCs and better patients' condition with less frequent histories of infections. Of note, SCGT trials for another SCID disease, i.e., X-linked severe combined immunodeficiency (X-SCID) indicated a possible age-related limitation for treatment efficacy (Thrasher *et al.*, 2005). Although particularly young age (less than 6 month-old) may be correlated to the high incidence of leukemia-like adverse events reported in the French X-SCID gene therapy trial (Hacein-Bey-Abina *et al.*, 2003a), younger age should be considered as one of the critical factors for successful SCGT.

5.2. *Transduction Procedures*

Currently, all the clinical trials of SCGT for SCID have utilized retroviral vectors for gene transfer. Because cell division is a prerequisite for retroviral-mediated gene transduction, various combinations of cytokines are being used in each trial in order to stimulate BM $CD34^+$ cell proliferation. Ideal transduction procedures, we believe, should preserve the life-long repopulating/multi-differentiating capabilities of transduced stem cells without impairing good transduction efficiency. Our protocol is the sole one that utilizes IL-6 and sIL-6R besides the standard cytokines, SCF, TPO, and Flt-3L. Our protocol may thus be significantly different from all others.

干细胞之家www.stemcell8.cn ←点击进入

Addition of IL-6/sIL-6R was expected to enhance repopulating abilities of human $CD34^+$ cells based on experiments utilizing a NOD/SCID xenotransplantaion model (Ueda *et al.*, 2000) and therefore might have contributed to the favorable clinical outcomes of our patients. As a newer xenotransplantation model, NOD/SCID/IL-2Rγ chain KO mice, has recently become available with its improved capability to allow human T cell development (Shultz *et al.*, 2005; Ishikawa *et al.*, 2005). Further refinements of cytokine combinations may thus be experimented that might be instrumental for the future clinical trials.

5.3. *Cell Doses*

Accumulation of worldwide clinical experience has generally demonstrated a positive correlation between higher cell doses and better engraftment (Davies *et al.*, 2000; Bittencourt *et al.*, 2002). A large number of murine experiments have also supported this correlation (Rao *et al.*, 1997; Mauch and Hellman, 1989; Glass *et al.*, 1993). The relatively low doses of $CD34^+$ cells ($\sim 1 \times 10^6$ cells/kg) transplanted into our patients may thus explain, at least partially, the much slower hematopoietic recovery than those observed in the other successful SCGT cases (Hacein-Bey-Abina *et al.*, 2002; Aiuti *et al.*, 2002a). In the SCGT settings, however, the cell dose effect on clinical outcome should be carefully discussed. During a 4–5 day course of gene transduction, $CD34^+$ hematopoietic cells usually expand to certain extents, which will vary according to the difference in cytokine cocktails. Extreme expansion of $CD34^+$ cells, however, does not necessarily lead to HSC expansion but rather may compromise the quality and/or quantity of HSCs. It is therefore important to consider the balance between maintenance of good repopulating abilities and total cell expansion when evaluating the appropriateness of culture conditions. Since single injections of relatively small numbers of gene-corrected cells have led to the development of hematopoietic cells capable of maintaining ADA levels sufficient for systemic detoxification, we may assume high repopulating abilities in the infused $CD34^+$ cell populations. Obviously, higher numbers of transduced cells with a similar repopulation potential would enhance hematopoietic and immunological reconstitution with our SCGT protocol.

5.4. *Use of PEG-ADA*

PEG-ADA has been, and will remain indispensable as a treatment option for ADA-deficiency. It especially has great significance in that the drug can

干细胞之家www.stemcell8.cn ←点击进入

be used to promptly stabilize patients' condition when HLA-matched suitable donors turn out to be lacking (Booth *et al.*, 2007). PEG-ADA, however, does have a disadvantage in terms of "selective growth/survival advantage for gene-corrected cells," which must be taken into account in the settings of SCGT. Since PEG-ADA helps growth/survival of both uncorrected and gene-corrected patient cells, concomitant use of the enzyme replacement is believed to lower the treatment efficacy by impairing the advantage otherwise assigned to ADA-normalized cells. This idea is supported by the expansion of small numbers of spontaneously gene-corrected T cells in the reported case of *in vivo* gene-reversion (Hirschhorn *et al.*, 1996) and the results of earlier clinical gene therapy trials for ADA-deficiency (Aiuti *et al.*, 2002b; Kohn *et al.*, 1998). The idea that we could expect better selective expansion of gene-corrected cells if PEG-ADA is not given to patients at the time of SCGT, does now translate into PEG-ADA-free SCGT trials for ADA-deficiency.

5.5. *Cytoreductive Preconditioning*

Apparently, the most remarkable difference between our protocol and the other currently ongoing SCGT clinical studies is in the use of cytoreductive preconditioning. The trials running in Italy, the UK, and the US are all utilizing a chemotherapeutic reagent before infusion of gene-transduced BM cells (Gaspar *et al.*, 2006; Engel *et al.*, 2007; Aiuti *et al.*, 2002a), while we did not give any preconditioning treatment to both patients enrolled in our trial. Although the above-mentioned factors, including age and cell doses may also have affected our patients' outcome, it may be natural to consider that the absence of preconditioning has significantly influenced the kinetics of hematopoietic reconstitution. Conditioning regimen, including "no preconditioning" as well, is a matter of debate in the SCGT field for primary immunodeficiency diseases that include not only ADA-deficiency but also other disorders. The conditioning issue will greatly affect the evaluation of the risk/benefit balance in each SCGT trial. We will, therefore, pay particular attention to this issue and discuss the significance of our own trial in light of the current situation in the SCGT field in the following section.

6. Myelopreparative Treatment: Roles in Stem Cell Gene Therapy

6.1. *Concept of Myelopreparation*

It has been a widely accepted concept that the use of cytoreductive treatment as conditioning makes "space" in recipient BM to give transfused HSCs a

干细胞之家www.stemcell8.cn ←点击进入

better chance to engraft (Vriesendorp, 2003). It is also true, however, that engraftment of long-term repopulating HSCs does not necessarily require marrow-conditioning as shown in many murine experimental studies (Stewart *et al.*, 1993; Bhattacharya *et al.*, 2006). Quesenberry *et al.* have shown that the replacement of HSCs likely occurs according to "equilibration" between host- and donor-cell populations in a setting of syngeneic BMT (Colvin *et al.*, 2004). We thus may think that the main effect of conditioning, i.e., total body irradiation or chemotherapeutic drugs, is not "to make space" but "to reduce competitive counterparts" (Fig. 2). According to this theory, the use of conditioning in SCGT may have particular significance. As shown in Fig. 2, we may regard SCGT after conditioning as "reductive"

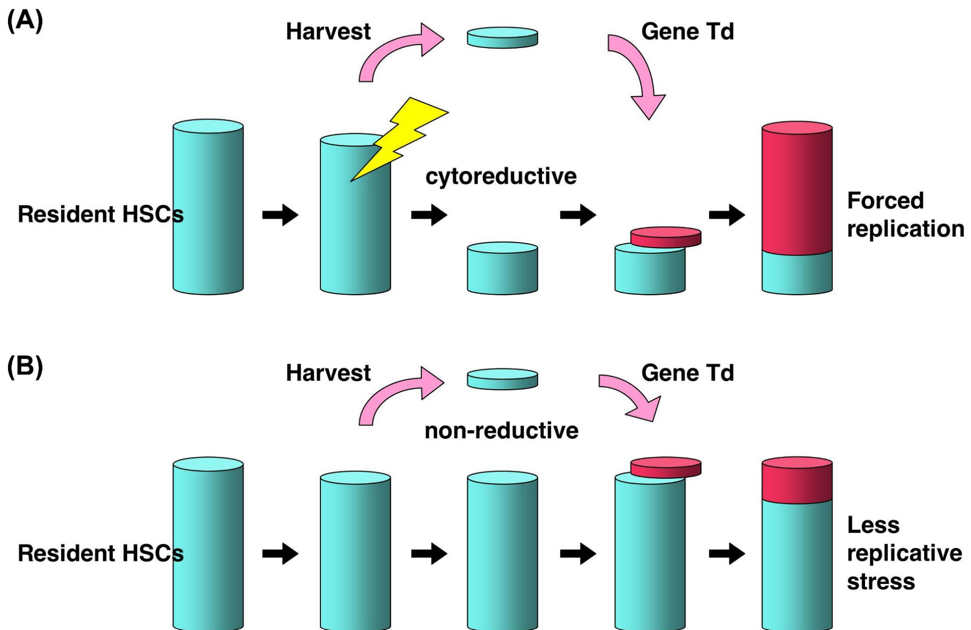


Fig. 2. Schematic representation for modeling of replicative stresses forced on HSCs in stem cell gene therapy. **(A)** Treatment with cytoreductive conditioning. The blue column on the left represents a total pool of patient HSCs. A small portion of the pool (a thin slice of blue column at the top) is removed from patient BM (Harvest), and then transduced with the therapeutic vectors (Gene Td). Gene-corrected HSCs (a thin slice of red column) is given back to BM. Because of cytoreductive conditioning, total mass of the original HSC pool is reduced (smaller blue columns). As a consequence, gene-corrected HSCs are most likely forced to rapidly replicate, resulting in substantial loss of their repopulation abilities. **(B)** Treatment with no conditioning. In the absence of conditioning, the resident HSC pool remains relatively untouched (blue columns). Transfused gene-corrected HSCs (a thin slice of red column) now have to compete for replication with resident HSCs, but are thought to be less susceptible to a replication-related loss of repopulation abilities.

干细胞之家www.stemcell8.cn ←点击进入

and SCGT with no conditioning as "non-reductive" with regard to the total number of HSCs. In light of the recent advances in stem cell research, senescence of HSCs has become an important issue (Wang *et al.*, 2006; Allsopp *et al.*, 2003). As replicative stress is thought to be one of the major factors that promote HSC senescence in transplantation (Harrison *et al.*, 1990), non-reductive SCGT may have an advantage in terms of the preservation of long-term repopulation potential in HSCs.

6.2. *Conditioning in SCGT: comparison between ADA-SCID and X-SCID*

Primary immunodeficiency represents the exceptional example regarding the choice of conditioning in transplantation medicine, especially for the cases of SCID. First, in the settings of allogeneic HSCT, preconditioning as a measure of immune suppression can be precluded since the patients' immunity is already impaired. Second, as SCGT utilizes patient's autologous hematopoietic cells, it also precludes the need for immunosuppressive drugs unless the strong immune reaction against the transgene product becomes a issue. Finally, selected SCID diseases such as X-SCID may not necessitate a high chimerism of donor- or gene-corrected-cells from the use of cytoreductive conditioning because of creditable selective growth/survival advantages assigned to normal or normalized cells over diseased cells. In fact, it is believed that such advantages have played a major role in achieving successful reconstitution in patients' immune in the X-SCID gene therapy trials (Cavazzana-Calvo *et al.*, 2000; Gaspar *et al.*, 2004). We may expect similar selective advantage in SCGT for ADA-deficiency, as well, considering the reported ADA-SCID cases in which spontaneously gene-corrected (revertant) T cells showed preferential expansion in the context of ADA-deficient counterparts (Ariga *et al.*, 2001; Hirschhorn *et al.*, 1996). Recent studies that used ADA-deficient mouse models also support this idea (Mortellaro *et al.*, 2006). Nonetheless, T cell reconstitution after transplantation without conditioning seems less satisfactory in the cases of ADA-deficiency than those of X-SCID in both HSCT (Haddad *et al.*, 1998; Buckley *et al.*, 1999) and SCGT settings. As suggested in a recent report, this advantage for X-SCID may be attributed to the absence of resident T cell precursors and the intact thymic environment (Cavazzana-Calvo *et al.*, 2007). Since the developmental block of T cells is not a feature of ADA-deficiency while it is the case for X-SCID, resident T cell precursors likely exist in ADA-deficient thymic environment with the potential to compete with incoming "normal" precursors. In addition, the thymic environment itself may be somehow dysfunctional

干细胞之家www.stemcell8.cn ←点击进入

in the absence of ADA, thereby hampering the prompt repopulation of incoming T cell precursors. Finally, since ADA-transduced transplanted cells have a systemic therapeutic action (toxic metabolite clearing) in addition to their specific HSC repopulating activity (see above), slow initial kinetics of immune reconstitution might reflect a bi-phasic process in which early systemic detoxification interferes with full immune recovery. By contrast, for X-SCID, the selective advantage of gene-corrected cells should theoretically remain constant throughout the treatment period after SCGT. For B cell development, however, the selective advantage situation appears to be very different between ADA-deficiency and X-SCID. In general, the B cell compartment of X-SCID patients rarely shows high chimerism of donor-derived or gene-corrected B cells without conditioning in both HSCT and SCGT situations (Cavazzana-Calvo *et al.*, 2000; Hacein-Bey-Abina *et al.*, 2002; Buckley *et al.*, 1999; Haddad *et al.*, 1998). This is most likely due to constraints of "normal" B cell development in BM because of competition with resident X-SCID B cell precursors as suggested in murine experiments (Liu *et al.*, 2006; Otsu *et al.*, 2000). In contrast, one patient in our trial has shown steady increase of B lymphocytes, ~60% of which were estimated to be gene-corrected. This observation supports the idea that due to sufficient selective advantage for gene-corrected cells, a limited number of normalized HSCs still have the potential to repopulate B cell compartments to meaningful levels in ADA-deficient SCID patients even in the absence of conditioning.

7. Future of SCGT for ADA-deficiency

Currently, for patients with ADA-deficiency who do not have a HLA-identical sibling donor, SCGT may be regarded as the first treatment option to be tested either before or after stabilization with the use of PEG-ADA. As has been reported above, over 10 ADA-deficient SCID patients have been treated worldwide with the latest SCGT protocols since the first successful cases in Italy, but there have been no treatment-related severe adverse events reported so far. Insertional leukemogenesis has been recognized as an inherited risk of retroviral-mediated gene transfer as evidenced in the French X-SCID gene therapy trial (Hacein-Bey-Abina *et al.*, 2003b and 2003a). In comparison to the cases of X-SCID gene therapy, gene-corrected HSCs expressing the ADA transgene may be less prone to leukemogenic events that are believed to occur due to cooperation between vector-insertional effects and the transgene expression (Thrasher *et al.*, 2006). Careful follow-ups of treated patients are absolutely essential for further development of ADA-SCGT.

干细胞之家www.stemcell8.cn ←点击进入

Establishment of the ideal treatment protocol for ADA-deficiency is obviously the ultimate goal. The recently developed gene-editing technique using a set of zinc finger nucleases (Urnov *et al.*, 2005) may be utilized for SCGT with the expectation that it can eliminate the risk of insertional leukemogenesis. Even if this becomes close to reality, however, the choice of conditioning will still remain the issue of debate. As long as *ex vivo* manipulation is inevitable in the currently available SCGT protocols, balancing selective advantages given to gene-corrected cells against competition between infused- and resident-HSCs continues to be critical to maximize treatment effectiveness. With the advancement in deciphering HSC-homing/lodgment mechanisms (Quesenberry *et al.*, 2005; Lapidot *et al.*, 2005; Broxmeyer, 2005) and with the improvement in technologies of HSC manipulation (Hofmeister *et al.*, 2007; Zheng *et al.*, 2003), we may expect further enhancement of treatment efficacy of ADA-SCGT even in the absence of conditioning. We believe that worldwide cooperations will culminate in the development of a risk-free SCGT protocol that can grant prompt, sufficient, and life-long immune reconstitution to all the ADA-deficient SCID patients in the not too far future.

References

Aiuti A, Slavin S, Aker M, *et al.* (2002a) Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. *Science* **296**: 2410–13.

Aiuti A, Vai S, Mortellaro A, *et al.* (2002b) Immune reconstitution in ADA-SCID after PBL gene therapy and discontinuation of enzyme replacement. *Nat Med* **8**: 423–25.

Allsopp RC, Morin GB, DePinho R, *et al.* (2003) Telomerase is required to slow telomere shortening and extend replicative lifespan of HSCs during serial transplantation. *Blood* **102**: 517–20.

Ariga T, Oda N, Yamaguchi K, *et al.* (2001) T-cell lines from 2 patients with adenosine deaminase (ADA) deficiency showed the restoration of ADA activity resulted from the reversion of an inherited mutation. *Blood* **97**: 2896–99.

Bhattacharya D, Rossi DJ, Bryder D, Weissman IL. (2006) Purified hematopoietic stem cell engraftment of rare niches corrects severe lymphoid deficiencies without host conditioning. *J Exp Med* **203**: 73–85.

Bittencourt H, Rocha V, Chevret S, *et al.* (2002) Association of CD34 cell dose with hematopoietic recovery, infections, and other outcomes after HLA-identical sibling bone marrow transplantation. *Blood* **99**: 2726–33.

Blaese RM, Culver KW, Miller AD, *et al.* (1995) T lymphocyte-directed gene therapy for ADA-SCID: initial trial results after 4 years. *Science* **270**: 475–80.

干细胞之家www.stemcell8.cn ←点击进入

Booth C, Hershfield M, Notarangelo L, *et al.* (2007) Management options for adenosine deaminase deficiency, *Proc EBMT Satellite Workshop*, Hamburg, March 2006. *Clin Immunol* **123**: 139–47.

Bordignon C, Notarangelo LD, Nobili N, *et al.* (1995) Gene therapy in peripheral blood lymphocytes and bone marrow for ADA- immunodeficient patients. *Science* **270**: 470–75.

Broxmeyer HE. (2005) Biology of cord blood cells and future prospects for enhanced clinical benefit. *Cytotherapy* **7**: 209–18.

Buckley RH. (2004) Molecular defects in human severe combined immunodeficiency and approaches to immune reconstitution. *Annu Rev Immunol* **22**: 625–55.

Buckley RH, Schiff RI, Schiff SE, *et al.* (1997) Human severe combined immunodeficiency: genetic, phenotypic, and functional diversity in one hundred eight infants. *J Pediatr* **130**: 378–87.

Buckley RH, Schiff SE, Schiff RI, *et al.* (1999) Hematopoietic stem-cell transplantation for the treatment of severe combined immunodeficiency. *N Engl J Med* **340**: 508–16.

Cavazzana-Calvo M, Carlier F, Le Deist F, *et al.* (2007) Long-term T-cell reconstitution after hematopoietic stem-cell transplantation in primary T-cell-immunodeficient patients is associated with myeloid chimerism and possibly the primary disease phenotype. *Blood* **109**: 4575–81.

Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, *et al.* (2000) Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science* **288**: 669–72.

Chan B, Wara D, Bastian J, *et al.* (2005) Long-term efficacy of enzyme replacement therapy for adenosine deaminase (ADA)-deficient severe combined immunodeficiency (SCID). *Clin Immunol* **117**: 133–43.

Colvin GA, Lambert JF, Abedi M, *et al.* (2004) Murine marrow cellularity and the concept of stem cell competition: geographic and quantitative determinants in stem cell biology. *Leukemia* **18**: 575–83.

Davies SM, Kollman C, Anasetti C, *et al.* (2000) Engraftment and survival after unrelated-donor bone marrow transplantation: a report from the National Marrow Donor Program. *Blood* **96**: 4096–102.

Engel BC, Podsakoff GM, Ireland JL, *et al.* (2007) Prolonged pancytopenia in a gene therapy patient with ADA-deficient SCID and trisomy 8 mosaicism: a case report. *Blood* **109**: 503–6.

Fischer A, Le Deist F, Hacein-Bey-Abina S, *et al.* (2005) Severe combined immunodeficiency. A model disease for molecular immunology and therapy. *Immunol Rev* **203**: 98–109.

Gaspar HB, Bjorkegren E, Parsley K, *et al.* (2006) Successful reconstitution of immunity in ADA-SCID by stem cell gene therapy following cessation of PEG-ADA and use of mild preconditioning. *Mol Ther* **14**: 505–13.

干细胞之家www.stemcell8.cn ←点击进入

Gaspar HB, Parsley KL, Howe S, *et al.* (2004) Gene therapy of X-linked severe combined immunodeficiency by use of a pseudotyped gammaretroviral vector. *Lancet* **364**: 2181–87.

Giblett ER, Anderson JE, Cohen F, *et al.* (1972) Adenosine-deaminase deficiency in two patients with severely impaired cellular immunity. *Lancet* **2**: 1067–69.

Glass B, Uharek L, Gaska T, *et al.* (1993) The influence of graft-versus-host reactivity, lymphocyte depletion, and cell dose on allogeneic bone marrow engraftment. *Bone Marrow Transplant* **12**(3): S41–S47.

Hacein-Bey-Abina S, Le Deist F, Carlier F, *et al.* (2002) Sustained correction of X-linked severe combined immunodeficiency by *ex vivo* gene therapy. *N Engl J Med* **346**: 1185–93.

Hacein-Bey-Abina S, von Kalle C, Schmidt M, *et al.* (2003a) A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *N Engl J Med* **348**: 255–56.

Hacein-Bey-Abina S, Von Kalle C, Schmidt M, *et al.* (2003b) LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* **302**: 415–19.

Haddad E, Landais P, Friedrich W, *et al.* (1998) Long-term immune reconstitution and outcome after HLA-nonidentical T-cell-depleted bone marrow transplantation for severe combined immunodeficiency: a European retrospective study of 116 patients. *Blood* **91**: 3646–53.

Harrison DE, Stone, M, Astle, CM. (1990) Effects of transplantation on the primitive immunohematopoietic stem cell. *J Exp Med* **172**: 431–37.

Hershfield MS. (1995) PEG-ADA: an alternative to haploidentical bone marrow transplantation and an adjunct to gene therapy for adenosine deaminase deficiency. *Hum Mutat* **5**: 107–12.

Hirschhorn R. (1993) Overview of biochemical abnormalities and molecular genetics of adenosine deaminase deficiency. *Pediatr Res* **33**: S35–S41.

Hirschhorn R, Yang DR, Puck JM, *et al.* (1996) Spontaneous *in vivo* reversion to normal of an inherited mutation in a patient with adenosine deaminase deficiency. *Nat Genet* **13**: 290–95.

Hofmeister CC, Zhang J, Knight KL, *et al.* (2007) *Ex vivo* expansion of umbilical cord blood stem cells for transplantation: growing knowledge from the hematopoietic niche. *Bone Marrow Transplant* **39**: 11–23.

Hoogerbrugge PM, van Beusechem VW, Fischer A, *et al.* (1996) Bone marrow gene transfer in three patients with adenosine deaminase deficiency. *Gene Ther* **3**: 179–83.

Ishikawa F, Yasukawa M, Lyons B, *et al.* (2005) Development of functional human blood and immune systems in NOD/SCID/IL2 receptor {gamma} chain (null) mice. *Blood* **106**: 1565–73.

Kawamura N, Ariga T, Ohtsu M, *et al.* (1999) *In vivo* kinetics of transduced cells in peripheral T cell-directed gene therapy: role of CD8+ cells in improved

干细胞之家www.stemcell8.cn ←点击进入

immunological function in an adenosine deaminase (ADA)-SCID patient. *J Immunol* **163**: 2256–61.

Kawamura N, Ariga T, Ohtsu M, *et al.* (1998) Elevation of serum IgE level and peripheral eosinophil count during T lymphocyte-directed gene therapy for ADA deficiency: implication of Tc2-like cells after gene transduction procedure. *Immunol Lett* **64**: 49–53.

Kohn DB, Hershfield MS, Carbonaro D, *et al.* (1998) T lymphocytes with a normal ADA gene accumulate after transplantation of transduced autologous umbilical cord blood $CD34^+$ cells in ADA-deficient SCID neonates. *Nat Med* **4**: 775–80.

Kohn DB, Weinberg KI, Nolta JA, *et al.* (1995) Engraftment of gene-modified umbilical cord blood cells in neonates with adenosine deaminase deficiency. *Nat Med* **1**: 1017–23.

Lapidot T, Dar A, Kollet O. (2005) How do stem cells find their way home? *Blood* **106**: 1901–10.

Liu A, Vosshenrich CA, Lagresle-Peyrou C, *et al.* (2006) Competition within the early B-cell compartment conditions B-cell reconstitution after hematopoietic stem cell transplantation in nonirradiated recipients. *Blood* **108**: 1123–28.

Malacarne F, Benicchi T, Notarangelo LD, *et al.* (2005) Reduced thymic output, increased spontaneous apoptosis and oligoclonal B cells in polyethylene glycol-adenosine deaminase-treated patients. *Eur J Immunol* **35**: 3376–86.

Markert ML. (1994) Molecular basis of adenosine deaminase deficiency. *Immunodeficiency* **5**: 141–57.

Mauch P, Hellman S. (1989) Loss of hematopoietic stem cell self-renewal after bone marrow transplantation. *Blood* **74**: 872–75.

Mortellaro A, Hernandez RJ, Guerrini MM, *et al.* (2006) *Ex vivo* gene therapy with lentiviral vectors rescues adenosine deaminase (ADA)-deficient mice and corrects their immune and metabolic defects. *Blood* **108**: 2979–88.

Myers LA, Patel DD, Puck JM, Buckley RH. (2002) Hematopoietic stem cell transplantation for severe combined immunodeficiency in the neonatal period leads to superior thymic output and improved survival. *Blood* **99**: 872–78.

Onodera M, Ariga T, Kawamura N, *et al.* (1998a) Successful peripheral T-lymphocyte-directed gene transfer for a patient with severe combined immune deficiency caused by adenosine deaminase deficiency. *Blood* **91**: 30–36.

Onodera M, Nelson DM, Yachie A, *et al.* (1998b) Development of improved adenosine deaminase retroviral vectors. *J Virol* **72**: 1769–74.

Otsu M, Sugamura K, Candotti F. (2000) *In vivo* competitive studies between normal and common gamma chain-defective bone marrow cells: implications for gene therapy. *Hum Gene Ther* **11**: 2051–56.

Quesenberry PJ, Colvin G, Abedi M. (2005) Perspective: fundamental and clinical concepts on stem cell homing and engraftment: a journey to niches and beyond. *Exp Hematol* **33**: 9–19.

干细胞之家www.stemcell8.cn ←点击进入

Rao SS, Peters SO, Crittenden RB, *et al.* (1997) Stem cell transplantation in the normal nonmyeloablated host: relationship between cell dose, schedule, and engraftment. *Exp Hematol* **25**: 114–21.

Schmidt M, Carbonaro DA, Speckmann C, *et al.* (2003) Clonality analysis after retroviral-mediated gene transfer to CD34^{+} cells from the cord blood of ADA-deficient SCID neonates. *Nat Med* **9**: 463–68.

Shultz LD, Lyons BL, Burzenski LM, *et al.* (2005) Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells. *J Immunol* **174**: 6477–89.

Stephan JL, Vlekova V, Le Deist F, *et al.* (1993) Severe combined immunodeficiency: a retrospective single-center study of clinical presentation and outcome in 117 patients. *J Pediatr* **123**: 564–72.

Stewart FM, Crittenden RB, Lowry PA, *et al.* (1993) Long-term engraftment of normal and post-5-fluorouracil murine marrow into normal nonmyeloablated mice. *Blood* **81**: 2566–71.

Thrasher AJ, Gaspar HB, Baum C, *et al.* (2006) Gene therapy: X-SCID transgene leukaemogenicity. *Nature* **443**: E5–E6; discussion E6–E7.

Thrasher AJ, Hacein-Bey-Abina S, Gaspar HB, *et al.* (2005) Failure of SCID-X1 gene therapy in older patients. *Blood* **105**: 4255–57.

Ueda T, Tsuji K, Yoshino H, *et al.* (2000) Expansion of human NOD/SCID-repopulating cells by stem cell factor, Flk2/Flt3 ligand, thrombopoietin, IL-6, and soluble IL-6 receptor. *J Clin Invest* **105**: 1013–21.

Urnov FD, Miller JC, Lee YL, *et al.* (2005) Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature* **435**: 646–51.

Vriesendorp HM. (2003) Aims of conditioning. *Exp Hematol* **31**: 844–54.

Wang Y, Schulte BA, LaRue AC, *et al.* (2006) Total body irradiation selectively induces murine hematopoietic stem cell senescence. *Blood* **107**: 358–66.

Zheng Y, Watanabe N, Nagamura-Inoue T, *et al.* (2003) *Ex vivo* manipulation of umbilical cord blood-derived hematopoietic stem/progenitor cells with recombinant human stem cell factor can up-regulate levels of homing-essential molecules to increase their transmigratory potential. *Exp Hematol* **31**: 1237–46.

干细胞之家www.stemcell8.cn ←点击进入

Chapter 2

Targeted Insertion of Transgene into a Specific Site on Chromosome 19 by Using Adeno-Associated Virus Integration Machinery

Masashi Urabe*, Yoko Obara, Takayuki Ito, Hiroaki Mizukami, Akihiro Kume and Keiya Ozawa*

Site-specific integration of the therapeutic transgene is favorable for gene therapy applications since it minimizes the risk of insertional mutagenesis and thereby prevents target cells from developing tumors. Adeno-associated virus (AAV), a member of parvovirus, is unique in that it integrates its genome into a specific site termed the AAVS1 locus (19q13.4) in the human genome. A non-structural replication initiator protein of AAV, Rep78 or Rep68, binds the inverted terminal repeat (ITR) sequence at either end of the AAV genome via tandem repeats of the GAGC/GCTC motif. A homologous sequence exists at the AAVS1 site. The Rep protein recognizes it and drives the integration of the AAV genome into AAVS1. The ITR is a *cis* element sufficient for AAVS1-specific integration. The incorporation of the ITR sequence into plasmid DNA is thus discussed in terms of Rep-mediated site-specific integration and of AAVS1 as a hazard-free target for transgene integration. Therefore, the use of the AAV integration machinery should allow us to develop a safer gene delivery system.

Keywords: Site-specific transgene integration; adeno-associated virus; Rep protein; AAVS1 locus.

*Corresponding authors. murabe@jichi.ac.jp, kozawa@ms2.jichi.ac.jp
Division of Genetic Therapeutics, Center for Molecular Medicine,
Jichi Medical University, Tochigi 329-0498, Japan

干细胞之家www.stemcell8.cn ←点击进入

1. Introduction

Stem cells such as hematopoietic stem cells, embryonic stem (ES) cells and mesenchymal stem cells (MSCs) are attractive targets for gene therapy since they replicate themselves and differentiate into various cell lineages. To manipulate genes in these cells, it is especially important to utilize a system to introduce therapeutic DNA with a minimal risk of insertional mutagenesis. Insertion of the gene of interest into a defined site in the human chromosome is desirable. However, current strategies that achieve the integration of transgene into host chromosomal DNA insert it randomly, which is an insertional mutagenic/oncogenic hazard as shown with retroviral vectors. It is undoubtedly a milestone in gene therapy that 10 patients with X-linked severe combined immune deficiency, a lethal inherited disease characterized by an early block in T and natural killer lymphocyte differentiation due to mutations of the gene encoding the γc cytokine receptor subunit, were treated by infusion of autologous CD34$^+$ hematopoietic stem cells transduced with a murine retrovirus vector encoding the common γ chain (Cavazzana-Calvo *et al.*, 2000; Hacein-Bey-Abina *et al.*, 2002). Unfortunately T-cell leukemia developed in four patients approximately three years after gene therapy (Hacein-Bey-Abina *et al.*, 2003; Baum, 2007). The vector sequence was integrated into the upstream region or the first intron of the LMO2 gene, which is known to be a T-cell proto-oncogene. The activation of the LMO2 gene by chromosomal translocation has been reported in patients with T-cell acute leukemia (Nam and Rabbitts, 2006). The integrated vector promoter, the long terminal repeat (LTR) enhanced the activity of the LMO2 promoter and resulted in aberrant LMO2 expression and premalignant cell proliferation (Hacein-Bey-Abina *et al.*, 2003). To prevent such an adverse event, it is absolutely necessary to employ a strategy that introduces foreign DNA specifically into a predefined safe region of chromosomal DNA.

Prokaryotic site-specific recombinases such as Cre (Sauer and Henderson, 1988) or Flt (Andrews *et al.*, 1985) are widely used to introduce DNA into a site that their respective recognition sequence in eukaryotic cells as well as prokaryotic cells. However, the recognition sequence must be inserted into target chromosomal DNA in advance. Phage phiC31 integrase recognizing phage *attP* and *attB* sites has been shown to mediate site-specific DNA integration in human genome at native "pseudo" *attP* sites (Thyagarajan *et al.*, 2001).

AAV integrates its genome into a particular site in human chromosome 19, termed AAVS1 (19q13.4) (Kotin *et al.*, 1992; Samulski *et al.*, 1991), through the activity of a specific replicase/integrase protein, Rep. Taking advantage

干细胞之家www.stemcell8.cn ←点击进入

of the AAV integration machinery, systems for AAVS1-specific integration of therapeutic DNA have been developed (Balague *et al.*, 1997; Surosky *et al.*, 1997). The systems are particularly valuable for *ex vivo* gene therapy applications for stem cells. Here the authors will review the current trend of the development of Rep-mediated AAVS1-targeted integration as well as basic biology of the site-specific integration of AAV.

2. Overview of AAV

The adeno-associated virus (AAV) belongs to the family *Parvoviridae* and is classified into the genus *Dependovirus*. A number of AAV serotypes have been reported so far. The AAV serotype 2 was sequenced more than 20 years ago and has been most extensively studied (for general review, see: Muzyczka, 2001; Smith, 2002). The type 2 is referred here as AAV. The AAV depends for its replication and propagation on a helper virus such as adenovirus, herpes virus, papilloma virus and vaccinia virus. When the AAV infects cells alone, it enters a latent infection phase and integrates into the human genome preferentially into the AAVS1 locus on chromosome 19 (19q14.2) (Kotin *et al.*, 1992; Samulski *et al.*, 1991). The integrated genome can be activated and rescued by subsequent superinfection by a helper virus (Fig. 1). There is no disease reported associated with AAV infection. More than 80% of adults are seropositive for antibodies against the AAV. The AAV is used as a gene-transfer vector particularly for long-term gene expression in livers, neurons and muscles. However, AAV vectors that are devoid of the *rep* gene fail to integrate into AAVS1 (see below for details).

The wild-type AAV has been reported to integrate into the AAVS1 site in immortalized cell lines at a frequency of 68% (Kotin *et al.*, 1990) or 94% (Kearns *et al.*, 1996). The insertion of AAV DNA into the AAVS1 site was also reported in human testis tissue (Mehrle *et al.*, 2004). The AAV vector genome has also been shown to persist extrachromosomally in cells (Afione *et al.*, 1996; Duan *et al.*, 1998; Nakai *et al.*, 2001). A recent study reported that the AAV genome in humans appears to persist as episomal forms, not as integrated forms; an attempt to detect a junction DNA sequence between the AAV genome and host chromosome by PCR from clinical tonsil-adenoid samples obtained from children was made. The junction sequence, however, could not be amplified, suggesting that the AAV genome existed in the respiratory tissues as an episomal form (Schnepp *et al.*, 2005). Thus, the mode of persistence of the AAV genome may be different among cell types.

The AAV genome is a single-stranded DNA of 4.8 kb in size. Either end of the genome shows a unique T-shaped hairpin configuration, which is

干细胞之家www.stemcell8.cn ←点击进入

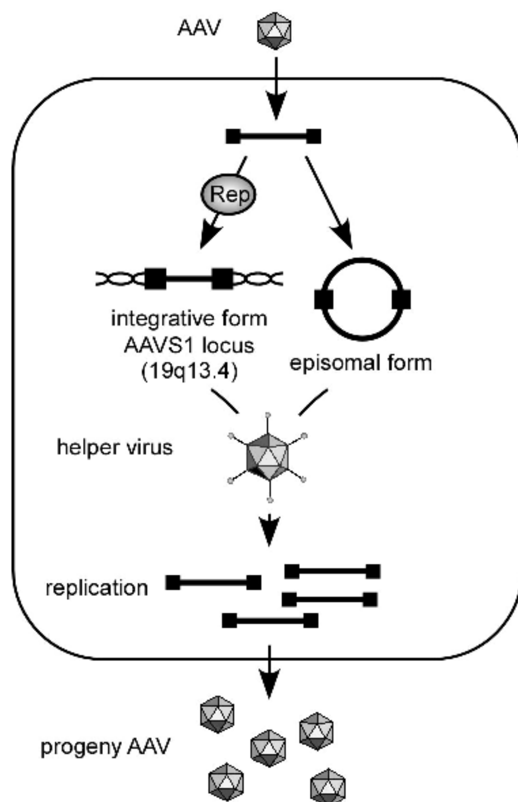


Fig. 1. Life cycle of AAV. Following infection, the AAV genome persists as an integrative or episomal form. The AAV genome preferentially integrates into the AAVS1 site (19q13.4) in the presence of the Rep protein. When AAV and a helper virus (e.g., adenovirus) coinfect cells or a helper virus superinfects the cells harboring the AAV genome, burst replication of AAV occurs.

called an inverted terminal repeat (ITR). The ITR serves as an origin of AAV genome replication. Between the ITRs are two open reading frames corresponding to *rep* and *cap* [Fig. 2(A)]. The *rep* gene encodes four overlapping nonstructural proteins, Rep78, Rep68, Rep52 and Rep40. The *cap* gene codes for structural proteins, VP1, VP2 and VP3. On the genome are three promoters, p5, p19, and p40, designated according to their map positions. The unspliced and spliced transcripts from the p5 promoter encode Rep78 and Rep 68, respectively, while Rep52 and Rep40 are translated from p19-unspliced and -spliced transcripts. Rep78 or Rep68 is a replication initiator of the AAV genome, which possesses site-specific, strand-specific endonuclease activity, ATP-dependent helicase activity (Im and Muzyczka, 1990), and ligase activity (Smith and Kotin, 2000). The Rep78 and Rep68 proteins bind

干细胞之家www.stemcell8.cn ←点击进入

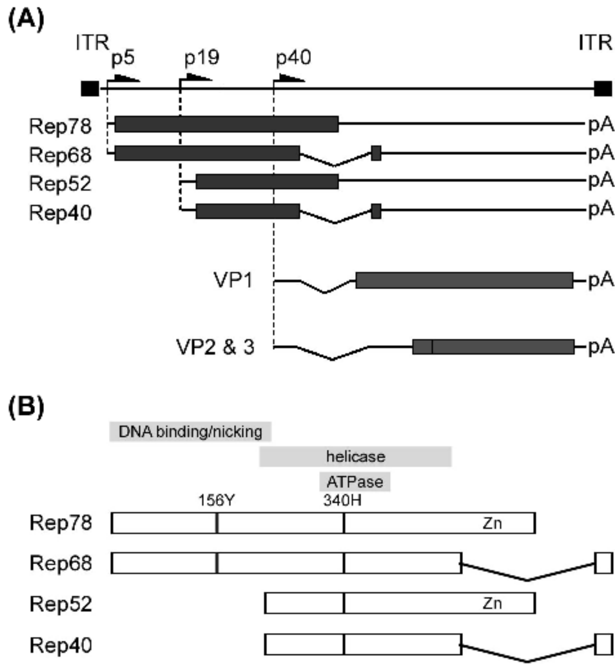


Fig. 2. AAV genome and Rep proteins. **(A)** Map of the AAV genome. Three promoters, p5, p19, and p40 drive transcription of Rep78/68, Rep52/40, and VP proteins, respectively. Spliced transcript from p5 and p19 encode Rep68 and Rep40 proteins, respectively. **(B)** Map of Rep proteins. The domains responsible for DNA binding/nicking, helicase and ATPase domains are indicated. The C-terminal portion of Rep 78 or Rep52 comprises a Zn-finger like motif. The tyrosine and histidine residues at position 156 and 340 are indicated, respectively.

the ITR via the Rep binding site (RBS) consisting of five tandem repeats of the GAGC/GCTC tetranucleotide, self-associate to form a hexameric complex (Hickman *et al.*, 2004; Smith *et al.*, 1997), extrude the terminal resolution site (*trs*), thereby forming a stem-loop structure via their helicase activity (Brister and Muzyczka, 1999) and nicking at the *trs* between the thymidine residues. The protrusion of the *trs* is a prerequisite for nicking at this site by the Rep protein. The site-specific nicking event is followed by unwinding of the terminal hairpin. The smaller Rep52 and Rep40 proteins also have a helicase activity (Collaco *et al.*, 2003; Smith and Kotin, 1998) and are involved in the packaging of the AAV genome into viral capsids (King *et al.*, 2001).

A number of mutational studies and the determination of the three-dimensional structure of the Rep protein (Hickman *et al.*, 2002 and 2004) revealed that the N-terminal half of the large Rep polypeptide is responsible for DNA binding and endonuclease activity. The central portion is essential for helicase activity, ATPase activity and multimerization of Rep proteins.

干细胞之家www.stemcell8.cn ←点击进入

The lysine residue at position 340 associates with ATP. The tyrosine residue at position 156 covalently links to the 5′-end of single stranded DNA, a product derived from the nicking reaction mediated by the Rep protein [Fig. 2(B)]. Charged amino acids that are important for the site-specific integration were identified in the N-terminal half of Rep78 (Urabe *et al.*, 1999).

Either large Rep protein is capable of supporting the replication of the AAV genome (Holscher *et al.*, 1995). However, functional differences between the two have been reported. Rep68 shows a stronger nicking activity than its counterpart and is more efficient in processing dimers to monomer duplex DNA (Ni *et al.*, 1998 and 1994), which is an intermediate replicative form of the AAV genome. The helicase activity of Rep78 has been reported to be stronger (Wollscheid *et al.*, 1997). Rep78 suppresses CREB-dependent transcription by the interaction of a domain unique to Rep78 with protein kinase A (PKA) (Chiorini *et al.*, 1998; Di Pasquale and Stacey, 1998) which implies that Rep78 (or Rep52) indirectly inhibits adenoviral nuclear transport by PKA (Suomalainen *et al.*, 2001) and CREB-dependent adenovirus promoters E1A, E2 and E4 (Leza and Hearing, 1989). Rep68, but not Rep78, associates with 14-3-3 proteins through phosphorylated serine at position 535 and its interaction may affect the life cycle of AAV (Han *et al.*, 2004).

3. Mechanism of AAVS1-Targeted Integration of the AAV Genome

There are similar RBS and *trs* sequences in the AAVS1, which correspond to the first exon and intron of the *MBS85* gene (Fig. 3). The Rep protein binds the RBS, which is the first event during the replication and amplification of the AAV genome. The Rep protein has been shown to mediate complex formation between AAV DNA and AAVS1 DNA (Weitzman *et al.*, 1994). It has been reported that the Rep protein asymmetrically amplifies the AAVS1 sequence (Urcelay *et al.*, 1995), suggesting that a similar event observed on the ITR during the replication of the AAV genome takes place on the AAVS1 locus as well. A model of deletion-substitution mechanism has been proposed for the mechanism of the site-specific integration of the AAV genome (Linden *et al.*, 1996a).

The ITR sequence alone appears to enhance the integration into host chromosomal DNA (Lieber *et al.*, 1999; Philip *et al.*, 1994), albeit not site-specifically. The minimal *cis* element for AAVS1-specific integration is controversial. A series of analyses of AAV integration events on an EBV-based episomal vector harboring the human AAVS1 sequence in the HEK293 cell

干细胞之家www.stemcell8.cn ←点击进入

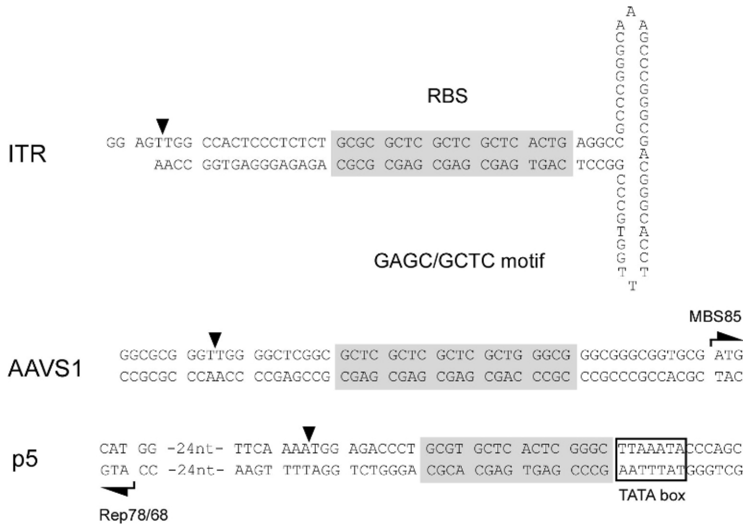


Fig. 3. Comparison of the ITR, AAVS1 and p5 promoter. Terminal resolution site (*trs*) or nicking site is indicated by an arrowhead. A motif consisting of five or four tandem repeats of the GAGC/GCTC tetranucleotide constitutes a Rep binding site (RBS). The initiation codon for myosin binding subunit 85 (MBS85) or for Rep78/68 is indicated by an arrow. The TATA box on the p5 promoter is indicated by a box.

line expressing EBNA-1, revealed that both the RBS and the *trs* sequences are required for AAVS1-targeted integration (Linden *et al.*, 1996a and 1996b). Removal of the sequence upstream of the *trs* appears to result in a decrease in the frequency of AAVS1 specific-integration (Linden *et al.*, 1996a and 1996b). The eight-base spacer sequence between the RBS and *trs* also appears important for site-specific integration, as partial replacement with unrelated nucleotides in the spacer sequence greatly reduces the integration frequency (Meneses *et al.*, 2000). On the other hand, a recent study reported that the RBS motif alone is sufficient for the Rep-mediated AAVS1-specific insertion of a GFP/Neo plasmid into HEK293 cells (Feng *et al.*, 2006). Seven out of 19 (37%) G418-resistant clones were considered to have the GFP/Neo transgene integrated at the AAVS1 site by Southern analysis. The RBS motif within the p5 promoter could direct the GFP/Neo gene into AAVS1 albeit with a lower frequency (2 of 13 clones).

Although the RBS in the ITR is the primary binding site for the Rep protein, a tip of the T-shaped hairpinned structure of the ITR is identified as another Rep association site (Ryan *et al.*, 1996). It has been shown that the affinity of the Rep protein for the RBS in the ITR is higher than for the RBS analog in the p5 promoter, as revealed by electrophoretic mobility shift assay

干细胞之家www.stemcell8.cn ←点击进入

(Glauser *et al.*, 2005). The ITR thus is the perfect substrate for Rep binding and seems to mediate AAVS1-specific integration most efficiently. The Rep protein is capable of associating with transcription factor Sp1 (Hermonat *et al.*, 1996; Pereira and Muzyczka, 1997) and the TATA binding protein (TBP) (Francois *et al.*, 2005; Hermonat *et al.*, 1998; Su *et al.*, 2000), in both cases binding to the p5 promoter region. The association of Rep with Sp1 and TBP may stabilize the binding of Rep to the p5 element, compensate for Rep's lower affinity for the DNA substrate, and enhance the site-specific integration.

Besides driving transcription of Rep78 and Rep68 mRNA, the p5 promoter sequence also serves as an origin of replication that promotes the amplification of integrated AAV genome (Francois *et al.*, 2005; Nony *et al.*, 2001). The p5 sequence element also enhances the AAVS1-specific integration and is sufficient for the site-specific integration (Philpott *et al.*, 2002a and 2002b). The p5 promoter comprises a RBS and *trs* homolog like the ITR and the AAVS1 site. The Rep protein binds the p5-RBS (Kyostio *et al.*, 1995) and nicks at the *trs* homolog (Francois *et al.*, 2005). The analysis of junction sequences between Rep-mediated integrants and host AAVS1 site revealed that some break points occur at the p5 promoter (Tsunoda *et al.*, 2000), which corroborates the presence of a *cis* element for AAVS1-specific integration.

Cellular factors involved in Rep-mediated integration into AAVS1 have not been fully identified although the Rep protein is a major player. A protein capable of associating with the Rep protein may be involved in the targeted integration. The DNA-dependent protein kinase, which is involved in the repair of double-stranded DNA break and in V(D)J recombination by nonhomologous end-joining, is responsible for the circularization and concatemerization of the AAV genome in cells and inhibits the site-specific integration into host DNA (Song *et al.*, 2004). Recently the TAR RNA-binding protein of 185 kDa (TRP-185), which was identified as a protein binding to the TAR RNA loop of human immunodeficiency virus type 1 (Wu *et al.*, 1991), has been shown to bind to the RBS region. Inhibiting the integration at the RBS of the AAV genome, TRP-185 appears to promote the hexamerization of the Rep protein and to enhance the integration downstream of the RBS (Yamamoto *et al.*, 2007).

4. AAV Target Site: AAVS1 Locus (19q13.4)

The AAVS1 locus (19q13.4) overlaps with the first exon of the *MBS85* gene encoding myosin binding subunit 85 or protein phosphatase 1 regulatory

干细胞之家www.stemcell8.cn ←点击进入

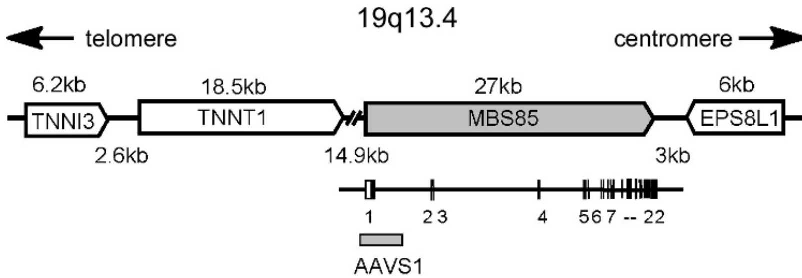


Fig. 4. Location of the AAVS1 site. The AAVS1 site of approximately 2 kb in length overlaps with the first exon and intron of *MBS85*, a hot spot of AAV integration. TNNI3, cardiac troponin I; TNNT1, slow skeletal troponin T1; MBS85, myosin binding subunit 85; EPS8L1, epidermal growth factor receptor pathway substrate 8-like protein 1. (Adapted from a figure by Dutheil *et al.* 2004.)

subunit 12C (Fig. 4). MBS85 is one of the effector molecules in Rho signaling pathway and is involved in the assembly of myosin chains (Tan *et al.*, 2001). Rho is a small molecule with GTPase activity (Bishop and Hall, 2000). RhoA associates with a kinase and the kinase phosphorylates MBS85. The phosphorylated MBS85 inhibits the myosin light chain phosphatase and a net increase in myosin light chain phosphorylation activates myosin and finally induces stress fiber formation. A region upstream of the initiation codon for MBS85 is homologous to the AAV origin of replication, at which are two elements essential for AAV integration: a RBS homolog and a *trs* homolog.

A sequence homologous to human AAVS1 is also found in the simian (Amiss *et al.*, 2003) as well as rodent genome (Dutheil *et al.*, 2004). Integration of the AAV genome into the simian AAVS1 ortholog was identified in a cell line, COS7 (Amiss *et al.*, 2003). Rep68 that are derived from serotype 4 AAV, which was originally isolated from monkey, has a higher affinity for simian RBS than AAV2 Rep68. A survey in genomic banks revealed the presence of an AAVS1 ortholog in the mouse genome too (Dutheil *et al.*, 2004). *In vitro* studies showed that a recombinant type 2 Rep68 can bind to mouse RBS and nick at *trs*, suggesting that the AAV genome would target the mouse AAVS1 locus if the genome is delivered to the nucleus.

The integration of the AAV genome occurs mostly within approximately 2 kb downstream of the RBS (Linden *et al.*, 1996a). The integration of the AAV genome or a transgene into AAVS1 is accompanied by deletion of the target site. After nicking at the *trs*, the Rep protein unwinds target DNA progressively and the DNA with the ITR sequences replaces the existing host DNA, which is contrasted to the microdeletion accompanying the retrovirus-mediated integration event.

干细胞之家www.stemcell8.cn ←点击进入

5. Multifunctional Rep Protein

The Rep proteins are "sticky" and have been reported to associate with a number of cellular proteins, including TBP (Hermonat *et al.*, 1998; Su *et al.*, 2000); Sp1 (Hermonat *et al.*, 1996; Pereira and Muzyczka, 1997); E2F-1 (Batchu *et al.*, 2001), p53 (Batchu *et al.*, 1999), pRB(Batchu *et al.*, 2002); a topoisomerase I binding protein (Topors) (Weger *et al.*, 2002); protein kinase A (PKA); a protein kinase (PrKX) (Chiorini *et al.*, 1998; Di Pasquale and Stacey, 1998); transcription-positive cofactor 4 (PC4) (Muramatsu *et al.*, 1998; Weger *et al.*, 1999); 14-3-3 proteins (association with Rep68 via phosphrylated serine at position 535) (Han *et al.*; 2004); UBC9 (E2 conjugating enzyme for the small ubiquitin-related polypeptide SUMO-1) (Weger *et al.*, 2004); TRP-185 (Yamamoto *et al.*, 2007); and high mobility group chromosomal protein 1 (HMG1) (Costello *et al.*, 1997).

The anti-proliferative action of the Rep protein is partially explained by the association of the Rep protein with TBP (Hermonat *et al.*, 1998; Su *et al.*, 2000), which is required for the assembly of the transcription initiation complex. The CREB protein (cAMP responsive element binding protein) is a transcription factor and is activated by PKA, which plays a central role in cell growth and development. Thus, the inhibiton of PKA by the Rep protein eventually suppresses the CREB-dependent transcriptional activation (Chiorini *et al.*, 1998; Di Pasquale and Stacey, 1998). PC4 is involved in the downregulation of the p5, p19, and p40 promoters in the absence of a helper virus (Weger *et al.*, 1999). Coinfection of adenovirus relieves the suppression of the AAV promoters (Weger *et al.*, 1999) and enhances the replication of the AAV genome (Muramatsu *et al.*, 1998). UBC9 is involved in the attachment of small ubiquitin-like modifier (SUMO-1), which protects a protein from ubiquitin-mediated degradation. A prolonged life span of the Rep protein (being protected by UBC9) may establish AAV latency in cells (Weger *et al.*, 2004). The association of adenovirus E1b with p53 induces ubiquitin-mediated degradation of the p53 tumor suppressor gene product and this disturbs the cell cycle pathway (Ciechanover *et al.*, 1994). AAV Rep78 associates with p53 and prevents it from being degradated (Batchu *et al.*, 1999). Topors appears to enhance the expression of Rep78, Rep52 and capsid proteins as a transcriptional regulator in the absence of a helper virus (Weger *et al.*, 2002). The 14-3-3 proteins are associated with a number of cellular proteins and implicated in their modification (Fu *et al.*, 2000). The interaction between Rep68 and 14-3-3 proteins results in reduced DNA binding activity of Rep68. However, the significance of the interaction has to be elucidated (Han *et al.*, 2004). A transcription factor E2F-1 is suppressed by its interaction

干细胞之家www.stemcell8.cn ←点击进入

with the retinoblastoma protein (pRB). Rep78 associates with E2F-1 and stabilizes the E2F-1-pRB complex (Batchu *et al.*, 2002). The Rep protein has been reported to interact with Sp1 and to inhibit a promoter bearing an Sp1 binding motif (Hermonat *et al.*, 1996). The Rep protein also binds to the E2 transactivator of human papilloma virus (HPV) type 16, disrupts the binding of E2 to the cellular transcriptional coactivator p300, and inhibits the replication and transforming activities of HPV (Marcello *et al.*, 2000).

Although the consensus Rep binding site on the ITRs is a five tandem repeat of the GAGC/GCTC tetranucleotide, the Rep proteins can bind to imperfect RBS motifs (Chiorini *et al.*, 1995). Within the AAV p5, p19, and p40 promoter regions, incomplete RBS motifs were identified and the Rep protein has been shown to bind there (Kyostio *et al.*, 1995). The Rep protein regulates the AAV p5, p19, and p40 promoter activity. In latent infection, the large Rep protein suppresses the p5 promoter, which is suitable for persistence of the AAV genome. In the presence of adenovirus, the large Rep protein trans-activates the p19 and p40 promoters.

Systematic *in vitro* studies indicated that the Rep protein can binds to many cellular promoter regions, including the c-*sis* proto-oncogene, glucose transporter, and KIP2 (cyclin-dependent kinase inhibitor) (Wonderling and Owens, 1996 and 1997). The Rep polypeptides have been shown to down-regulate the promoters of a number of genes such as c-H-*ras* , c-*fos*, c-*myc* (Batchu *et al.*, 1994; Hermonat, 1991 and 1994); c-*sis* (Wonderling and Owens, 1996), E2F-1 (Batchu *et al.*, 2001); and the LTR promoter of the human immunodeficiency virus 1 (Batchu and Hermonat, 1995; Oelze *et al.*, 1994). A detailed study showed that Rep suppresses the HPV type 16 p97 promoter by inhibiting the binding of TBP to the p97 promoter (Su *et al.*, 2000) or by directly binding the p97 promoter region (Zhan *et al.*, 1999). While most promoter activities are down-regulated by Rep78 or Rep68, some promoters, such as the human cytomegalovirus (CMV) immediate early promoter (Wonderling *et al.*, 1997) and the c-*sis* promoter (Wonderling and Owens, 1996) are trans-activated. The Rep protein also suppresses translation (Takeuchi *et al.*, 2000).

AAV inhibits the replication of viruses (Bantel-Schaal and zur Hausen, 1988) and cellular transformation by SV40 or Ad E1a plus *ras* oncogene (Khleif *et al.*, 1991). Establishment of cellular transformants by plasmid transfection was also inhibited (Labow *et al.*, 1987). A cellular factor induced by AAV infection that suppresses cell proliferation has been reported (Bantel-Schaal, 2001). It is reported that AAV infection results in an increase in p21 level, a cyclin-dependent kinase inhibitor (Hermanns *et al.*, 1997). Rep68 and Rep78 induce cell cycle arrest in G1 and G2 phases and, in addition,

干细胞之家www.stemcell8.cn ←点击进入

Rep78 inhibits S-phase progression by accumulating active hypophosphorylated pRb (Saudan *et al.*, 2000), a negative regulator of transcription factors (Weinberg, 1995). Infection with AAV suppresses indirectly the HPV18 promoter activity in transgenic mice (Walz *et al.*, 2002). The Rep protein has been reported to suppress the expression of c-*myc*, c-*myb*, and pRb whereas it up-regulates c-*fos* expression (Klein-Bauernschmitt *et al.*, 1992).

6. The Use of the AAV Integration Machinery to Achieve Site-Specific Integration

Since the elements required for AAVS1-specific integration of AAV are Rep78 or Rep68, and the ITR sequences, it is possible to insert any DNA sequence linked to an ITR sequence into AAVS1 if the Rep78 or Rep68 protein is expressed simultaneously. Early studies demonstrated that a reporter gene was efficiently inserted into the AAVS1 site in cultured cells such as HeLa cells and HEK293 cells, although the frequency of the site-specific integration differed (Balague *et al.*, 1997; Shelling and Smith, 1994; Surosky *et al.*, 1997). Table 1 summarizes the studies of AAVS1-targeted integration in cultured cells.

Shelling and Smith inserted a *Neo* gene downstream of the p40 promoter, the original promoter for capsid protein expression (Shelling and Smith, 1994). The plasmid construct harbored the coding sequence for the p5-, p19-*rep* genes and a p40-driven *Neo* gene flanked by the ITRs (Tratschin *et al.*, 1985). Since the open reading frames (ORFs) of *rep78* and *rep68* extend over the p40 promoter, the Rep proteins expressed from this plasmid were C-terminally truncated. After transfecting it into HeLa cells and HEK293 cells, they analyzed G418-resistant clones by Southern blot and showed that nine out of 12 clones had the *Neo* gene integrated in AAVS1. They also generated an AAV vector with the same *rep* and *Neo* genes. The AAVS1-specific integration efficiency was 82% (nine out of 11 clones).

Balague *et al.* adopted a similar strategy, but the difference is that a *rep78* gene was placed outside the ITR-flanked *GFP* transgene portion on the plasmid. One day after transfecting HEK293 cells, they sorted fluorescent cells and plated them at 1 cell per well, thereby isolating single-cell clones without selection for stable transgene expression analysis. Forty five percent (103 out of 227) of the clones were GFP-positive as compared to eight out of 167 with control *rep78*-free plasmid. Southern analysis showed that in five out of nine (55%) clones integration of the GFP gene was into AAVS1. The results indicated that Rep78 enhances the chromosomal integration of an ITR plasmid both site-specifically and randomly (Balague *et al.*, 1997).

Table 1 Studies of AAVS1-targeted Integration in Cultured Cells

Authors	Year	Cells	Transfection[1] (*n* Plasmid[2])	Rep Expression: Promoter (Protein)	Transgene (*cis* Element)	AASV1/Total[3] (% Targeted)	Analysis of Integration
Shelling *et al.*	1994	HeLa/293	C (1)	p5 (Rep78/68)	Neo (ITR)	9/12 (75)	Southern
Balagué *et al.*	1997	293	C (1)	p5 (Rep78)	Neo (ITR)	5/9 (45)	Southern
Surosky *et al.*	1997	293	C (2)	RSV (Rep78/68)	LacZ (ITR)	6/7 (86)	Southern/FISH
Pieroni *et al.*	1998	HeLa	C (1)	p5 (Rep78/68)	GFP/Neo (ITR)	15/100 (15)	Southern/FISH
		Huh-7	L (1)	p5 (Rep78/68)	GFP/Neo (ITR)	5/55 (9)	Southern/PCR
Lamartina *et al.*	1998	HeLa	L (1)	CMV (Rep78)	Neo (ITR)	9/37 (24)	Southern
		HeLa	L (1)	CMV (Rep68)	Neo (ITR)	8/37 (22)	Southern
		HeLa	L (1)	Rep68 protein[4]	Neo (ITR)	N.A.[5]	PCR
Tsunoda *et al.*	2000	HeLa	L (1)	p5 (Rep78/68)	Neo (ITR/p5)	22/36 (61)	Southern/PCR
Rinaudo *et al.*	2000	HeLa	C (2)	CMV (Rep78/68-PR[6])	Neo (ITR)	7/28 (25)	Southern
Kogure *et al.*	2001	K562	L (2)	MMTV/IRES (Rep78)	Neo (ITR)	6/17 (22)	Southern/FISH
		K562	E (2)	CMV (Rep78)	Neo (ITR)	8/25 (32)	Southern/FISH
Huttner *et al.*	2003	HeLa	C (rAAV[7])	p5 (Rep78/68)	GFP/Hygro (ITR)	7/10 (70)	Southern/FISH
Urabe *et al.*	2003	293	C (2)	CMV (Rep78)	Neo (ITR)	4/17 (24)	Southern/FISH
		293	C (2)	CMV (Rep68)	Neo (ITR)	8/20 (40)	Southern/FISH
Philpott *et al.*	2004	HeLa	E (1)	p5 (Rep78/68)	GFP (ITR/p5)	82/95 (86)	Southern
		HeLa	E (1)	p5 (Rep78/68)	GFP (p5)	44/47 (94)	Southern
		HeLa	E (2)	T7 (Rep78/68)	CAT (p5)	9/10 (90)	Southern
Wong, Jr. *et al.*	2006	293	C (rAAV2[7])	CMV (Rep78/68-VP22[8])	SEAP (ITR)	N.A.	PCR
Feng *et al.*	2006	293	C (2)	p5 (Rep78/Rep68)	GFP/Neo (RBS)	7/19 (37)	Southern/PCR

[1]Method used: C, calcium phosphate method; L, lipofection; E, electroporation.
[2]Number of plasmids used for transfection. "1" indicates that a *rep* gene and a transgene cassette were on the same plasmid. "2" indicates that a *rep* plasmid and a transgene plasmid were cotransfected.
[3]Number of clones with AAVS-targeted integration/total number of clones analyzed.
[4]Purified recombinant Rep68 protein was transfected instead of a Rep68 expression cassette.
[5]N.A., not available.
[6]PR (ligand binding domain of the progesterone receptor) was fused to Rep78/68.
[7]AAV vector transduction was used instead of plasmid transfection.
[8]VP22 (a tegument protein of herpes simplex virus) was fused to Rep78/68.

千细胞之家www.stemcell8.cn ←点击进入

In order to reduce the frequency of the chromosomal integration of the *rep* gene, Surosky *et al.* used a two-plasmid system: one plasmid is for the expression of the Rep protein and the other is an ITR-linked transgene plasmid (Surosky *et al.*, 1997). The idea is that the frequency of integration of a *rep* plasmid is lower than that of an ITR-plasmid when the Rep protein is supplied *in trans*. They concluded that six out of seven LacZ-positive colonies had the LacZ transgene into the AAVS1 site as revealed by Southern blot analysis. Fluorescent *in situ* hybridization (FISH) confirmed that two out of six clones had a LacZ signal on chromosome 19. They also showed that Rep78 or Rep68 alone was sufficient for promoting AAVS1-specific integration and that one ITR sequence could target integration to the AAVS1 site. The full sequence of one AAVS1-integrant was determined and it was thus shown that the whole plasmid was integrated at AAVS1 in tandem array.

The work by Pieroni *et al.* described the transfection of HeLa cells or Huh-7 cells with a plasmid harboring the *p5-rep* gene and an ITR-flanked GFP/Neo cassette, with the resulting generation of clones with the transgene integrated in AAVS1 (7 to 25%), which were then analyzed by Southern blot. AAVS1-specific integration was confirmed in one Huh-7 clone by PCR amplification of the ITR-AAVS1 junction sequence and in three HeLa cell clones by FISH colocalizaiton of the GFP/Neo and AAVS1 signals (Pieroni *et al.*, 1998).

By using an AAV vector plasmid similar to that of Shelling and Smith, Tsunoda *et al.* tested site-specific integration in HeLa cells (Tsunoda *et al.*, 2000). In addition, their plasmid had a hygromycin resistance gene beside the ITR-flanked *p5-rep* and *Neo* gene cassette. Southern blot analysis showed that 22 out of 36 (61%) clones had the *GFP/Neo* gene in AAVS1. The junction sequence between the ITR and AAVS1 was amplified by PCR and this showed that the junction occurred in the p5 promoter region. A RBS homolog and *trs* homolog exist in the p5 promoter region, where Rep78 or Rep68 binds and regulates the p5 promoter activity. The Rep-mediated amplification of the AAV genome occurs via the RBS homolog (Nony *et al.*, 2001; Tessier *et al.*, 2001; Tullis and Shenk, 2000), which is important for efficient production of AAV progeny. In addition, the p5 element has been shown to be sufficient for AAVS1-specific integration (Philpott *et al.*, 2002a and 2002b). All the junction sequences amplified had a partially deleted p5 portion, not an ITR sequence (Tsunoda *et al.*, 2000). This is probably partially because cell survival depends on the inactivation of the *rep* gene. The Rep protein is cytotoxic and anti-proliferative (see above). Tsunoda *et al.* also speculated that the plasmid backbone was also simultaneously inserted into AAVS1 as

干细胞之家www.stemcell8.cn ←点击进入

well as the ITR-flanked *rep/Neo* cassette, an observation consistent with the result of Surosky *et al.* (Surosky *et al.*, 1997).

Kogure *et al.* applied the AAVS1-targeting system to K562 cells, a hematopoietic cell line (Kogure *et al.*, 2001). To limit the expression of Rep78, they used a weak promoter derived from the long terminal repeat (LTR) of mouse mammary tumor virus (MMTV) driving bicistronic expression of GFP and Rep78. They transfected the *rep* plasmid and an ITR-Neo plasmid into K562 cells, analyzed 17 clones by Southern blot and found that six clones (22%) harbored the *Neo* gene in AAVS1. FISH analysis confirmed that five out of six clones had a *Neo* gene on chromosome 19.

Urabe *et al.* used a CMV-driven *rep78* gene plasmid for AAVS1-specific integration in HEK 293 cells (Urabe *et al.*, 2003). To regulate the expression level of Rep78, they used decreasing amounts of *rep78* plasmid. They observed that approximately 20% of the clones analyzed by Southern blot showed integration of the *Neo* gene into AAVS1. They also compared the efficiency of targeted integration mediated by Rep78 and Rep68, and found that Rep68 was superior to Rep78 in their experiment. Southern analysis of G418-resistant clones obtained from transfection with the *rep68* plasmid showed that eight out of 20 clones (40%) had the integration of the *Neo* gene into AAVS1. The presence of the *Neo* gene on chromosome 19 was confirmed in all six clones.

Philpott *et al.* utilized the p5 promoter instead of the ITR as a *cis* element for AAVS1-specific integration and successfully introduced the *GFP* gene into AAVS1 at a frequency of 94% (out of 47 HeLa cell clones), a result comparable to data obtained with an ITR-plasmid (86%) (Philpott *et al.*, 2004). They observed that clones obtained by transfection of a plasmid harboring both a *rep* cassette and a *GFP* cassette showed a gradual decrease in the transgene expression over 18 weeks, while clones obtained from transfection with two plasmids (one for Rep expression, and the other for transgene delivery) stably expressed the transgene over time.

Recombinant AAV vector is widely used as a gene transfer vector and is being evaluated for some human applications including coagulation factor IX deficiency, lipoprotein lipase deficiency, and Parkinson's disease. Conventional AAV vectors harbor the gene of interest flanked by the ITRs and are thus devoid of the *rep* gene. Since the *rep* gene product is essential to AAVS1-specific integration, these AAV vectors are not able to preferentially integrate into AAVS1. To achieve the AAVS1-specific integration of the AAV vector genome, Huttner *et al.* infected HeLa cells with an AAV vector after

干细胞之家www.stemcell8.cn ←点击进入

transfection with a Rep-expression plasmid and reported that seven out of 10 clones showed site-specific integration of the AAV vector genome (Huttner *et al.*, 2003). Wong, Jr. *et al.* also performed Rep-mediated insertion of rAAV by using a fusion protein consisting of Rep and VP22, a tegument protein of herpes simplex virus (Wong *et al.*, 2006). VP22 traffics intercellularly and spreads over adjacent cells (Elliott and O'Hare, 1997). The Rep protein has a nuclear localization signal (NLS) sequence, which inhibits the spread of the Rep-VP22 fusion protein. Removal of the NLS allowed the fusion protein to traffic intercellularly. The advantage of this strategy is that it can be applied to cells easy to transduce with AAV vectors and hard to transfect with plasmid DNA.

7. How to Regulate Rep Expression

The Rep protein affects numerous cellular phenomena as mentioned above. In addition, infection with wild-type AAV or Rep-directed transgene insertion into AAVS1 appears to accompany the disruption and rearrangement of the AAVS1 locus (Balague *et al.*, 1997; Hamilton *et al.*, 2004; Kotin *et al.*, 1990; Shelling and Smith, 1994; Urabe *et al.*, 2003). Thus, it is necessary to regulate the expression level of the Rep protein to as low as possible for applications aimed at AAVS1-specific integration.

The regulation of Rep protein expression at the DNA level includes the bacterial Cre/*loxP* system (Sauer and Henderson, 1988) and the yeast FLP system (O'Gorman *et al.*, 1991). The former was applied to the regulation of the Rep protein (Satoh *et al.*, 2000). The p5 promoter was moved downstream of the *rep* ORF on a plasmid and a *loxP* sequence was placed upstream of the *rep* ORF and another was placed downstream of the p5 promoter. Cre removes a stuffer sequence between the two *loxP* sites and the *rep* cassette is circularized such that the p5 promoter is placed just upstream of the *rep* ORF. Co-transfection of HEK293 cells was performed with a Rep-expression plasmid, a Cre expression plasmid, and an AAV vector plasmid on which a *Neo* gene and an expression cassette for a secreted form of alkaline phosphatase (SEAP) were placed between the ITRs. Only by the use of a *Cre-expression* plasmid could they obtain G418-resistant clones where the *Neo/SEAP* gene was targeted into the AAVS1 locus.

Regulation of the *rep* gene at the transcription level includes the use of a weak promoter. The bacteriophage T7 promoter (Recchia *et al.*, 1999) and the combination of the mouse mammary tumor virus (MMTV) LTR promoter and internal ribosome entry site (IRES) sequence of the encephalomyocarditis virus (Kogure *et al.*, 2001) have been used for Rep expression.

干细胞之家www.stemcell8.cn ←点击进入

The native p5 promoter is weak enough for Rep expression. However, since the p5 promoter encompasses an imperfect RBS and is thus a *cis* element of AAVS1-specific integration (see above), it is desirable to avoid using the p5 promoter for Rep expression in order to minimize the frequency of *rep* gene integration.

In general, RNA is more labile than DNA. Transfection of RNA encoding Rep proteins may thus reduce the prolonged cytostatic effects of the Rep protein. The delivery of the Rep protein may limit the duration of Rep cytostatic action even more. Lamartina *et al.* transfected HeLa cells with a mixture of a recombinant Rep protein and an ITR-flanked plasmid by the use of a lipid reagent (Lamartina *et al.*, 1998). The analysis of pooled transfected cells by PCR showed that AAVS1 site-specific integration occured in many cells. Three junctions were sequenced and confirmed the site-specific integration.

Regulation of a protein function by a molecular switch is an attractive tool for Rep protein. Rinaudo *et al.* developed a chimeric protein between Rep and the truncated form of the ligand binding domain (LBD) of the progesterone receptor (Rinaudo *et al.*, 2000). The progesterone receptor (PR) associates with heat shock proteins hsp70, hsp90, and several co-chaperone proteins via its LBD. Binding of progesterone promotes conformational changes in PR, resulting in its release from the chaperone complex, and then its nuclear transport (McKenna *et al.*, 1999). The truncated LBD does not bind endogenous progesterone but a synthetic antagonist, RU486 (Rinaudo *et al.*, 2000). In the absence of RU486, C-terminally truncated Rep (residue 1-491) fused to the LBD is predominantly in the cytoplasm, whereas in the presence of RU485, the fusion protein moves into the nucleus. Following cotransfection of HeLa cells with a Rep-LBD expression plasmid and an ITR-flanked *Neo* plasmid and a 24-hour treatment with RU486, they obtained G418-resistant clones. Southern analysis showed that seven out of 28 clones harbored the *Neo* gene in the AAVS1 site. In addition, generation of AAVS1 rearrangement without insertion of the *Neo* gene was markedly reduced. Another regulation system for the Rep protein was reported. The Rep protein functions as a hexameric complex. The Rep domain responsible for DNA binding and nicking fused to a protein that multimerizes can target an ITR-linked DNA into AAVS1. Oligomerization of Rep molecules on the RBS is a prerequisite for Rep enzymatic activities, including nicking, helicase, and ATPase activities. The N-terminal two thirds portion (1–224) of the Rep protein is able to target site-specific integration when it is fused C-terminally to artificial multimerizing proteins (Cathomen *et al.*, 2000).

干细胞之家www.stemcell8.cn ←点击进入

8. Vehicles to Deliver the Rep Gene into Cells

A number of strategies have been developed to incorporate DNA into target cells. Plasmid transfection with the calcium phosphate precipitation method or with lipofection is the simplest way to introduce DNA as well as electroporation. For "hard-to-transfect" cells, a viral vector is the second choice.

The Rep proteins are cytostatic as mentioned above and transient or regulated expression of the Rep protein is favored. A number of attempts have been made to create an adenoviral vector harboring the *rep* gene, which turned to be unsuccessful due to low yields and instability of recombinant *rep*-Ad vectors. AAV Rep proteins inhibit the replication of adenovirus at different steps. The Rep78 and Rep68 proteins associate with the single-stranded DNA binding protein, an E2A gene product of adenovirus (Stracker *et al.*, 2004). Rep68 has been shown to bind the E2a promoter region (Casper *et al.*, 2005) and suppresses transcription (Casper *et al.*, 2005; Jing *et al.*, 2001; Nada and Trempe, 2002). In addition Rep78 represses E1a, E2a, E4 promoter activity, but trans-activates E1b and E3 promoters. By contrast, in the presence of E1a protein, Rep78 repressed all the promoters (Jing *et al.*, 2001). Rep78 and Rep68 inhibit the transcription from the Ad major late promoter by the association with the TATA-box binding protein and binding to sites adjacent to the TATA box (Needham *et al.*, 2006). The regulation of *rep* gene expression by the Cre/*LoxP* system (Ueno *et al.*, 2000) or the tetracycline inducible system (Recchia *et al.*, 2004) succeeded in generating an Ad vector with the *rep* gene. In addition, a promoter derived from bacteriophage T7 (Recchia *et al.*, 1999) and the locus control region (LCR) of the human β-globin gene (Wang and Lieber, 2006), which functions very weakly in mammalian cells, have been shown to drive expression levels of the Rep protein compatible with Ad vector production.

Another viral vector tested for incorporation of the Rep expression cassette is herpes simplex virus (HSV) vector. The details are presented by Fraefel *et al.* in the following chapter. HSV is also a helper virus for AAV and support AAV replication. HSV appears to tolerate the anti-viral effect of the Rep protein more than the adenovirus. A p5 promoter-driven Rep cassette could be successfully packaged into an HSV mini-amplicon vector although the titer of the recombinant HSVs was low. By using a hybrid amplicon vector with a *rep* gene and a transgene cassette between the ITR sequences, AAVS1-targeted integration of the transgene was achieved, which was confirmed by amplification of junction sequences in HEK293 cells (Heister *et al.*, 2002) or by Southern blot and FISH analysis in fibroblasts obtained from transgenice mice bearing human AAVS1 sequence (Bakowska *et al.*, 2003).

干细胞之家www.stemcell8.cn ←点击进入

Baculovirus, an invertebrate virus that is widely used for the production of recombinant proteins is also able to harbor the *rep* gene and to mediate AAVS1-specific integration (Palombo *et al.*, 1998). It was reported that the *rep* gene and the ITRs were stable in the baculovirus genome and that titers of *rep*-baculovirus were comparable to wild-type ones (Urabe *et al.*, 2002).

9. Future Direction

Insertion of foreign DNA into a specific chromosome at a predetermined site will become a prerequisite for human gene manipulation in the future. AAV offers an attractive tool to achieve site-specific integration. Currently it is impossible to insert transgene into AAVS1 in all transfected cells. *Ex vivo* gene therapy is a practical strategy to apply for AAVS1-targeted integration since a cell clone that harbors the therapeutic transgene at the AAVS1 site can be selected and expanded for use.

Some challenges to develop better AAVS1-targeted integration systems include: 1) increasing the frequency of AAVS1-specific integration; 2) decreasing the frequency of disruption of non-AAVS1 sites; and 3) reducing the cytotoxicity of the Rep protein. Better understanding of AAV biology will help us to refine the system.

References

Afione SA, Conrad CK, Kearns WG, *et al.* (1996) *In vivo* model of adeno-associated virus vector persistence and rescue. *J Virol* **70**(5): 3235–41.

Amiss TJ, McCarty DM, Skulimowski A, *et al.* (2003) Identification and characterization of an adeno-associated virus integration site in CV-1 cells from the African green monkey. *J Virol* **77**(3): 1904–15.

Andrews BJ, Proteau GA, Beatty LG, *et al.* (1985) The FLP recombinase of the 2 micron circle DNA of yeast: interaction with its target sequences. *Cell* **40**(4): 795–803.

Bakowska JC, Di Maria MV, Camp SM, *et al.* (2003) Targeted transgene integration into transgenic mouse fibroblasts carrying the full-length human AAVS1 locus mediated by HSV/AAV rep(+) hybrid amplicon vector. *Gene Ther* **10**(19): 1691–702.

Balague C, Kalla M, Zhang WW. (1997) Adeno-associated virus Rep78 protein and terminal repeats enhance integration of DNA sequences into the cellular genome. *J Virol* **71**(4): 3299–306.

Bantel-Schaal U. (2001) Integration of adeno-associated virus 2 DNA in human MKR melanoma cells induces a peptide with oncosuppressive properties. *Int J Cancer* **92**(4): 537–44.

干细胞之家www.stemcell8.cn ←点击进入

Bantel-Schaal U, zur Hausen H. (1988) Adeno-associated viruses inhibit SV40 DNA amplification and replication of herpes simplex virus in SV40-transformed hamster cells. *Virology* **164**(1): 64–74.

Batchu RB, Hermonat PL. (1995) The trans-inhibitory Rep78 protein of adeno-associated virus binds to TAR region DNA of the human immunodeficiency virus type 1 long terminal repeat. *FEBS Lett* **367**(3): 267–71.

Batchu RB, Kotin RM, Hermonat PL. (1994) The regulatory rep protein of adeno-associated virus binds to sequences within the c-H-ras promoter. *Cancer Lett* **86**(1): 23–31.

Batchu RB, Shammas MA, Wang JY, *et al.* (2002) Adeno-associated virus protects the retinoblastoma family of proteins from adenoviral-induced functional inactivation. *Cancer Res* **62**(10): 2982–85.

Batchu RB, Shammas MA, Wang JY, *et al.* (1999) Interaction of adeno-associated virus Rep78 with p53: implications in growth inhibition. *Cancer Res* **59**(15): 3592–95.

Batchu RB, Shammas MA, Wang JY, *et al.* (2001) Dual level inhibition of E2F-1 activity by adeno-associated virus Rep78. *J Biol Chem* **276**(26): 24315–22.

Baum C. (2007) Fourth case of leukaemia in the first SCID-X1 gene therapy trial, and the diversity of gene therapy. *http://www.esgct.org/upload/4th_CaseofLeukemial.pdf*

Bishop AL, Hall A. (2000) Rho GTPases and their effector proteins. *Biochem J* **348** (Pt 2): 241–55.

Brister JR, Muzyczka N. (1999) Rep-mediated nicking of the adeno-associated virus origin requires two biochemical activities, DNA helicase activity and transesterification. *J Virol* **73**(11): 9325–36.

Casper JM, Timpe JM, Dignam JD, *et al.* (2005) Identification of an adeno-associated virus Rep protein binding site in the adenovirus E2a promoter. *J Virol* **79**(1): 28–38.

Cathomen T, Collete D, Weitzman MD. (2000) A chimeric protein containing the N-terminus of the adeno-associated virus Rep protein recognizes its target site in an *in vivo* assay. *J Virol* **74**(5): 2372–82.

Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, *et al.* (2000) Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science* **288**(5466): 669–72.

Chiorini JA, Yang L, Safer B, *et al.* (1995) Determination of adeno-associated virus Rep68 and Rep78 binding sites by random sequence oligonucleotide selection. *J Virol* **69**(11): 7334–38.

Chiorini JA, Zimmermann B, Yang L, *et al.* (1998) Inhibition of PrKX, a novel protein kinase, and the cyclic AMP-dependent protein kinase PKA by the regulatory proteins of adeno-associated virus type 2. *Mol Cell Biol* **18**(10): 5921–29.

Ciechanover A, Shkedy D, Oren M, *et al.* (1994) Degradation of the tumor suppressor protein p53 by the ubiquitin-mediated proteolytic system requires a novel species of ubiquitin-carrier protein, E2. *J Biol Chem* **269**(13): 9582–89.

干细胞之家www.stemcell8.cn ←点击进入

Collaco RF, Kalman-Maltese V, Smith AD, *et al.* (2003) A biochemical characterization of the adeno-associated virus Rep40 helicase. *J Biol Chem* **278**(36): 34011–17.

Costello E, Saudan P, Winocour E, *et al.* (1997) High mobility group chromosomal protein 1 binds to the adeno-associated virus replication protein (Rep) and promotes Rep-mediated site-specific cleavage of DNA, ATPase activity and transcriptional repression. *EMBO J* **16**(19): 5943–54.

Di Pasquale G, Stacey SN. (1998) Adeno-associated virus Rep78 protein interacts with protein kinase A and its homolog PRKX and inhibits CREB-dependent transcriptional activation. *J Virol* **72**(10): 7916–25.

Duan D, Sharma P, Yang J, *et al.* (1998) Circular intermediates of recombinant adeno-associated virus have defined structural characteristics responsible for long-term episomal persistence in muscle tissue. *J Virol* **72**(11): 8568–77.

Dutheil N, Yoon-Robarts M, Ward P, *et al.* (2004) Characterization of the mouse adeno-associated virus AAVS1 ortholog. *J Virol* **78**(16): 8917–21.

Elliott G, O'Hare P. (1997) Intercellular trafficking and protein delivery by a herpesvirus structural protein. *Cell* **88**(2): 223–33.

Feng D, Chen J, Yue Y, *et al.* (2006) A 16bp Rep binding element is sufficient for mediating Rep-dependent integration into AAVS1. *J Mol Biol* **358**(1): 38–45.

Francois A, Guilbaud M, Awedikian R, *et al.* (2005) The cellular TATA binding protein is required for rep-dependent replication of a minimal adeno-associated virus type 2 p5 element. *J Virol* **79**(17): 11082–94.

Fu H, Subramanian RR, Masters SC. (2000) 14-3-3 proteins: structure, function, and regulation. *Ann Rev Pharmacol Toxicol* **40**: 617–47.

Glauser DL, Saydam O, Balsiger NA, *et al.* (2005) Four-dimensional visualization of the simultaneous activity of alternative adeno-associated virus replication origins. *J Virol* **79**(19): 12218–30.

Hacein-Bey-Abina S, Le Deist F, Carlier F, *et al.* (2002) Sustained correction of X-linked severe combined immunodeficiency by *ex vivo* gene therapy. *N Engl J Med* **346**(16): 1185–93.

Hacein-Bey-Abina S, Von Kalle C, Schmidt M, *et al.* (2003) LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* **302**(5644): 415–19.

Hamilton H, Gomos J, Berns KI, *et al.* (2004) Adeno-associated virus site-specific integration and AAVS1 disruption. *J Virol* **78**(15): 7874–82.

Han SI, Kawano MA, Ishizu K, *et al.* (2004) Rep68 protein of adeno-associated virus type 2 interacts with 14-3-3 proteins depending on phosphorylation at serine 535. *Virology* **320**(1): 144–55.

Heister T, Heid I, Ackermann M, *et al.* (2002) Herpes simplex virus type 1/adeno-associated virus hybrid vectors mediate site-specific integration at the adeno-associated virus preintegration site, AAVS1, on human chromosome 19. *J Virol* **76**(14): 7163–73.

干细胞之家www.stemcell8.cn ←点击进入

Hermanns J, Schulze A, Jansen-Db1urr P, *et al.* (1997) Infection of primary cells by adeno-associated virus type 2 results in a modulation of cell cycle-regulating proteins. *J Virol* **71**(8): 6020–27.

Hermonat PL. (1991) Inhibition of H-ras expression by the adeno-associated virus Rep78 transformation suppressor gene product. *Cancer Res* **51**(13): 3373–77.

Hermonat PL. (1994) Down-regulation of the human c-fos and c-myc proto-oncogene promoters by adeno-associated virus Rep78. *Cancer Lett* **81**(2): 129–36.

Hermonat PL, Santin AD, Batchu RB. (1996) The adeno-associated virus Rep78 major regulatory/transformation suppressor protein binds cellular Sp1 *in vitro* and evidence of a biological effect. *Cancer Res* **56**(22): 5299–304.

Hermonat PL, Santin AD, Batchu RB, *et al.* (1998) The adeno-associated virus Rep78 major regulatory protein binds the cellular TATA-binding protein *in vitro* and *in vivo*. *Virology* **245**(1): 120–27.

Hickman AB, Ronning DR, Kotin RM, *et al.* (2002) Structural unity among viral origin binding proteins: crystal structure of the nuclease domain of adeno-associated virus Rep. *Mol Cell* **10**(2): 327–37.

Hickman AB, Ronning DR, Perez ZN, *et al.* (2004) The nuclease domain of adeno-associated virus rep coordinates replication initiation using two distinct DNA recognition interfaces. *Mol Cell* **13**(3): 403–14.

Holscher C, Kleinschmidt JA, Burkle A. (1995) High-level expression of adeno-associated virus (AAV) Rep78 or Rep68 protein is sufficient for infectious-particle formation by a rep-negative AAV mutant. *J Virol* **69**(11): 6880–85.

Huttner NA, Girod A, Schnittger S, *et al.* (2003) Analysis of site-specific transgene integration following cotransduction with recombinant adeno-associated virus and a rep encodingplasmid. *J Gene Med* **5**(2): 120–29.

Im DS, Muzyczka N. (1990) The AAV origin binding protein Rep68 is an ATP-dependent site-specific endonuclease with DNA helicase activity. *Cell* **61**(3): 447–57.

Jing XJ, Kalman-Maltese V, Cao X, *et al.* (2001) Inhibition of adenovirus cytotoxicity, replication, and E2a gene expression by adeno-associated virus. *Virology* **291**(1): 140–51.

Kearns WG, Afione SA, Fulmer SB, *et al.* (1996) Recombinant adeno-associated virus (AAV-CFTR) vectors do not integrate in a site-specific fashion in an immortalized epithelial cell line. *Gene Ther* **3**(9): 748–55.

Khleif SN, Myers T, Carter BJ, *et al.* (1991) Inhibition of cellular transformation by the adeno-associated virus rep gene. *Virology* **181**(2): 738–41.

King JA, Dubielzig R, Grimm D, *et al.* (2001) DNA helicase-mediated packaging of adeno-associated virus type 2 genomes into preformed capsids. *EMBO J* **20**(12): 3282–91.

Klein-Bauernschmitt P, zur Hausen H, Schlehofer JR. (1992) Induction of differentiation-associated changes in established human cells by infection with adeno-associated virus type 2. *J Virol* **66**(7): 4191–200.

干细胞之家www.stemcell8.cn ←点击进入

Kogure K, Urabe M, Mizukami H, *et al.* (2001) Targeted integration of foreign DNA into a defined locus on chromosome 19 in K562 cells using AAV-derived components. *Int J Hematol* **73**(4): 469–75.

Kotin RM, Linden RM, Berns KI. (1992) Characterization of a preferred site on human chromosome 19q for integration of adeno-associated virus DNA by nonhomologous recombination. *EMBO J* **11**(13): 5071–78.

Kotin RM, Siniscalco M, Samulski RJ, *et al.* (1990) Site-specific integration by adeno-associated virus. *Proc Natl Acad Sci USA* **87**(6): 2211–15.

Kyostio SR, Wonderling RS, Owens RA. (1995) Negative regulation of the adeno-associated virus (AAV) P5 promoter involves both the P5 rep binding site and the consensus ATP-binding motif of the AAV Rep68 protein. *J Virol* **69**(11): 6787–96.

Labow MA, Graf LH Jr, Berns KI. (1987) Adeno-associated virus gene expression inhibits cellular transformation by heterologous genes. *Mol Cell Biol* **7**(4): 1320–25.

Lamartina S, Roscilli G, Rinaudo D, *et al.* (1998) Lipofection of purified adeno-associated virus Rep68 protein: toward a chromosome-targeting nonviral particle. *J Virol* **72**(9): 7653–58.

Leza MA, Hearing P. (1989) Independent cyclic AMP and E1A induction of adenovirus early region 4 expression. *J Virol* **63**(7): 3057–64.

Lieber A, Steinwaerder DS, Carlson CA, *et al.* (1999) Integrating adenovirus-adeno-associated virus hybrid vectors devoid of all viral genes. *J Virol* **73**(11): 9314–24.

Linden RM, Ward P, Giraud C, *et al.* (1996a) Site-specific integration by adeno-associated virus. *Proc Natl Acad Sci USA* **93**(21): 11288–94.

Linden RM, Winocour E, Berns KI. (1996b) The recombination signals for adeno-associated virus site-specific integration. *Proc Natl Acad Sci USA* **93**(15): 7966–72.

Marcello A, Massimi P, Banks L, *et al.* (2000) Adeno-associated virus type 2 rep protein inhibits human papillomavirus type 16 E2 recruitment of the transcriptional coactivator p300. *J Virol* **74**(19): 9090–98.

McKenna NJ, Lanz RB, O'Malley BW. (1999) Nuclear receptor coregulators: cellular and molecular biology. *Endocr Rev* **20**(3): 321–44.

Mehrle S, Rohde V, Schlehofer JR. (2004) Evidence of chromosomal integration of AAV DNA in human testis tissue. *Virus Genes* **28**(1): 61–69.

Meneses P, Berns KI, Winocour E. (2000) DNA sequence motifs which direct adeno-associated virus site-specific integration in a model system. *J Virol* **74**(13): 6213–16.

Muramatsu S, Handa A, Kajigaya S, *et al.* (1998) Transcription-positive cofactor 4 enhances rescue of adeno-associated virus genome from an infectious clone. *J Gen Virol* **79** (Pt 9): 2157–61.

Muzyczka N, Berns KI. (2001) Parvoviridae: the viruses and their replication. In: *Fields' Virology*, Knipe DM, Howley PM, Griffin DE, *et al.* (eds.), Lippincott Williams & Wilkins, Philadelphia, 4th edn., pp. 2327–2359.

Nada S, Trempe JP. (2002) Characterization of adeno-associated virus rep protein inhibition of adenovirus E2a gene expression. *Virology* **293**(2): 345–55.

干细胞之家www.stemcell8.cn ←点击进入

Nakai H, Yant SR, Storm TA, *et al.* (2001) Extrachromosomal recombinant adeno-associated virus vector genomes are primarily responsible for stable liver transduction *in vivo*. *J Virol* **75**(15): 6969–76.

Nam CH, Rabbitts TH. (2006) The role of LMO2 in development and in T cell leukemia after chromosomal translocation or retroviral insertion. *Mol Ther* **13**(1): 15–25.

Needham PG, Casper JM, Kalman-Maltese V, *et al.* (2006) Adeno-associated virus rep protein-mediated inhibition of transcription of the adenovirus major late promoter *in vitro*. *J Virol* **80**(13): 6207–17.

Ni TH, McDonald WF, Zolotukhin I, *et al.* (1998) Cellular proteins required for adeno-associated virus DNA replication in the absence of adenovirus coinfection. *J Virol* **72**(4): 2777–87.

Ni TH, Zhou X, McCarty DM, *et al.* (1994) *In vitro* replication of adeno-associated virus DNA. *J Virol* **68**(2): 1128–38.

Nony P, Tessier J, Chadeuf G, *et al.* (2001) Novel cis-acting replication element in the adeno-associated virus type 2 genome is involved in amplification of integrated rep-cap sequences. *J Virol* **75**(20): 9991–94.

O'Gorman S, Fox DT, Wahl GM. (1991) Recombinase-mediated gene activation and site-specific integration in mammalian cells. *Science* **251**(4999): 1351–55.

Oelze I, Rittner K, Sczakiel G. (1994) Adeno-associated virus type 2 rep gene-mediated inhibition of basal gene expression of human immunodeficiency virus type 1 involves its negative regulatory functions. *J Virol* **68**(2): 1229–33.

Palombo F, Monciotti A, Recchia A, *et al.* (1998) Site-specific integration in mammalian cells mediated by a new hybrid baculovirus-adeno-associated virus vector. *J Virol* **72**(6): 5025–34.

Pereira DJ, Muzyczka N. (1997) The adeno-associated virus type 2 p40 promoter requires a proximal Sp1 interaction and a p19 CArG-like element to facilitate Rep transactivation. *J Virol* **71**(6): 4300–309.

Philip R, Brunette E, Kilinski L, *et al.* (1994) Efficient and sustained gene expression in primary T lymphocytes and primary and cultured tumor cells mediated by adeno-associated virus plasmid DNA complexed to cationic liposomes. *Mol Cell Biol* **14**(4): 2411–18.

Philpott NJ, Giraud-Wali C, Dupuis C, *et al.* (2002a) Efficient integration of recombinant adeno-associated virus DNA vectors requires a p5-rep sequence in cis. *J Virol* **76**(11): 5411–21.

Philpott NJ, Gomos J, Berns KI, *et al.* (2002b) A p5 integration efficiency element mediates Rep-dependent integration into AAVS1 at chromosome 19. *Proc Natl Acad Sci USA* **99**(19): 12381–85.

Philpott NJ, Gomos J, Falck-Pedersen E. (2004) Transgene expression after rep-mediated site-specific integration into chromosome 19. *Hum Gene Ther* **15**(1): 47–61.

干细胞之家www.stemcell8.cn ←点击进入

Pieroni L, Fipaldini C, Monciotti A, *et al.* (1998) Targeted integration of adeno-associated virus-derived plasmids in transfected human cells. *Virology* **249**(2): 249–59.

Recchia A, Parks RJ, Lamartina S, *et al.* (1999) Site-specific integration mediated by a hybrid adenovirus/adeno-associated virus vector. *Proc Natl Acad Sci USA* **96**(6): 2615–20.

Recchia A, Perani L, Sartori D, *et al.* (2004) Site-specific integration of functional transgenes into the human genome by adeno/AAV hybrid vectors. *Mol Ther* **10**(4): 660–70.

Rinaudo D, Lamartina S, Roscilli G, *et al.* (2000) Conditional site-specific integration into human chromosome 19 by using a ligand-dependent chimeric adeno-associated virus/Rep protein. *J Virol* **74**(1): 281–94.

Ryan JH, Zolotukhin S, Muzyczka N. (1996) Sequence requirements for binding of Rep68 to the adeno-associated virus terminal repeats. *J Virol* **70**(3): 1542–53.

Samulski RJ, Zhu X, Xiao X, *et al.* (1991) Targeted integration of adeno-associated virus (AAV) into human chromosome 19. *EMBO J* **10**(12): 3941–50.

Satoh W, Hirai Y, Tamayose K, *et al.* (2000) Site-specific integration of an adeno-associated virus vector plasmid mediated by regulated expression of rep based on Cre-loxP recombination. *J Virol* **74**(22): 10631–38.

Saudan P, Vlach J, Beard P. (2000) Inhibition of S-phase progression by adeno-associated virus Rep78 protein is mediated by hypophosphorylated pRb. *EMBO J* **19**(16): 4351–61.

Sauer B, Henderson N. (1988) Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. *Proc Natl Acad Sci USA* **85**(14): 5166–70.

Schnepp BC, Jensen RL, Chen CL, *et al.* (2005) Characterization of adeno-associated virus genomes isolated from human tissues. *J Virol* **79**(23): 14793–803.

Shelling AN, Smith MG. (1994) Targeted integration of transfected and infected adeno-associated virus vectors containing the neomycin resistance gene. *Gene Ther* **1**(3): 165–69.

Smith RH, Kotin, RM. (1998) The Rep52 gene product of adeno-associated virus is a DNA helicase with 3′-to-5′ polarity. *J Virol* **72**(6): 4874–81.

Smith RH, Kotin RM. (2000) An adeno-associated virus (AAV) initiator protein, Rep78, catalyzes the cleavage and ligation of single-stranded AAV ori DNA. *J Virol* **74**(7): 3122–29.

Smith RH, Kotin RM. (2002) Adeno-associated virus. In: *Mobile DNA II*, Craig N, Craigie R, Gellert M, Lambowitz AM (eds.), ASM Press, Washington, DC, pp. 905–923.

Smith RH, Spano AJ, Kotin RM. (1997) The Rep78 gene product of adeno-associated virus (AAV) self-associates to form a hexameric complex in the presence of AAV ori sequences. *J Virol* **71**(6): 4461–71.

干细胞之家www.stemcell8.cn ←点击进入

Song S, Lu Y, Choi YK, *et al.* (2004) DNA-dependent PK inhibits adeno-associated virus DNA integration. *Proc Natl Acad Sci USA* **101**(7): 2112–16.

Stracker TH, Cassell GD, Ward P, *et al.* (2004) The Rep protein of adeno-associated virus type 2 interacts with single-stranded DNA-binding proteins that enhance viral replication. *J Virol* **78**(1): 441–53.

Su PF, Chiang SY, Wu CW, *et al.* (2000) Adeno-associated virus major Rep78 protein disrupts binding of TATA-binding protein to the p97 promoter of human papillomavirus type 16. *J Virol* **74**(5): 2459–65.

Suomalainen M, Nakano MY, Boucke K, *et al.* (2001) Adenovirus-activated PKA and p38/MAPK pathways boost microtubule-mediated nuclear targeting of virus. *EMBO J* **20**(6): 1310–19.

Surosky RT, Urabe M, Godwin SG, *et al.* (1997) Adeno-associated virus Rep proteins target DNA sequences to a unique locus in the human genome. *J Virol* **71**(10): 7951–59.

Takeuchi T, Kozuka T, Nakagawa K, *et al.* (2000) Adeno-associated virus type 2 nonstructural protein Rep78 suppresses translation *in vitro*. *Virology* **266**(1): 196–202.

Tan I, Ng CH, Lim L, *et al.* (2001) Phosphorylation of a novel myosin binding subunit of protein phosphatase 1 reveals a conserved mechanism in the regulation of actin cytoskeleton. *J Biol Chem* **276**(24): 21209–16.

Tessier J, Chadeuf G, Nony P, *et al.* (2001) Characterization of adenovirus-induced inverted terminal repeat-independent amplification of integrated adeno-associated virus rep-cap sequences. *J Virol* **75**(1): 375–83.

Thyagarajan B, Olivares EC, Hollis RP, *et al.* (2001) Site-specific genomic integration in mammalian cells mediated by phage phiC31 integrase. *Mol Cell Biol* **21**(12): 3926–34.

Tratschin JD, Miller IL, Smith MG, *et al.* (1985) Adeno-associated virus vector for high-frequency integration, expression, and rescue of genes in mammalian cells. *Mol Cell Biol* **5**(11): 3251–60.

Tsunoda H, Hayakawa T, Sakuragawa N, *et al.* (2000) Site-specific integration of adeno-associated virus-based plasmid vectors in lipofected HeLa cells. *Virology* **268**(2): 391–401.

Tullis GE, Shenk T. (2000) Efficient replication of adeno-associated virus type 2 vectors: a cis-acting element outside of the terminal repeats and a minimal size. *J Virol* **74**(24): 11511–21.

Ueno T, Matsumura H, Tanaka K, *et al.* (2000) Site-specific integration of a transgene mediated by a hybrid adenovirus/adeno-associated virus vector using the Cre/loxP-expression-switching system. *Biochem Biophys Res Commun* **273**(2): 473–78.

Urabe M, Ding C, Kotin RM. (2002) Insect cells as a factory to produce adeno-associated virus type 2 vectors. *Hum Gene Ther* **13**(16): 1935–43.

干细胞之家www.stemcell8.cn ←点击进入

Urabe M, Hasumi Y, Kume A, *et al.* (1999) Charged-to-alanine scanning mutagenesis of the N-terminal half of adeno-associated virus type 2 Rep78 protein. *J Virol* **73**(4): 2682–93.

Urabe M, Kogure K, Kume A, *et al.* (2003) Positive and negative effects of adeno-associated virus Rep on AAVS1-targeted integration. *J Gen Virol* **84**(Pt 8): 2127–32.

Urcelay E, Ward P, Wiener SM, *et al.* (1995) Asymmetric replication *in vitro* from a human sequence element is dependent on adeno-associated virus Rep protein. *J Virol* **69**(4): 2038–46.

Walz CM, Correa-Ochoa MM, Muller M, *et al.* (2002) Adenoassociated virus type 2-induced inhibition of the human papillomavirus type 18 promoter in transgenic mice. *Virology* **293**(1): 172–81.

Wang H, Lieber A. (2006) A helper-dependent capsid-modified adenovirus vector expressing adeno-associated virus rep78 mediates site-specific integration of a 27-kilobase transgene cassette. *J Virol* **80**(23): 11699–709.

Weger S, Hammer E, Heilbronn R. (2002) Topors, a p53 and topoisomerase I binding protein, interacts with the adeno-associated virus (AAV-2) Rep78/68 proteins and enhances AAV-2 gene expression. *J Gen Virol* **83**(Pt 3): 511–16.

Weger S, Hammer E, Heilbronn R. (2004) SUMO-1 modification regulates the protein stability of the large regulatory protein Rep78 of adeno associated virus type 2 (AAV-2). *Virology* **330**(1): 284–94.

Weger S, Wendland M, Kleinschmidt JA, *et al.* (1999) The adeno-associated virus type 2 regulatory proteins rep78 and rep68 interact with the transcriptional coactivator PC4. *J Virol* **73**(1): 260–69.

Weinberg RA. (1995) The retinoblastoma protein and cell cycle control. *Cell* **81**(3): 323–30.

Weitzman MD, Kyostio SR, Kotin RM, *et al.* (1994) Adeno-associated virus (AAV) Rep proteins mediate complex formation between AAV DNA and its integration site in human DNA. *Proc Natl Acad Sci USA* **91**(13): 5808–12.

Wollscheid V, Frey M, Zentgraf H, *et al.* (1997) Purification and characterization of an active form of the p78Rep protein of adeno-associated virus type 2 expressed in *Escherichia coli*. *Protein Expr Purif* **11**(3): 241–49.

Wonderling RS, Kyostio SR, Walker SL, *et al.* (1997). The Rep68 protein of adeno-associated virus type 2 increases RNA levels from the human cytomegalovirus major immediate early promoter. *Virology* **236**(1): 167–76.

Wonderling RS, Owens RA. (1996) The Rep68 protein of adeno-associated virus type 2 stimulates expression of the platelet-derived growth factor B c-sis proto-oncogene. *J Virol* **70**(7): 4783–86.

Wonderling RS, Owens RA. (1997) Binding sites for adeno-associated virus Rep proteins within the human genome. *J Virol* **71**(3): 2528–34.

Wong KK Jr, Chatterjee S, Conrad J, *et al.* (2006) AAV2 Rep protein fusions. *US Patent 7122348*: *http://www.uspto.gov/web/patents/patog/week42/OG/html/1311-3/US07122348-20061017.html*.

干细胞之家www.stemcell8.cn ←点击进入

Wu F, Garcia J, Sigman D, *et al.* (1991) Tat regulates binding of the human immunodeficiency virus trans-activating region RNA loop-binding protein TRP-185. *Genes Dev* **5**(11): 2128–40.

Yamamoto N, Suzuki M, Kawano MA, *et al.* (2007) Adeno-associated virus site-specific integration is regulated by TRP-185. *J Virol* **81**(4): 1990–2001.

Zhan D, Santin AD, Liu Y, *et al.* (1999) Binding of the human papillomavirus type 16 p97 promoter by the adeno-associated virus Rep78 major regulatory protein correlates with inhibition. *J Biol Chem* **274**(44): 31619–24.

干细胞之家www.stemcell8.cn ←点击进入

Chapter 3

Herpes Simplex Virus Type 1/Adeno-Associated Virus Hybrids as Site-Specific Integrating Vectors

Cornel Fraefel*, Daniel L. Glauser, Thomas Heister
and Mathias Ackermann

Herpes simplex virus type 1 (HSV-1) amplicons can accommodate foreign DNA of any size between 1 kbp and 150 kbp and, therefore, give room for extensive combinations of genetic elements. Genomic sequences as well as cDNA, large transcriptional regulatory sequences for cell type-specific expression, multiple transgenes, and genetic elements from other viruses to create hybrid vectors may be inserted in a modular fashion. Hybrid amplicons use genetic elements from HSV-1 that allow replication and packaging of the vector DNA into HSV-1 virions, and genetic elements from other viruses that either direct integration of transgene sequences into the host genome or allow the vector to replicate autonomously as an episome. Thus, the advantages of the HSV-1 amplicon system, such as large transgene capacity, broad host range, strong nuclear localization, and availability of a helper virus-free packaging system are retained and combined with those of heterologous viral elements that confer genetic stability to the vector DNA within transduced cells. In this respect, adeno-associated virus (AAV) has the unique capability of integrating its genome into a specific site, designated AAVS1, on human chromosome 19. The viral *rep* gene and the inverted terminal repeats (*ITRs*), that flank the AAV genome, or the p5 promoter sequence, driving expression of *rep*, are sufficient for this process. HSV-1 amplicons have thus been designed that incorporate the AAV *rep* gene on the backbone and the AAV *ITRs* on the boundaries of the transgene cassette. These HSV/AAV hybrid vectors direct site-specific integration of transgene sequences into AAVS1 and support long-term transgene expression. However, production of these hybrid vectors is hampered by *rep* expression. Optimization of these Rep-dependent site-specific integrating vectors is discussed in light of the molecular mechanisms of AAV-mediated inhibition of HSV-1 replication and is

*Corresponding author. cornelf@vetvir.uzh.ch
Institute of Virology, University of Zurich, Switzerland

干细胞之家www.stemcell8.cn ←点击进入

aimed at eliminating both background random-integration and *rep* interference on vector packaging.

Keywords: Herpes simplex virus type 1; adeno-associated virus; HSV/AAV hybrid vector; long-term gene expression; site-specific integration.

1. Introduction

Viral vectors that exist as nonreplicating, extrachromosomal molecules, such as herpes simplex virus type 1 (HSV-1) or adenovirus (Ad) derivatives, do not support long-term gene expression, at least in dividing cells. By contrast, wild-type (wt) adeno-associated virus (AAV) has the unique ability to preferentially integrate its DNA into a site on human chromosome 19, designated AAVS1, while causing no apparent cytopathic effects (Kotin *et al.*, 1990; Linden *et al.*, 1996a; Muzyczka and Berns, 2001). Such a property is highly desirable in a long-term gene delivery vector, as it provides for stable transgene expression (under appropriate design conditions) while avoiding hazardous insertional mutagenesis inherent to random-integrating vectors (retroviruses: see Thrasher *et al.*, 2006). Two genetic elements from wt AAV, the inverted terminal repeats (*ITRs*) which flank the AAV genome and the *rep* gene, in particular *rep68/78*, are sufficient to mediate this site-specifc integration (Surosky *et al.*, 1997). Alternatively, the p5 promoter sequence can also serve as a substrate for Rep-mediated site-specific vector integration (Philpott *et al.*, 2002b). Unfortunately, AAV vectors have a small transgene capacity (~4.6 kb), which, in general, does not allow the inclusion of the *rep* gene in addition to a therapeutic transgene. In the absence of *rep*, AAV vectors do not integrate site-specifically (Balague *et al.*, 1997).

A new generation of gene delivery viral vectors has been introduced to overcome the limitations of pre-existing viral vector systems. These so-called hybrid viral vectors are designed to combine selected properties of the current vector systems (e.g., Johnston *et al.*, 1997; Palombo *et al.*, 1998; Recchia *et al.*, 1999), such as the large transgene capacity of HSV-1 (~150 kbp), or of Ad (~50 kbp), with the capability for site-specific genomic integration of AAV.

Interestingly, the combination of genetic elements from different viruses to gain new biological properties may not be entirely artificial, but rather mimicking a naturally occurring process of virus-virus interaction in eukaryotic cells. For example, the human herpes virus 6 (HHV-6) *U94* gene encodes a 490-amino-acid polypeptide homologous to AAV Rep (Thomson *et al.*, 1991). Moreover, HHV-6 is a helper for AAV replication and HHV-6 *U94* can complement replication of *rep*-deficient AAV (Thomson *et al.*, 1994). As

干细胞之家www.stemcell8.cn ←点击进入

no cellular or (non-parvo-)viral homologues of *rep* are known, HHV-6 *U*94 was perhaps acquired from AAV, and this acquisition likely had important and advantageous consequences for the life cycle of HHV-6 (Thomson *et al.*, 1991).

This chapter discusses aspects of HSV-1 and AAV biology that are relevant to the construction of vector systems, followed by a description of the design and biological properties of HSV/AAV hybrid vectors.

2. HSV-1 and HSV-based Vectors

2.1. *HSV-1: Virion, Genome, and Life Cycle*

All the viruses of the family *Herpesviridae* have a large genome of linear, double-stranded DNA (120 kbp–250 kbp) and share the ability to establish both lytic and latent infections. Based on certain biological properties, including host range, duration of replication cycle, cytopathology and characteristics of latent infection, the family can be divided into three subfamilies: *Alpha-, Beta- and Gammaherpesvirinae* (Roizman and Pellett, 2001). HSV-1 belongs to the *Alphaherpesvirinae* and is one of the eight human herpesviruses identified to date.

The HSV-1 virion has a diameter of approximately 300 nm and consists of an icosadeltahedral capsid containing the viral genome. The capsid, which has a diameter of approximately 100 nm, is surrounded by a proteinaceous layer, the tegument, which is in turn enclosed by a lipid envelope carrying the viral glycoproteins [Fig. 1(A)]. The virus genome is approximately 152 kbp in size and is composed of two unique segments, U_L and U_S, which are each flanked by inverted repeats, IR_L and IR_S [Fig. 2(A)]. The HSV-1 genome contains three origins of DNA replication, ori_L, located within U_L, and ori_S, which is found within IR_s and is therefore duplicated (Stow, 1982; Lockshon and Galloway, 1986). DNA cleavage/packaging signals (*pac*) are situated at both termini of the genome, as well as at the junction between the long and the short segments. *Ori* and *pac* are the sole *cis*-acting elements necessary for HSV-1 replication and packaging.

HSV-1 infection [Fig. 3(A)] starts with the attachment of virion glycoproteins C and B (gC, gB) to glycosaminoglycans, preferentially heparan sulfate, on the cellular surface of skin or mucosa (Campadelli-Fiume *et al.*, 1990; Herold *et al.*, 1991; Lycke *et al.*, 1991; Shieh *et al.*, 1992; Laquerre *et al.*, 1998). Glycoproteins B, D, and the gH-gL complex mediate fusion of the virion envelope with the cell membrane (Cai *et al.*, 1988; Desai *et al.*, 1988; Ligas and Johnson, 1988). At least 3–4 specific cell surface receptors that interact

干细胞之家www.stemcell8.cn ←点击进入

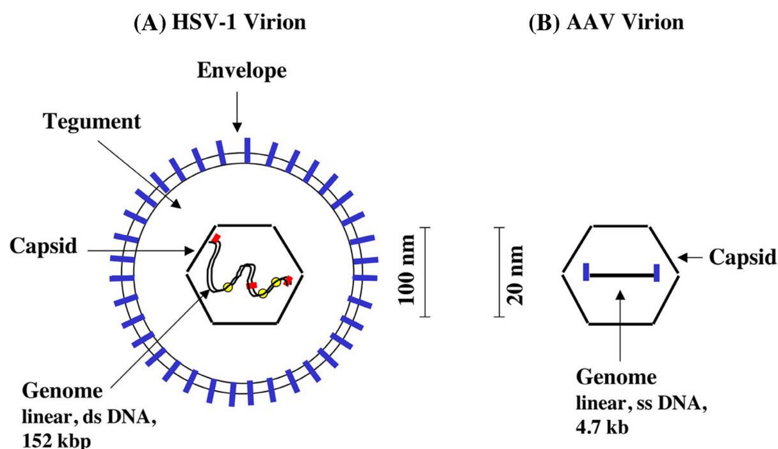


Fig. 1. Virion structures (not to scale). (**A**) The HSV-1 virion consists of a very large icosahedral capsid containing the linear, double-stranded DNA genome. The capsid is surrounded by the tegument, a proteinaceous layer, which in turn is enclosed by a lipid envelope that carries viral glycoptoteins. **(B)** The AAV virion consists of a tiny icosahedral capsid which contains the linear, single-stranded DNA genome. (Based on Fig. 1 of: Fraefel *et al.*, *Gene Ther Regul* **2**: 7–28 (2003), with permission of VSP/Brill.)

with gD have been identified and designated herpes virus entry mediators (Montgomery *et al.*, 1996; Geraghty *et al.*, 1998; Terry-Allison *et al.*, 1998; Shukla *et al.*, 1999; Perez *et al.*, 2005). After entry into the cytoplasm, the capsid and tegument proteins are transported along microtubules to the nuclear pores (Sodeik *et al.*, 1997), where the DNA is released into the nucleoplasm and circularizes.

During lytic infection, approximately 80 HSV-1 genes are transcribed in a tightly regulated cascade with three temporal phases: immediate-early, early, and late (Roizman and Knipe, 2001). Transcription of the immediate-early genes is stimulated by virion tegument protein VP16, which enters the nucleus along with the viral genome. The immediate-early proteins have regulatory functions and activate transcription of the early and late genes. Early genes encode some of the tegument proteins and envelope glycoproteins, as well as enzymes required for DNA metabolism, including thymidine kinase, ribonucleotide reductase, and DNA ploymerase. Accumulation of these enzymes initiates the replication of the virus genome, which in turn is required for the expression of some of the late genes. The late genes encode most of the structural components of the virion.

HSV-1 replication occurs in nuclear domains termed replication compartments (Maul *et al.*, 1996; Sourvinos and Everett, 2002). Replication of the

干细胞之家www.stemcell8.cn ←点击进入

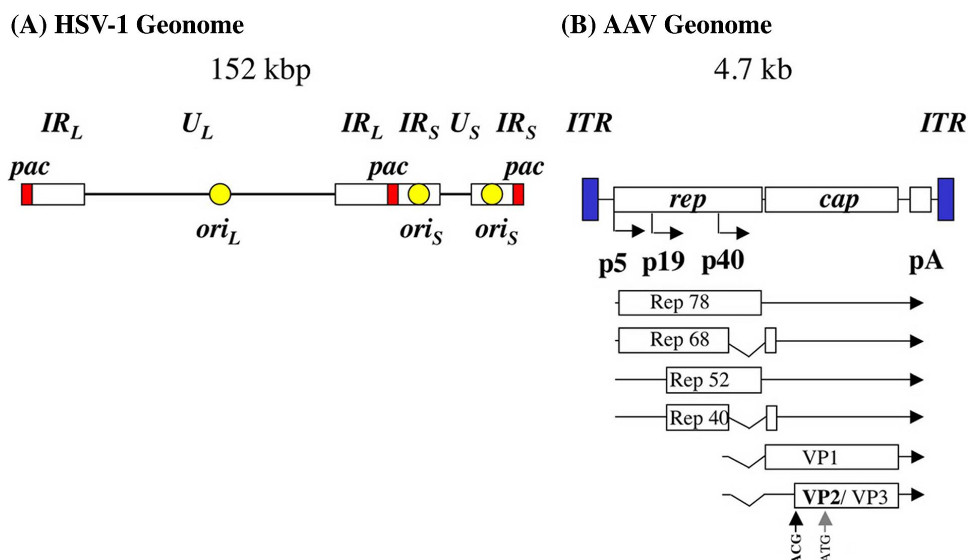


Fig. 2. Virus genomes (not to scale). **(A)** The HSV-1 genome (152 kbp) is composed of unique long (U_L) and unique short (U_S) segments, which are each flanked by inverted repeats, IR_L, IR_S. The DNA cleavage/packaging signals (*pac*) and the origins of DNA replication (ori_L, ori_S) are indicated. **(B)** The AAV genome is a linear, single-stranded DNA of 4.7 kb containing 145-base inverted terminal repeats (*ITR*) at both ends, flanking two clusters of genes, *rep* and *cap*. The *ITRs* contain the origin of DNA replication and the packaging signal. The *rep* genes encode four overlapping proteins, Rep78, 68, 52 and 40, from two different promoters, p5 and p19. The *cap* gene encodes three overlapping proteins, VP1, 2 and 3, from a single promoter, p40. Translation of VP2 starts from an ACG start codon located 66 codons upstream of the VP3 ATG start codon. All *rep* and *cap* transcripts share a common polyadenylation signal (pA). (Based on Fig. 2 of: Fraefel *et al.*, *Gene Ther Regul* **2**: 7–28 (2003), with permission of VSP/Brill.)

HSV-1 genome starts at the origins of DNA replication and produces large, branched concatemers of head-to-tail linked genomes (Zhang *et al.*, 1994; Severini *et al.*, 1996). Concatemers are cleaved into unit-length genomes at the *pac* signals after filling pre-formed capsids (Jacob and Roizman, 1977; Vlazny *et al.*, 1982; Deiss *et al.*, 1986). Different models of HSV-1 maturation, envelopment and egress have been proposed. One model suggests that the virus acquires some tegument proteins and a primary envelope when budding through the inner nuclear membrane. The enveloped virions fuse with the outer nuclear envelope releasing free nucleocapsids at the cytoplasm that will be re-enveloped in a Golgi-related compartment. Re-enveloped virion particles are then secreted by a vesicular route (Mettenleiter, 2002). Another model proposes two distinct pathways: the first pathway involves

干细胞之家www.stemcell8.cn ←点击进入

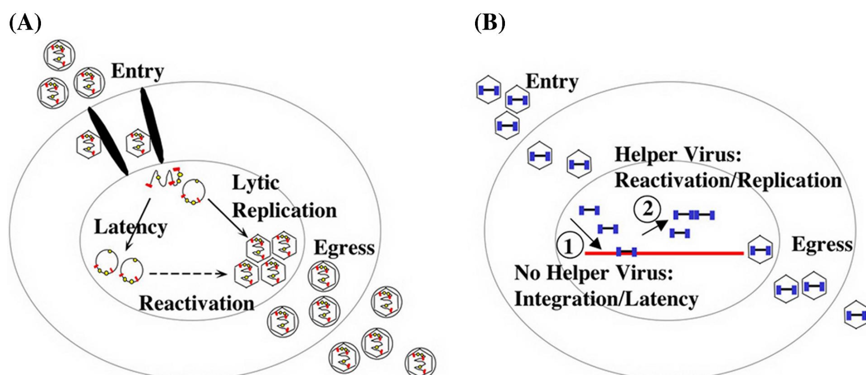


Fig. 3. Lytic and latent infections. **(A)** Lytic and latent HSV-1 infection. HSV-1 envelope glycoproteins mediate attachment and fusion of the virion membrane with the cell membrane. After entry into the cytoplasm, capsid and tegument are transported along microtubules to nuclear pores, where the virus genome is released into the nucleoplasm and circularizes. During lytic infection, approximately 80 HSV-1 genes are transcribed; the genome is replicated, and progeny virus is produced. In neurons, HSV-1 can enter latency. Latent viral genomes exist as circular episomes, and gene expression is limited to the so-called latency-associated transcripts. Latent HSV-1 can periodically reactivate in response to a variety of stimuli. **(B)** Lytic and latent AAV-1 infection. Following receptor-mediated endocytosis, AAV virions accumulate in the perinuclear space and then in the nucleus. AAV can enter both latent (1) or productive (2) infections, depending on the presence or absence of a helper virus. In the absence of helper virus, the AAV genome integrates into the human genome, preferentially at AAVS1 on chromosome 19. Superinfection of latently infected cells with helper virus induces the expression of the AAV genes, the excision of the AAV genome from the host genome, and the onset of DNA replication and packaging into AAV virions. (Based on Fig. 3 of: Fraefel *et al.*, *Gene Ther Regul* **2**: 7–28 (2003), with permission of VSP/Brill.)

envelopment at the inner nuclear membrane followed by intraluminal transport to Golgi cisternae, where transport vacuoles are formed. Alternatively, capsids leave the nucleus via impaired nuclear pores and are enveloped at the cytoplasmic membranes of the rough endoplasmic reticulum (Wild *et al.*, 2002; Leuzinger *et al.*, 2005).

Lytic HSV-1 infection usually causes only mild symptoms, such as cold sores, and is rapidly controlled by the immune system. As an alternative to the lytic cycle, HSV-1 can enter latency, usually in sensory neurons in the trigeminal ganglia, thereby avoiding elimination by the immune system (Stevens, 1975; Rock *et al.*, 1987). Latent viral genomes exist as circular episomes, and gene expression is limited to the so-called latency-associated transcripts (LAT). Latent HSV-1 can periodically reactivate in response to a variety of stimuli and enter a new lytic cycle, usually at the site of the primary infection.

干细胞之家www.stemcell8.cn ←点击进入

2.2. *HSV-1-Based Vector Systems*

The development of vectors based on HSV-1 has been stimulated by the need for methods to deliver therapeutic genes to cells of the nervous system. HSV-1 has many features that make it suitable for this task: (a) the entire sequence of the HSV-1 genome is known (McGeoch *et al.*, 1988); (b) more than 20% of the viral genes are non-essential for replication in cell culture and can, therefore, be replaced by foreign DNA; (c) HSV-1 has a wide host range and efficiently infects both dividing and non-dividing cells; and (d) during latency in neurons, the HSV-1 genome remains in a relatively stable state that supports at least some transcriptional activity.

Over the past two decades, two fundamentally different HSV-1-based vector systems, recombinant and amplicon, have been developed to exploit the numerous biological properties that make this virus such an efficient gene delivery vehicle.

Recombinant HSV-1 vectors are created by replacing one or several virus genes with transgene sequences. Depending on the virus genes that are replaced, recombinant HSV-1 vectors can be replication-competent, conditional (Forrester *et al.*, 1992; Boviatsis *et al.*, 1994a and 1994b; Coffin *et al.*, 1996), or-defective (McCarthy *et al.*, 1989; Samaniego *et al.*, 1998). The choice of the replicative state of a vector depends on the purpose of gene delivery and the target tissue, e.g., replication-defective for gene replacement therapy (Glorioso and Fink, 2002; Goss *et al.*, 2002; Natsume *et al.*, 2002) or replication-competent/conditional for cancer gene therapy and vaccination (Herrlinger *et al.*, 2000; Loudon *et al.*, 2001; Markert *et al.*, 2001; Pawlik *et al.*, 2002; Rees *et al.*, 2002).

2.3. *HSV-1 Amplicon*

In contrast to recombinant HSV-1 vectors, amplicons contain less than 1% of the HSV-1 genome, in particular an origin of DNA replication (*ori*) and a DNA cleavage/packaging signal [*pac*; Fig. 4(A)]. The idea of the amplicon originated from the detection of defective viruses in HSV-1 stocks that have been serially passaged at high multiplicities of infection (Spaete and Frenkel, 1982). The genomes of these naturally occurring defective viruses had a size of ~150 kbp and consisted of multiple reiterations of a small subset of viral sequences, including *pac* and *ori*. Transfection of a bacterial plasmid that contained *ori* and *pac*, termed amplicon, into HSV-1 infected cells, resulted in the replication of the seed amplicon and subsequent packaging

干细胞之家www.stemcell8.cn ←点击进入

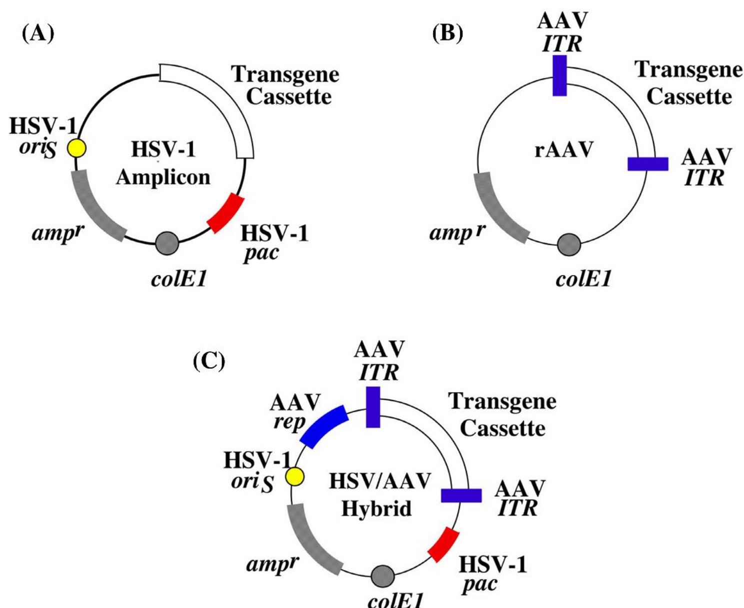


Fig. 4. Vector designs. **(A)** The standard HSV-1 amplicon is composed of three types of genetic elements: (i) sequences from bacteria, including an origin of DNA replication (*colEI*) and an antibiotic resistance gene (*amp*^{r}); (ii) sequences from HSV-1, in particular an origin of DNA replication (*ori*$_S$) and a DNA cleavage/packaging signal (*pac*); and (iii) a transgene cassette with one or several genes of interest. **(B)** The rAAV vector is a bacterial plasmid that contains a transgene cassette flanked by the AAV *ITRs*. **(C)** In addition to the standard amplicon elements, HSV/AAV hybrid vectors contain the AAV *rep* gene and a transgene cassette that is flanked by the AAV *ITRs*. (Based on Fig. 4 of: Fraefel *et al.*, *Gene Ther Regul* **2**: 7–28 (2003), with permission of VSP/Brill.)

of 150 kbp, head-to-tail-linked, concatemeric replication products into HSV-1 virions (Spaete and Frenkel, 1982).

The general design of the HSV-1 amplicon has not changed over the past 20 years. However, safety issues have initiated the development of innovative means to provide helper functions required for amplicon replication and packaging. First, wt HSV-1 has been replaced by replication-conditional mutants of HSV-1 as helper viruses (Geller and Breakefield, 1988; Geller *et al.*, 1990; Johnson *et al.*, 1992; During *et al.*, 1994; Ho *et al.*, 1995; Lim *et al.*, 1996). This has increased the safety of the amplicon vector system, but many helper virus-associated problems remained and have limited the usefulness of amplicon vectors for gene transfer/therapy experiments (Johnson *et al.*, 1992; Ho *et al.*, 1995). These problems include: (a) acute cytopathic effects and pronounced immune responses induced by gene expression from the helper

干细胞之家www.stemcell8.cn ←点击进入

virus; (b) reversion of the helper virus to wt HSV-1 phenotype; (c) potential interactions with endogenous viruses, such as reactivation and recombination; and (d) instability of transgene expression. Many of these limitations have been reduced or excluded by the development of helper virus-free packaging systems (Fraefel *et al.*, 1996; Saeki *et al.*, 1998; Stavropoulos and Strathdee, 1998; Horsburgh *et al.*, 1999; Saeki *et al.*, 2001). Helper virus-free packaging systems use replication-competent, packaging-defective HSV-1 genomes to provide the functions necessary for replication and packaging of amplicon DNA [Fig. 5(A)]. The prototype helper virus-free packaging system used a set of five cosmids that contained the entire HSV-1 genome, with

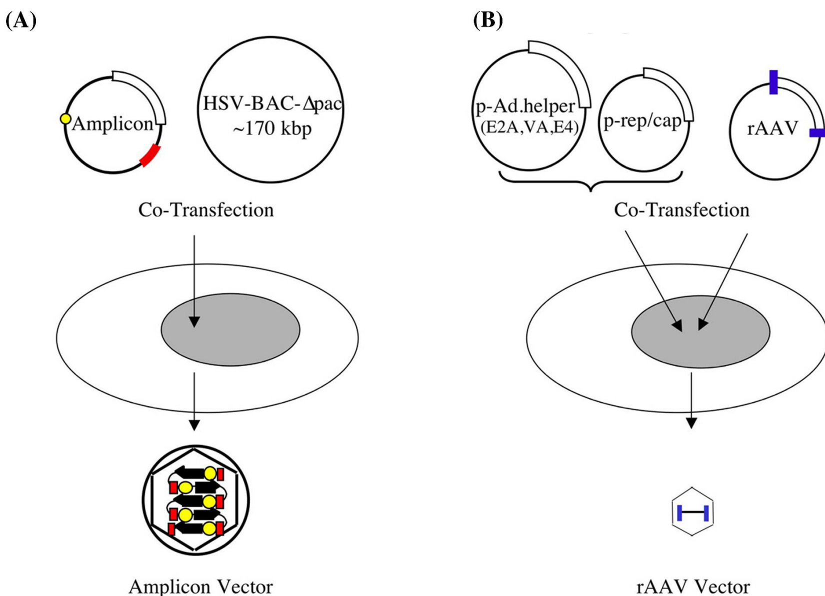


Fig. 5. Packaging of HSV-1 amplicons and rAAV. **(A)** Packaging of HSV-1 Amplicons. Cells that are permissive for HSV-1 replication are co-transfected with amplicon DNA and replication-competent, packaging-defective HSV-1 helper DNA (HSV-BAC-Δpac). The helper DNA provides all the functions necessary for replication and packaging of the amplicon DNA, but is itself not packaged owing to the absence of the *pac* signals. **(B)** Packaging of rAAV vectors. Conventionally, the rAAV plasmid and a second plasmid that provides *rep* and *cap* in *trans* are co-transfected into helper virus (Ad or HSV-1) infected cells. As shown in the figure, helper virus-free protocols are now available in which helper virus functions (e.g., adenoviral *E2A*, *VA* and *E4* genes) are provided by a third plasmid (triple transfection strategy) (Xiao *et al.*, 1998) or incorporated in the *rep-cap* construct (double transfection strategy) (Grimm *et al.*, 1998). The *ITR*-flanked transgene cassette is replicated and single-stranded replication products are packaged into AAV capsids. (Based on Fig. 5 of: Fraefel *et al.*, *Gene Ther Regul* **2**: 7–28 (2003), with permission of VSP/Brill.)

干细胞之家www.stemcell8.cn ←点击进入

pac signals deleted (Fraefel *et al.*, 1996). Upon transfection into cells, these five cosmids can form a complete, replication-competent HSV-1 genome, via homologous recombination between the individual clones, and provide all helper functions necessary for the replication and packaging of co-transfected amplicon DNA. Packaging of the reconstituted HSV-1 genomes is, however, prevented by the absence of the *pac* signals. To simplify this cosmid-based packaging system, the entire HSV-1 genome, deleted for the *pac* signals, has been cloned as a bacterial artificial chromosome (BAC) in *E. coli* (Saeki *et al.*, 1998; Stavropoulos and Strathdee, 1998; Horsburgh *et al.*, 1999; Saeki *et al.*, 2001). Amplicon vector stocks, produced by using helper virus-free packaging systems, have titers of up to 10^7 transducing units (t.u.) per ml of cell culture medium (and up to 10^9 t.u./ml following purification and concentration) and can efficiently transduce many different cell types, including post-mitotic neurons and non-dividing hepatocytes both in culture and *in vivo* (Fraefel *et al.*, 1997; Costantini *et al.*, 1999; Persichetti *et al.*, 1999; Wang *et al.*, 2000; Hocknell *et al.*, 2002; Wang *et al.*, 2002b; Fraefel *et al.*, 2005; Saydam *et al.*, 2005). Furthermore, these vectors cause minimal to no cytopathic effects at multiplicities of infection of at least 50 t.u. per cell and no detectable immune responses at doses of at least 10^7 t.u. (Costantini *et al.*, 1999).

A novel strategy for producing large amounts of amplicon stocks with low helper virus contamination has recently been reported by Logvinoff and Epstein (Logvinoff and Epstein, 2001). This system does not depend on DNA transfection because it utilizes a recombinant HSV-1 helper virus that contains a *pac* signal flanked by loxP sites. This recombinant is replication- and packaging-competent in normal cells, but when amplicon packaging is performed in *cre*-recombinase expressing cells, the *pac* signals are efficiently deleted from the helper virus genome.

Numerous properties make the HSV-1 amplicon an attractive gene transfer vehicle: (a) this vector has a large transgene capacity (150 kbp) (Wade-Martins *et al.*, 2001); (b) replication of amplicon DNA proceeds by a rolling circle mechanism which creates long concatemers of head-to-tail linked amplicon units. For packaging into virions, these concatemers are cleaved at pairs of *pac* signals that are separated by ~150 kbp of DNA, which is the packaging capacity of the HSV-1 capsid. Therefore, the size of the seed amplicon determines the number of transgene copies packaged in one virion; (c) amplicon vectors can infect most cell types and various species, including humans, dogs, cats, elephants, and turtles; and (d) amplicon stocks can be produced that contain essentially no helper virus.

干细胞之家www.stemcell8.cn ←点击进入

However, HSV-1 amplicons have one major limitation: transgene expression is in general transient.

3. AAV and AAV-Based Vectors

3.1. *AAV: Virion, Genome, and Life Cycle*

The *Parvoviridae* have a virion diameter of 18–26 nm and are among the smallest of the DNA animal viruses. The virus family can be divided into two subfamilies: the *Parvovirinae*, which infect vertebrates, and the *Densovirinae*, which infect insects (Muzyczka and Berns, 2001). The *Parvovirinae* include three genera: parvoviruses, erythroviruses, and dependoviruses, which depend on a helper virus for productive replication (AAVs). The AAV virion consists of an icosahedral capsid, which is composed of a mixture of virion proteins (VP) 1, 2, and 3, and contains the virus genome [Fig. 1(B)]. To date, there are 11 known serotypes of primate AAVs, with the human AAV2 being the prototype species. The following section will focus on the biology of AAV2, which has been the subject of intensive research and is one of the best-characterized parvoviruses.

The AAV genome is a linear, single-stranded DNA of 4.7 kb containing 145-base *ITRs* at both ends, flanking two clusters of genes, *rep* and *cap* [Fig. 2(B)] (Lusby *et al.*, 1980; Srivastava *et al.*, 1983). The *ITRs* can form T-shaped secondary structures and comprise the Rep-binding site (RBS) and the terminal resolution site (TRS), which together act as a minimal origin of DNA replication and as packaging signal (Ward and Berns, 1995; Wang *et al.*, 1996; Xiao *et al.*, 1997; Ward *et al.*, 2001). The *rep* genes encode four overlapping proteins, Rep78, 68, 52 and 40, from two different promoters, p5 and p19. In particular, transcription from the p5 promoter leads to synthesis of the large Rep proteins, Rep68/78, while transcription from the p19 promoter results in the synthesis of the small Rep proteins, Rep40/52. Alternative splicing of an intron in the C-terminal half of the *rep* ORF leads to the production of both spliced (Rep68 and Rep40) and unspliced (Rep78 and Rep52) variants from each promoter [Fig. 2(B)]. The p5 promoter also contains a RBS (McCarty *et al.*, 1994), which is involved in the Rep-mediated regulation of p5 activity (Kyostio *et al.*, 1995; Pereira *et al.*, 1997), as well as a functional TRS (Wang and Srivastava, 1997), suggesting that it may contain an alternative origin of DNA replication. Indeed, several groups have reported on the replication origin activity of the p5 promoter sequence in the presence of both Ad and HSV-1 helper functions (Nony *et al.*, 2001; Musatov *et al.*, 2002; Glauser *et al.*, 2005). In addition, the p5 promoter sequence can also mediate,

干细胞之家www.stemcell8.cn ←点击进入

albeit inefficiently, the packaging of single-stranded, *ITR*-deficient *rep-cap* sequences into AAV particles (Nony *et al.*, 2003). The *cap* gene is transcribed from the p40 promoter. All three capsid proteins VP1, VP2, and VP3 are translated from two alternatively spliced mRNA species (Muralidhar *et al.*, 1994). The major spliced species is used to translate the major capsid protein VP3 from a conventional AUG codon and the minor capsid protein VP2 from an upstream in-frame ACG codon. The minor spliced species is translated from an upstream AUG codon, allowing the translation of the entire cap ORF and leading to synthesis of the minor capsid protein VP1. All the *rep* and *cap* transcripts share a common polyadenylation signal [Fig. 2(B)].

The first step of an AAV infection is the binding at cell surface receptors and entry into the cell by an endocytic pathway. AAV2, for instance, uses heparan sulfate proteoglycan as primary receptor for cell attachment (Summerford and Samulski, 1998), while fibroblast growth factor receptor-1 (Qing *et al.*, 1999), integrin alpha-V-beta-5 (Summerford *et al.*, 1999), and hepatocyte growth factor receptor (Kashiwakura *et al.*, 2005) act as co-receptors assisting virus internalization. Following receptor binding, AAV enters the cell via a dynamin-dependent clathrin-coated pit endocytosis pathway. AAV then escapes from the late endosome and accumulates in a perinuclear pattern, before the genome is delivered to the nucleus (Weitzman *et al.*, 1996; Duan *et al.*, 1999; Bartlett *et al.*, 2000; Hansen *et al.*, 2000; Sanlioglu *et al.*, 2000; Xiao *et al.*, 2002). The ssDNA genome is subsequently converted into a double-stranded template for transcription by the host cell DNA polymerase, a process facilitated by the T-shaped hairpin structure of the *ITR*, which provides the primer for synthesis of the complementary DNA strand. In the presence of a helper virus, the AAV genome is replicated following a single-strand displacement mechanism, which is also called the "rolling hairpin model" of DNA replication (Straus *et al.*, 1976; Tattersall and Ward, 1976; Hauswirth and Berns, 1977). The ssDNA replication products are subsequently packaged into the AAV capsid.

AAV can enter both productive and latent infections, depending on the presence or absence of a helper virus. *ITRs* and either Rep78 or Rep68 are sufficient for replication of the AAV genome in the presence of a helper virus, such as Ad or HSV-1 [Fig. 3(B)]. In the absence of the helper virus, *ITRs* and either Rep78 or 68 are sufficient to mediate the integration of the AAV genome into a specific site, termed AAVS1, on chromosome 19 of human cells (Kotin *et al.*, 1990; Samulski *et al.*, 1991; Linden *et al.*, 1996a; Surosky *et al.*, 1997). Rep68/78 bind to the RBSs located on both the AAV genome and the AAVS1 pre-integration site, and a nonhomologous deletion-insertion

干细胞之家www.stemcell8.cn ←点击进入

recombination results in the integration of the AAV genome (Giraud *et al.*, 1994; Weitzman *et al.*, 1994; Urcelay *et al.*, 1995; Linden *et al.*, 1996b; Dyall and Berns, 1998; Young *et al.*, 2000; Young and Samulski, 2001). The finding that p5 promoter sequences are frequently found in recombinant junctions formed by site-specific integration of wt AAV or recombinant AAV (rAAV) vectors into AAVS1 indicate that the p5 promoter sequence may play an equally important role in site-specific integration as the *ITRs* (Samulski *et al.*, 1991; Giraud *et al.*, 1995; Linden *et al.*, 1996a; Linden *et al.*, 1996b; Tsunoda *et al.*, 2000). Indeed, the p5 promoter sequence enhances Rep-mediated, site-specific integration of *ITR*-flanked transgene DNA (Philpott *et al.*, 2002a) and is in fact, sufficient to allow for site-specific integration of transgene DNA in the absence of *ITRs* (Philpott *et al.*, 2002b). Superinfection of latently infected cells with helper virus induces the expression of the AAV genes (Janik *et al.*, 1981; Laughlin *et al.*, 1982; Richardson and Westphal, 1984; Chang *et al.*, 1989; Geoffroy *et al.*, 2004), the rescue of the AAV genome from the host genome by a process coupled to DNA replication (Samulski *et al.*, 1983; Ward *et al.*, 1994 and 2003), and the onset of viral DNA synthesis and packaging into AAV virions.

3.2. *Recombinant AAV Vectors*

Many of its biological properties, in particular those regarding toxicity and immune response, stability of gene expression, and simplicity, make AAV an interesting platform for vector development (Goncalves, 2005): (a) AAV has not been associated with any human or animal disease (Muzyczka and Berns, 2001); (b) in the absence of a helper virus, AAV is replication-defective, enters latency and, thereby, has the potential to maintain transgene sequences in a physical state that supports long-term gene expression; (c) AAV can infect many different cell types, both dividing and non-dividing, without causing cytopathic effects; (d) the AAV genome is relatively simple, can be cloned in bacterial plasmids and can be easily manipulated to make room for transgene sequences; and (e) in the presence of a helper virus, integrated, double-stranded AAV genomes are rescued, replicated, and packaged into AAV virions. More importantly for vector production, the AAV genome is rescued not only from the genomes of latently infected cells, but also from bacterial plasmids (Laughlin *et al.*, 1983), and the *ITRs* are the only *cis* elements required for rescue, replication, and packaging (Samulski *et al.*, 1982 and 1983).

This last point allows the following overall strategy for rAAV vector design and production: the rAAV vector is a bacterial plasmid that contains

干细胞之家www.stemcell8.cn ←点击进入

a transgene flanked by the AAV *ITRs* [Fig. 4(B)]. Conventionally, the AAV replicative and structural genes (*rep* and *cap*) are provided in *trans* from a separate plasmid. Following co-transfection of the two plasmids into Ad or HSV-1 infected cells, the *ITR*-flanked transgene cassette is replicated and single-stranded replication products are packaged into AAV capsids. Infectious rAAV particles are separated from helper virus particles by density gradient centrifugation. There are many variations to this general packaging strategy with respect to cellular delivery of the three components, rAAV plasmid vector, *rep/cap*, and helper virus functions (Xiao *et al.*, 1998; Zhou and Muzyczka, 1998; Conway *et al.*, 1999; Feudner *et al.*, 2001; Zhang *et al.*, 2001). Helper virus-free packaging protocols [Fig. 5(B)] (Grimm *et al.*, 1998; Xiao *et al.*, 1998) associated with viral particle purification using affinity chromatography based on identified cellular receptors (Summerford and Samulski, 1998; Kaludov *et al.*, 2001) are becoming popular (e.g., Zolotukhin *et al.*, 2002).

Recombinant AAV vectors have been used for delivery of therapeutic genes into many different cell types. Studies in animals models of various diseases, including Parkinson's disease, diabetes and hemophilia B (for a review see: Summerford *et al.*, 2000) as well as in clinical trials (e.g., Wagner *et al.*, 2002) have been most promising.

However, rAAV vectors have also limitations. The small packaging capacity does not allow the insertion of large regulatory sequences, genomic transgene sequences, or even some cDNAs. Furthermore, rAAV vector genomes can persist for long periods and support long-term gene expression, but this is not due to site-specific integration, as *rep* is in general removed from the *ITR* cassette to make room for transgene sequences. In the absence of *rep*, rAAV remains episomally or integrates randomly (Balague *et al.*, 1997).

4. HSV/AAV Hybrid Vectors

4.1. *Rationale and Design*

The development of improved HSV-1 amplicon packaging systems has greatly reduced the toxicity and immunogenicity vectors, but had little effect on the stability of amplicon-mediated transgene expression, even if cell type-specific promoters were used (Fraefel *et al.*, 1997). On the other hand, classical rAAV vectors have a small transgene capacity (~4.6 kb) and, due to the replacement of the *rep* and *cap* genes by transgenic sequences, do not conserve the potential of the parent virus for site-specific integration. The

干细胞之家www.stemcell8.cn ←点击进入

rep gene can, however, be provided in *trans* and is included in the second-generation AAV-based vector systems (see Owens, 2002). HSV/AAV hybrid vectors have been designed to overcome these limitations (Johnston *et al.*, 1997; Heister *et al.*, 2002; Wang *et al.*, 2002a).

In addition to the standard HSV-1 amplicon elements, HSV/AAV hybrid vectors incorporate the AAV *rep* gene and a transgene that is flanked by AAV *ITRs* [Fig. 4(C)]. By placing the *rep* gene outside of the *ITR* cassette, it is not integrated into the host genome. Loss of *rep* after integration of the *ITR* cassette eliminates a potential source of toxicity and the risk of rescue/excision of integrated *ITR* cassettes. Because HSV/AAV hybrid vectors can be packaged into HSV-1 virions, they conserve the properties of the HSV-1 amplicon, including the high efficiency of gene transfer, the large transgene capacity, and availability of a helper virus-free packaging system. However, after delivery into the host cell nucleus, the vector has the potential to act like AAV with *rep*-mediated site-specific integration of the *ITR*-flanked transgene cassette into the AAVS1 element of human chromosome 19 (Heister *et al.*, 2002).

4.2. *Biological Properties*

The initial study HSV/AAV hybrid vectors demonstrated that these vectors can be packaged into HSV-1 virions by using either helper virus-dependent or helper virus-free packaging systems (Johnston *et al.*, 1997). Moreover, *ITR*-flanked transgene cassettes could efficiently be rescued from the amplicon backbone and packaged into AAV particles in the presence of a helper virus and *cap* expression, indicating that *rep* and *ITRs* are functional in the context of the hybrid vector. Following infection of cultured human glioma cells, hybrid vectors supported transgene retention and expression significantly longer than standard amplicons (Johnston *et al.*, 1997). Although the possibility of transgene integration had not been specifically addressed in that study, the percentage of cells expressing the transgene was consistently higher with hybrid vectors that contained the *rep* gene than with those without *rep*, or with standard amplicons, especially in the long term.

Two other studies have specifically addressed the question whether HSV/AAV hybrid vectors mediate genomic integration, both randomly or site-specifically at the AAVS1 site on human chromosome 19 (Heister *et al.*, 2002; Wang *et al.*, 2002a).

Heister and colleagues constructed HSV/AAV hybrid vectors that contained *rep68*, *rep78*, both *rep68* and *rep78*, all four *rep* “genes,” or no *rep* gene, and a green fluorescent protein (GFP) reporter gene flanked by the

干细胞之家www.stemcell8.cn ←点击进入

AAV *ITRs*. Replication assays demonstrated that both the AAV elements and the HSV-1 elements were functional in the context of the hybrid vector: in the presence of the HSV-1 helper DNA, all *rep*-containing hybrid vectors produced both monomeric and multimeric replication intermediates of the *ITR*-flanked transgene cassette and high molecular-weight concatemeric products of replication from the HSV-1 origin of DNA replication (Fig. 6). Importantly enough, all hybrid vectors could be packaged into HSV-1 virions, although those containing the *rep* sequences incurred a drastic (20 to 2100-fold) reduction in titers. The highest titer among the *rep*-containing hybrid vectors was obtained with the amplicon vector that comprises the four *rep* "genes." Site-specific integration at AAVS1 was directly demonstrated by PCR and sequence analysis of the *ITR*-AAVS1 junctions in transduced human 293 cells. The junctions were similar to those that had been identified in cells infected with wt AAV (Kotin *et al.*, 1990; Samulski *et al.*, 1991; Kotin *et al.*, 1992; Giraud *et al.*, 1994 and 1995; Linden *et al.*, 1996a and 1996b); bacculovirus/AAV hybrid vectors (Palombo *et al.*, 1998); or Ad/AAV

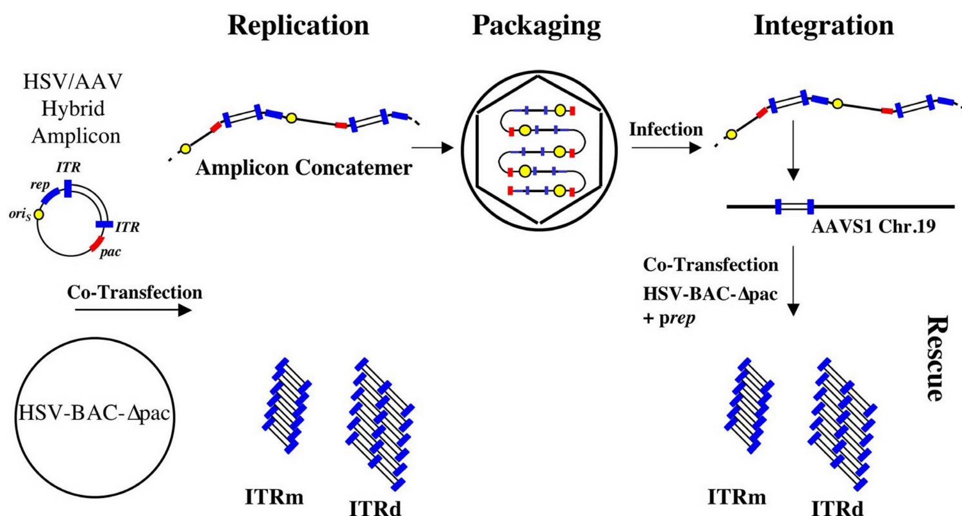


Fig. 6. Biological properties of the HSV/AAV hybrid amplicon. In the presence of HSV-1 helper DNA (HSV-BAC-Δpac), *rep*-containing HSV/AAV hybrid vectors produce both monomeric and dimeric replication intermediates of the *ITR*-flanked transgene cassette and high molecular-weight amplicon concatemers. The amplicon concatemers are packaged into HSV-1 particles. Upon infection of human cells, HSV/AAV hybrid vectors can mediate the integration of the *ITR* cassette into the AAVS1 site on chromosome 19. Co-transfection of HSV-1 helper DNA and a *rep*-expressing plasmid into cells with stably integrated *ITR* cassettes, results in the rescue and replication of the *ITR* cassette. (Based on Fig. 5 of: Fraefel *et al.*, *Gene Ther Regul* **2**: 7–28, 2003, with permission of VSP/Brill.)

干细胞之家www.stemcell8.cn ←点击进入

hybrid vectors (Recchia *et al.*, 1999). Cell clones that stably expressed the transgene could be easily isolated without genetic selection. Under non-selective growth conditions, these cells maintained transgene expression for at least 12 months. Although not quantitative, this study showed that clonal stability was usually associated with site-specific integration of the transgene cassette and that sequences outside of the *ITR* cassette such as the *rep* gene were usually absent. As expected, transfection of these clones with the HSV-1 helper DNA and a *rep* expressing plasmid resulted in the rescue/replication of the integrated *ITR* cassettes (Fig. 6). However, some of the clones were characterized by random integration of the *ITR* cassette alone or along with sequences from the vector backbone.

Similar results were obtained by Wang and colleagues who have also used 293 cells and extended the study to other cell lines, including glioma cells (gli36) and primary myoblasts (Wang *et al.*, 2002a). These investigators used HSV/AAV hybrid vectors that contained *rep68* and *rep78*, or no *rep*, and an *ITR*-flanked transgene cassette that consisted of a GFP reporter gene and a neomycin resistance gene. In order to overcome the low-titer packaging problem inherent to the *rep* gene, they worked on position/orientation effects and found that a decent amplicon vector titer is achieved when the *rep68/78* "gene" is placed downstream of the *ITR* cassette in the forward orientation. Rep-dependent transduction studies were restricted to this *rep68/78*-containing hybrid vector. It had a significantly improved efficiency of stable tranduction in all human cells tested, including 293 cells, glioma cells and primary myoblasts. Although neomycin selection was employed for cell cloning, 86% of the stably transduced glioma cells and 40% of the stably transduced 293 cells had the transgene sequences correctly integrated at the AAVS1 site.

In summary, inserting the AAV *ITRs* and *rep* "genes" into an HSV-1 amplicon considerably improved the frequency of stable transgene expression in various proliferating human cell types. Such a long-term transgene expression mediated by HSV/AAV hybrid vectors appears to rely on chromosomal integration of the transgene sequences, most frequently at the AAVS1 site.

5. Molecular Mechanisms of AAV-Mediated Inhibition of HSV/AAV Hybrid Vector Production

There are at least two potential mechanisms by which the genetic elements and the gene products of the two hybrid vector partners may interfere during hybrid vector production. First, the multiple origins of DNA replication

干细胞之家www.stemcell8.cn ←点击进入

present on HSV/AAV hybrid vectors, specifically the HSV-1 *ori*$_S$, the AAV *ITRs*, and the AAV p5 promoter sequences, might compete for the HSV-1 helper functions. If, for example, rescue and rolling-hairpin replication from the AAV *ITR* replication origins proceeded much more efficiently than the rolling-circle replication from the HSV-1 *ori*$_S$, this would inevitably result in lower amounts of packagable hybrid vector DNA, as the rescued *ITR* cassettes are not packagable into HSV-1 virions. Second, the expression of *rep*, which undoubtedly occurs during hybrid vector production, may have direct inhibitory effects on the HSV-1 *ori*$_S$ replication. Although the molecular mechanisms of AAV-mediated inhibition of HSV-1 replication are not yet fully understood, in recent years, some light has been shed on the interaction of the AAV and HSV-1 replication origins as well on the mechanisms by which AAV inhibits the replication of another helper virus, Ad. In addition, increasing knowledge about the pronounced effects of AAV on the host cell, in particular the DNA damage response and the cell-cycle (Saudan *et al.*, 2000; Raj *et al.*, 2001; Berthet *et al.*, 2005; Jurvansuu *et al.*, 2005), raises the question of whether these effects may also contribute to inhibition of HSV-1 replication.

The mechanisms by which AAV Rep inhibits the replication of Ad have been elucidated to some extent. Trempe and coworkers have shown that transfected AAV *rep* repressed expression from the Ad *E*1*a*, *E*2*a*, and *E*4 promoters but trans-activated the *E*1*b* and *E*3 promoters (Jing *et al.*, 2001). When AAV *rep* was co-transfected with Ad *E*1*a*, *rep* repressed expression from all the five promoters (Jing *et al.*, 2001). They also demonstrated that repression of *E*2*a* expression is due to Rep-mediated inhibition of Ad *E*2*a* transcription and that binding of Rep to a specific site in the *E*2*a* promoter contributes to the effect (Nada and Trempe, 2002; Casper *et al.*, 2005). The most recent work from the same group, however, suggested that the abovementioned effects of Rep on Ad early genes may in fact not be the main factors responsible for AAV-mediated inhibition of Ad replication (Timpe *et al.*, 2006). Specifically, this study demonstrated that AAV co-infection, in particular Rep78, mainly inhibited *E*4 and late transcription of Ad, and to a lesser extent, *E*1*a* and *E*2*a*. Interestingly, transfected Rep78 did not reduce *E*2*a* and *E*4 transcript levels prior to DNA replication nor did AAV co-infection affect *E*2*a* and *E*4 mRNA production in the presence of hydroxyurea. The authors concluded that AAV replication and/or *rep* gene expression inhibited Ad DNA replication and that the reduced early gene expression was a consequence rather than the cause of inhibited DNA replication (Timpe *et al.*, 2006). However, there are also indirect mechanisms which account for Rep-mediated inhibition of Ad

干细胞之家www.stemcell8.cn ←点击进入

replication. Two independent groups reported the interaction of AAV Rep78 and Rep52 proteins with the cellular cyclic AMP-dependent protein kinase A (PKA) and the novel protein kinase PrKX (Chiorini *et al.*, 1998; Di Pasquale and Stacey, 1998). The interaction led to inhibition of PKA and PrKX kinase activity with Rep78 acting as a competitive inhibitor (Chiorini *et al.*, 1998; Di Pasquale and Stacey, 1998; Schmidt *et al.*, 2002). Consistent with this finding, the PrKX and PKA inhibition domains of Rep78, which are located in the putative C-terminal zinc finger domain, were demonstrated to display homology to a cellular pseudosubstrate inhibitor of PKA, the protein kinase inhibitor PKI (Schmidt *et al.*, 2002). Interestingly, the PKI-like domain of Rep78 was not directly necessary for AAV replication and packaging but strongly contributed to AAV-mediated inhibition of Ad replication and consequently was important for AAV replication fitness during Ad co-infection (Di Pasquale and Chiorini, 2003).

Apart from the observations made in the context of HSV/AAV hybrid vectors (Heister *et al.*, 2002; Wang *et al.*, 2002a), AAV-mediated inhibition of HSV-1 replication was also observed in two earlier studies. In particular, co-infection with AAV inhibited replication of HSV-1 in simian virus 40-transformed hamster cells (Bantel-Schaal and zur Hausen, 1988), while co-transfection of AAV *rep*-expressing plasmids inhibited HSV-1 ori_S-dependent DNA replication (Heilbronn *et al.*, 1990). In a recent study, Glauser *et al.*, addressed the question whether the AAV *ITR* and p5 replication origins can be simultaneously active using live-cell visualization systems for *ITR*- and p5-mediated AAV replication (Glauser *et al.*, 2005). The results demonstrated that in the presence of AAV Rep and HSV-1 helper functions, these alternative replication origins are simultaneously active and form spatially separate replication compartments recruiting differential amounts of Rep protein. The differential recruitment of Rep was due to an approximately 100-fold lower affinity of Rep for the linear p5 sequence than for the hairpinned *ITR*. Rolling-circle plasmid replication from the p5 replication origin led to the accumulation of large, head-to-tail linked concatameric replication products, which could readily be packaged into HSV-1 virions if the HSV-1 packaging/cleavage signal was included in the plasmid (Glauser *et al.*, 2005). Taken together, these findings demonstrated, that the AAV *ITR* and p5 replication origins can be simultaneously active and that the AAV p5 replication origin can even substitute for the HSV-1 ori_S on the HSV/AAV hybrid vectors. In a subsequent study, the interaction between AAV and HSV-1 replication origins was addressed and some aspects of AAV-mediated inhibition of HSV-1 replication were elucidated (Glauser *et al.*, 2007). Live

干细胞之家www.stemcell8.cn ←点击进入

cell visualization assays for AAV and HSV-1 DNA replication revealed the formation of separate AAV and HSV-1 replication compartments and the inhibition of HSV-1 replication compartment formation in the presence of AAV. While AAV Rep proteins were recruited into AAV, but not HSV-1 replication compartments, the single-stranded DNA-binding protein HSV-1 ICP8 was found in both the AAV and HSV-1 compartments. The analysis of cells co-infected with HSV-1 and AAV revealed inhibition of HSV-1 DNA replication by wt AAV, but not by *rep*-negative rAAV. In addition, wt AAV was found to affect the levels of the HSV-1 immediate early and early gene products only modestly, but strongly inhibited the accumulation of HSV-1 late gene products. Furthermore, the presence of Rep in the absence of AAV DNA replication was sufficient for the inhibition of HSV-1. In particular, Rep68/78 proteins severely inhibited the formation of mature HSV-1 replication compartments and led to the accumulation of ICP8 at sites of cellular DNA synthesis, a phenomenon previously observed in the presence of viral polymerase inhibitors (Fig. 7). Taken together, these results suggested that, although AAV and HSV-1 replicate in separate compartments, competition for HSV-1 helper functions likely did not represent the main mechanism of inhibition, because the essential replication protein ICP8 was found in both the AAV and HSV-1 replication domains. Rather, the AAV Rep proteins, in particular Rep68/78, have an intrinsic ability to directly inhibit HSV-1 DNA replication, and fairly high levels of Rep are required for inhibition of HSV-1 replication. Specifically, expression of *rep* from its native promoters resulting in initially low levels of Rep, which gradually increase after helper-virus infection, is compatible with the co-existence of AAV and HSV-1 replication domains. However, expression of Rep from the strong CMV promoter almost completely precluded the formation of mature HSV-1 replication compartments (Glauser *et al.*, 2007; Fig. 7). As such, these observations may have important implications for the design of improved HSV/AAV hybrid vector systems as discussed in the following section.

6. Perspectives and Considerations for the Design of Novel HSV/AAV Hybrid Amplicons

The full potential of HSV/AAV hybrid vectors has now to be evaluated for site-specific integration of both large transgene constructs and genomic sequences. Importantly enough, such an evaluation is amenable to *in vivo* testing since transgenic animals are available that carry the human-specific AAVS1 genomic element (Rizzuto *et al.*, 1999). Moreover, as murine (Dutheil

干细胞之家www.stemcell8.cn ←点击进入

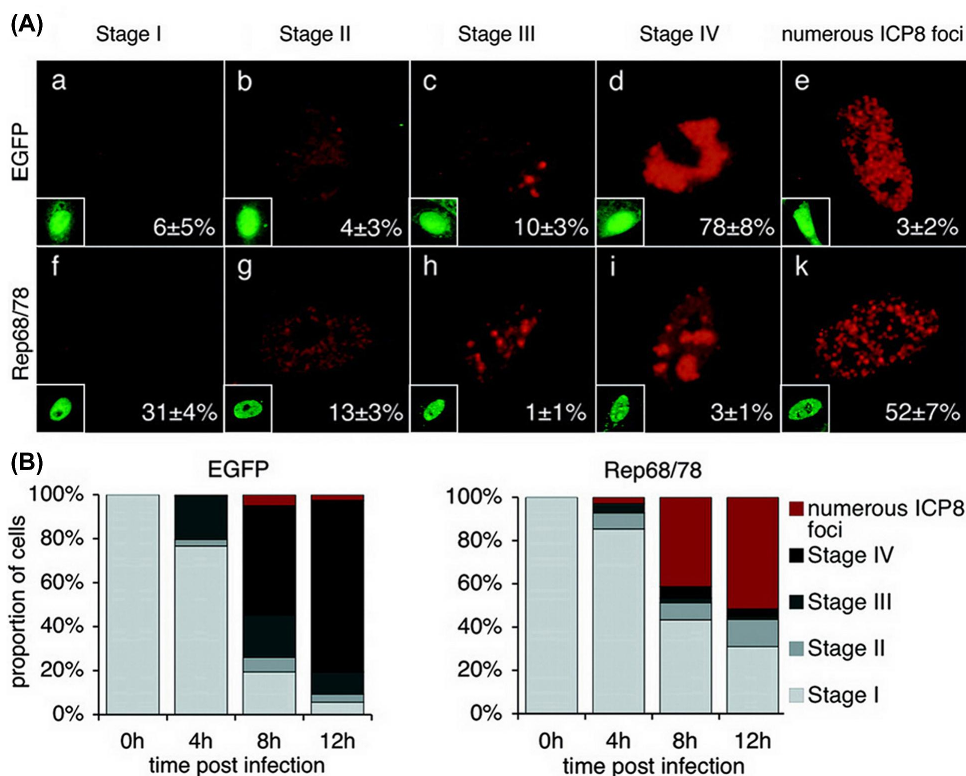


Fig. 7. Influence of AAV Rep68/78 proteins on HSV-1 replication compartment (RC) formation. **(A)** Vero cells were transfected with plasmid pCMVrep68/78_AL, encoding Rep68/78 under the control of the CMV promoter, or plasmid pEGFP-N3, containing the same vector backbone and encoding EGFP instead of Rep68/78. On the following day, the cells were infected with HSV-1 at an MOI of 10 PFU. Cells were fixed at 0, 4, 8, and 12 h post infection (p.i.) and stained with anti-ICP8 MAb 7381 and an AF594-conjugated secondary antibody (a to k; red). The cells in panels f to k were also stained with a rabbit serum specific for Rep and a FITC-conjugated secondary antibody (f to k; green). pEGFP-N3-tranfected cells were identified by EGFP fluorescence (a to e; green). Cells were observed by epifluorescence microscopy, and stages of HSV-1 replication were assessed according to the ICP8 staining pattern, as previously described (Burkham *et al.*, 1998; Lukonis *et al.*, 1997). The numbers indicate the proportions of cells in the respective stages at 12 h p.i. and are means ± standard deviations for triplicate experiments. **(B)** Time course of HSV-1 RC formation in transfected cells expressing EGFP or Rep68/78. The bars show the proportions of cells in the respective stages and represent the mean values for triplicate experiments. (Based on Fig. 7 of: Glauser *et al.*, *J Virol* **81**: 4732–4743, 2007, with permission of the American Society for Microbiology.)

et al., 2004) and simian (Amiss *et al.*, 2003) AAVS1 orthologs have been found, AAV2 likely can mediate site-specific integration in other species as well.

On the other hand, potential improvements of the HSV/AAV amplicon vectors may rely on the appropriate use of the p5 promoter sequence

干细胞之家www.stemcell8.cn ←点击进入

that has been shown to mediate Rep-dependent integration into the AAVS1 site (Philpott *et al.*, 2002b). Indeed, the p5 promoter driving the expression of the Rep78 and Rep68 proteins in the aforedescribed HSV/AAV hybrid vectors (Heister *et al.*, 2002; Wang *et al.*, 2002a) may promote prohibitable vector-backbone integration (Philpott *et al.*, 2002a) owing to its location outside of the therapeutic cassette. In addition, it may also interfere with site-specific integration of the p5-free *ITR*-flanked transgenes. Transferring the p5 promoter sequence from the Rep expression cassette to the transgenic one may thus be a straightforward way to solve the problem and thereby increase site-specific integration, while minimizing random integration (e.g., Ad/AAV hybrid vectors: Philpott *et al.*, 2002a and 2002b).

Importantly, removing the p5 promoter from the Rep expression cassette should provide an additional improvement path for HSV/AAV hybrid vectors. Indeed, Rep proteins are cytotoxic (Berns and Linden, 1995) and inhibit both Ad replication (Weitzman *et al.*, 1996) and HSV-1 DNA amplification (Glauser *et al.*, 2007; Heilbronn *et al.*, 1990). In the presence of HSV-1 or AAV helper viruses, the p5 promoter drives lytic/rescue expression levels of the Rep proteins [see Figs. 3(B) and 6], thereby promoting a drastic drop in packaging titers of both HSV/AAV (Heister *et al.*, 2002; Wang *et al.*, 2002a) and Ad/AAV (Recchia *et al.*, 1999) hybrid vectors. In the aforedescribed HSV/AAV amplicon vectors, the packaging hurdle has been partially overcome by either using the full-length p5-p19 *rep* gene (Heister *et al.*, 2002) or by working on position-orientation parameters of the p5-*rep* expression cassette on the hybrid vectors (Wang *et al.*, 2002a). In both cases, the vector packaging titers were far from optimal and therefore may request substantial improvements. Replacement of the p5 promoter with the phage T7 promoter has been successfully used to drive appropriate low level Rep expression in Ad/AAV hybrid vectors (Recchia *et al.*, 1999; Philpott *et al.*, 2002a). The use of the phage T7 promoter or even a more sophisticated promoter may therefore significantly contribute to the optimization of HSV/AAV hybrid vector packaging processes.

Liu *et al*. developed a different strategy to overcome the negative effect of AAV Rep on hybrid vector replication and packaging (Liu *et al.*, 2006). These investigators designed an HSV/AAV hybrid vector in such a way that little or no *rep* was expressed during packaging. However, *rep* was expressed in transduced cells if Cre-recombinase was provided; following site-specific integration, *rep* was suppressed again. These vectors mediated stable expression in 22% of transduced Cre-expressing 293 cells. Of those

干细胞之家www.stemcell8.cn ←点击进入

cells, approximately 70% transduction efficiency was achieved by Rep-mediated site-specific integration.

The finding that concatameric plasmid replication products from the AAV p5 replication origin are packaged into HSV-1 virions if HSV-1 *pac* is included on the plasmid (Glauser *et al.*, 2005), suggests that it is in principle possible to construct a novel generation of HSV/AAV hybrid amplicon vectors, which replicate from a heterologous origin of DNA replication, the AAV p5 replication origin, and still are packageable into HSV-1 particles. Such a vector system would have several advantages: first, as described by Philpott and coworkers, the AAV p5 element can efficiently mediate site-specific vector integration into AAVS1 on human chromosome 19 and support long term transgene expression (Philpott *et al.*, 2002b and 2004). It can therefore be expected that the potential for site-specific vector integration is also conferred to p5-containing HSV/AAV hybrid amplicon vectors. Second, the AAV p5 replication origin is not inhibited by, but instead depends on the presence of AAV Rep protein in the replication/packaging process (Nony *et al.*, 2001; Musatov *et al.*, 2002; Glauser *et al.*, 2005). Therefore, the incorporation of an AAV *rep* gene, which is required for site-specific vector integration in the target cell (Heister *et al.*, 2002; Wang *et al.*, 2002a), does not interfere with the vector replication/packaging process. However, expression of AAV *rep* is expected to also inhibit replication of the packaging defective HSV-1 helper genome used in the packaging process, resulting in suboptimal helper function. This problem may eventually be overcome by the incorporation of an AAV p5 or *ITR* replication origin on the packaging defective helper virus genome to facilitate its replication in presence of AAV *rep*.

The HSV-1 virion contains three proteinaceous compartments for delivery — envelope, tegument, and capsid – which could all be used to deliver functional foreign proteins by fusion with virion components. For example, AAV Rep could be fused with VP16, an abundant HSV-1 tegument protein that enters the cell nucleus along with the virus genome. This would allow eliminating the *rep* gene from the HSV/AAV hybrid vector genome, as Rep protein could enter the cell nucleus as a fusion with VP16 and there may mediate efficient site-specific integration of the transgene sequences via p5 or *ITRs*.

References

Amiss TJ, McCarty DM, Skulimowski A, Samulski RJ. (2003) Identification and characterization of an adeno-associated virus integration site in CV-1 cells from the African green monkey. *J Virol* **77**: 1904–15.

干细胞之家www.stemcell8.cn ←点击进入

Balague C, Kalla M, Zhang WW. (1997) Adeno-associated virus Rep78 protein and terminal repeats enhance integration of DNA sequences into the cellular genome. *J Virol* **71**: 3299–306.

Bantel-Schaal U, zur Hausen H. (1988) Adeno-associated viruses inhibit SV40 DNA amplification and replication of herpes simplex virus in SV40-transformed hamster cells. *Virology* **164**: 64–74.

Bartlett JS, Wilcher R, Samulski RJ. (2000) Infectious entry pathway of adeno-associated virus and adeno-associated virus vectors. *J Virol* **74**: 2777–85.

Berns KI, Linden RM. (1995) The cryptic life style of adeno-associated virus. *Bioessays* **17**: 237–45.

Berthet C, Raj K, Saudan P, Beard P. (2005) How adeno-associated virus Rep78 protein arrests cells completely in S phase. *Proc Natl Acad Sci USA* **102**: 13634–39.

Boviatsis EJ, Park JS, Sena-Esteves M, *et al*. (1994a) Long-term survival of rats harboring brain neoplasms treated with ganciclovir and a herpes simplex virus vector that retains an intact thymidine kinase gene. *Cancer Res* **54**: 5745–51.

Boviatsis EJ, Scharf JM, Chase M, *et al*. (1994b) Antitumor activity and reporter gene transfer into rat brain neoplasms inoculated with herpes simplex virus vectors defective in thymidine kinase or ribonucleotide reductase. *Gene Ther* **1**: 323–31.

Burkham J, Coen DM, Weller SK. (1998) ND10 protein PML is recruited to herpes simplex virus type 1 prereplicative sites and replication compartments in the presence of viral DNA polymerase. *J Virol* **72**: 10100–107.

Cai WH, Gu B, Person S. (1988) Role of glycoprotein B of herpes simplex virus type 1 in viral entry and cell fusion. [erratum appears in *J Virol* November 1988; **62**(11): 4438]. *J Virol* **62**: 2596–604.

Campadelli-Fiume G, Stirpe D, Boscaro A, *et al*. (1990) Glycoprotein C-dependent attachment of herpes simplex virus to susceptible cells leading to productive infection. *Virology* **178**: 213–22.

Casper JM, Timpe JM, Dignam JD, Trempe JP. (2005) Identification of an adeno-associated virus Rep protein binding site in the adenovirus E2a promoter. *J Virol* **79**: 28–38.

Chang LS, Shi Y, Shenk T. (1989) Adeno-associated virus P5 promoter contains an adenovirus E1A-inducible element and a binding site for the major late transcription factor. *J Virol* **63**: 3479–88.

Chiorini JA, Zimmermann B, Yang L, *et al*. (1998) Inhibition of PrKX, a novel protein kinase, and the cyclic AMP-dependent protein kinase PKA by the regulatory proteins of adeno-associated virus type 2. *Mol Cell Biol* **18**: 5921–29.

Coffin RS, MacLean AR, Latchman DS, Brown SM. (1996) Gene delivery to the central and peripheral nervous systems of mice using HSV1 ICP34.5 deletion mutant vectors. *Gene Ther* **3**: 886–91.

Conway JE, Rhys CM, Zolotukhin I, *et al*. (1999) High-titer recombinant adeno-associated virus production utilizing a recombinant herpes simplex virus type I vector expressing AAV-2 Rep and Cap. *Gene Ther* **6**: 986–93.

干细胞之家www.stemcell8.cn ←点击进入

Costantini LC, Jacoby DR, Wang S, *et al*. (1999) Gene transfer to the nigrostriatal system by hybrid herpes simplex virus/adeno-associated virus amplicon vectors. [see comment][erratum appears in *Hum Gene Ther* April 10, 2000; **11**(6): 981]. *Hum Gene Ther* **10**: 2481–94.

Deiss LP, Chou J, Frenkel N. (1986) Functional domains within the a sequence involved in the cleavage-packaging of herpes simplex virus DNA. *J Virol* **59**: 605–18.

Desai PJ, Schaffer PA, Minson AC. (1988) Excretion of non-infectious virus particles lacking glycoprotein H by a temperature-sensitive mutant of herpes simplex virus type 1: evidence that gH is essential for virion infectivity. *J Gen Virol* **69**: 1147–56.

Di Pasquale G, Chiorini JA. (2003) PKA/PrKX activity is a modulator of AAV/adenovirus interaction. *EMBO J* **22**: 1716–24.

Di Pasquale G, Stacey SN. (1998) Adeno-associated virus Rep78 protein interacts with protein kinase A and its homolog PRKX and inhibits CREB-dependent transcriptional activation. *J Virol* **72**: 7916–25.

Duan D, Li Q, Kao AW, *et al*. (1999) Dynamin is required for recombinant adeno-associated virus type 2 infection. *J Virol* **73**: 10371–76.

During MJ, Naegele JR, O'Malley KL, Geller AI. (1994) Long-term behavioral recovery in Parkinsonian rats by an HSV vector expressing tyrosine hydroxylase. [see comment]. *Science* **266**: 1399–403.

Dutheil N, Yoon-Robarts M, Ward P, *et al*. (2004) Characterization of the mouse adeno-associated virus AAVS1 ortholog. *J Virol* **78**: 8917–21.

Dyall J, Berns KI. (1998) Site-specific integration of adeno-associated virus into an episome with the target locus via a deletion-substitution mechanism. *J Virol* **72**: 6195–98.

Feudner E, de Alwis M, Thrasher AJ, *et al*. (2001) Optimization of recombinant adeno-associated virus production using an herpes simplex virus amplicon system. *J Virol Meth* **96**: 97–105.

Forrester A, Farrell H, Wilkinson G, *et al*. (1992) Construction and properties of a mutant of herpes simplex virus type 1 with glycoprotein H coding sequences deleted. *J Virol* **66**: 341–48.

Fraefel C, Jacoby DR, Lage C, *et al*. (1997) Gene transfer into hepatocytes mediated by helper virus-free HSV/AAV hybrid vectors. *Mol Med* **3**: 813–25.

Fraefel C, Mendes-Madeira A, Mabon O, *et al*. (2005) *In vivo* gene transfer to the rat retina using herpes simplex virus type 1 (HSV-1)-based amplicon vectors. *Gene Ther* **12**: 1283–88.

Fraefel C, Song S, Lim F, *et al*. (1996) Helper virus-free transfer of herpes simplex virus type 1 plasmid vectors into neural cells. *J Virol* **70**: 7190–97.

Geller AI, Breakefield XO. (1988) A defective HSV-1 vector expresses *Escherichia coli* beta-galactosidase in cultured peripheral neurons. *Science* **241**: 1667–69.

干细胞之家www.stemcell8.cn ←点击进入

Geller AI, Keyomarsi K, Bryan J, Pardee AB. (1990) An efficient deletion mutant packaging system for defective herpes simplex virus vectors: potential applications to human gene therapy and neuronal physiology. *Proc Natl Acad Sci USA* **87**: 8950–54.

Geoffroy MC, Epstein AL, Toublanc E, *et al*. (2004) Herpes simplex virus type 1 ICP0 protein mediates activation of adeno-associated virus type 2 rep gene expression from a latent integrated form. *J Virol* **78**: 10977–86.

Geraghty RJ, Krummenacher C, Cohen GH, *et al*. (1998) Entry of alphaherpesviruses mediated by poliovirus receptor-related protein 1 and poliovirus receptor. *Science* **280**: 1618–20.

Giraud C, Winocour E, Berns KI. (1994) Site-specific integration by adeno-associated virus is directed by a cellular DNA sequence. *Proc Natl Acad Sci USA* **91**: 10039–43.

Giraud C, Winocour E, Berns KI. (1995) Recombinant junctions formed by site-specific integration of adeno-associated virus into an episome. *J Virol* **69**: 6917–24.

Glauser DL, Saydam O, Balsiger NA, *et al*. (2005) Four-dimensional visualization of the simultaneous activity of alternative adeno-associated virus replication origins. *J Virol* **79**: 12218–30.

Glauser DL, Strasser R, Laimbacher AS, *et al*. (2007) Live covisualization of competing adeno-associated virus and herpes simplex virus type 1 DNA replication: molecular mechanisms of interaction. *J Virol* **81**: 4732–43.

Glorioso JC, Fink DJ. (2002) Use of HSV vectors to modify the nervous system. *Curr Opin Drug Discov Dev* **5**: 289–95.

Goncalves MAFV. (2005) Adeno-associated virus: from defective virus to effective vector. *Virol J* **2**: 43.

Goss JR, Goins WF, Lacomis D, *et al*. (2002) Herpes simplex-mediated gene transfer of nerve growth factor protects against peripheral neuropathy in streptozotocin-induced diabetes in the mouse. *Diabetes* **51**: 2227–32.

Grimm D, Kern A, Rittner K, Kleinschmidt JA. (1998) Novel tools for production and purification of recombinant adenoassociated virus vectors. *Hum Gene Ther* **9**: 2745–60.

Hansen J, Qing K, Kwon HJ, *et al*. (2000) Impaired intracellular trafficking of adeno-associated virus type 2 vectors limits efficient transduction of murine fibroblasts. *J Virol* **74**: 992–96.

Hauswirth WW, Berns KI. (1977) Origin and termination of adeno-associated virus DNA replication. *Virology* **78**: 488–99.

Heilbronn R, Burkle A, Stephan S, zur Hausen H. (1990) The adeno-associated virus rep gene suppresses herpes simplex virus-induced DNA amplification. *J Virol* **64**: 3012–18.

Heister T, Heid I, Ackermann M, Fraefel C. (2002) Herpes simplex virus type 1/adeno-associated virus hybrid vectors mediate site-specific integration at the adeno-associated virus preintegration site, AAVS1, on human chromosome 19. *J Virol* **76**: 7163–73.

干细胞之家www.stemcell8.cn ←点击进入

Herold BC, WuDunn D, Soltys N, Spear PG. (1991) Glycoprotein C of herpes simplex virus type 1 plays a principal role in the adsorption of virus to cells and in infectivity. *J Virol* **65**: 1090–98.

Herrlinger U, Woiciechowski C, Sena-Esteves M, *et al*. (2000) Neural precursor cells for delivery of replication-conditional HSV-1 vectors to intracerebral gliomas. *Mol Ther* **1**: 347–57.

Ho DY, Saydam TC, Fink SL, *et al*. (1995) Defective herpes simplex virus vectors expressing the rat brain glucose transporter protect cultured neurons from necrotic insults. *J Neurochem* **65**: 842–50.

Hocknell PK, Wiley RD, Wang X, *et al*. (2002) Expression of human immunodeficiency virus type 1 gp120 from herpes simplex virus type 1-derived amplicons results in potent, specific, and durable cellular and humoral immune responses. *J Virol* **76**: 5565–80.

Horsburgh BC, Hubinette MM, Qiang D, *et al*. (1999) Allele replacement: an application that permits rapid manipulation of herpes simplex virus type 1 genomes. *Gene Ther* **6**: 922–30.

Jacob RJ, Roizman B. (1977) Anatomy of herpes simplex virus DNA VIII. Properties of the replicating DNA. *J Virol* **23**: 394–411.

Janik JE, Huston MM, Rose JA. (1981) Locations of adenovirus genes required for the replication of adenovirus-associated virus. *Proc Natl Acad Sci USA* **78**: 1925–29.

Jing XJ, Kalman-Maltese V, Cao X, *et al*. (2001) Inhibition of adenovirus cytotoxicity, replication, and E2a gene expression by adeno-associated virus. *Virology* **291**: 140–51.

Johnson PA, Miyanohara A, Levine F, *et al*. (1992) Cytotoxicity of a replication-defective mutant of herpes simplex virus type 1. *J Virol* **66**: 2952–65.

Johnston KM, Jacoby D, Pechan PA, *et al*. (1997) HSV/AAV hybrid amplicon vectors extend transgene expression in human glioma cells. *Hum Gene Ther* **8**: 359–70.

Jurvansuu J, Raj K, Stasiak A, Beard P. (2005) Viral transport of DNA damage that mimics a stalled replication fork. *J Virol* **79**: 569–80.

Kaludov N, Brown KE, Walters RW, *et al*. (2001) Adeno-associated virus serotype 4 (AAV4) and AAV5 both require sialic acid binding for hemagglutination and efficient transduction but differ in sialic acid linkage specificity. *J Virol* **75**: 6884–93.

Kashiwakura Y, Tamayose K, Iwabuchi K, *et al*. (2005) Hepatocyte growth factor receptor is a coreceptor for adeno-associated virus type 2 infection. *J Virol* **79**: 609–14.

Kotin RM, Linden RM, Berns KI. (1992) Characterization of a preferred site on human chromosome 19q for integration of adeno-associated virus DNA by non-homologous recombination. *EMBO J* **11**: 5071–78.

Kotin RM, Siniscalco M, Samulski RJ, *et al*. (1990) Site-specific integration by adeno-associated virus. *Proc Natl Acad Sci USA* **87**: 2211–15.

干细胞之家www.stemcell8.cn ←点击进入

Kyostio SR, Wonderling RS, Owens RA. (1995) Negative regulation of the adeno-associated virus (AAV) P5 promoter involves both the P5 rep binding site and the consensus ATP-binding motif of the AAV Rep68 protein. *J Virol* **69**: 6787–96.
Laquerre S, Argnani R, Anderson DB, *et al.* (1998) Heparan sulfate proteoglycan binding by herpes simplex virus type 1 glycoproteins B and C, which differ in their contributions to virus attachment, penetration, and cell-to-cell spread. *J Virol* **72**: 6119–30.
Laughlin CA, Jones N, Carter BJ. (1982) Effect of deletions in adenovirus early region 1 genes upon replication of adeno-associated virus. *J Virol* **41**: 868–76.
Laughlin CA, Tratschin JD, Coon H, Carter BJ. (1983) Cloning of infectious adeno-associated virus genomes in bacterial plasmids. *Gene* **23**: 65–73.
Leuzinger H, Ziegler U, Schraner EM, *et al.* (2005) Herpes simplex virus 1 envelopment follows two diverse pathways. *J Virol* **79**: 13047–59.
Ligas MW, Johnson DC. (1988) A herpes simplex virus mutant in which glycoprotein D sequences are replaced by beta-galactosidase sequences binds to but is unable to penetrate into cells. *J Virol* **62**: 1486–94.
Lim F, Hartley D, Starr P, *et al.* (1996) Generation of high-titer defective HSV-1 vectors using an IE 2 deletion mutant and quantitative study of expression in cultured cortical cells. *Biotechniques* **20**: 460–69.
Linden RM, Ward P, Giraud C, *et al.* (1996a) Site-specific integration by adeno-associated virus. *Proc Natl Acad Sci USA* **93**: 11288–94.
Linden RM, Winocour E, Berns KI. (1996b) The recombination signals for adeno-associated virus site-specific integration. *Proc Natl Acad Sci USA* **93**: 7966–72.
Liu Q, Perez CF, Wang Y. (2006) Efficient site-specific integration of large transgenes by an enhanced herpes simplex virus/adeno-associated virus hybrid amplicon vector. *J Virol* **80**: 1672–79.
Lockshon D, Galloway DA. (1986) Cloning and characterization of oriL2, a large palindromic DNA replication origin of herpes simplex virus type 2. *J Virol* **58**: 513–21.
Logvinoff C, Epstein AL. (2001) A novel approach for herpes simplex virus type 1 amplicon vector production, using the Cre-loxP recombination system to remove helper virus. *Hum Gene Ther* **12**: 161–67.
Loudon PT, Blakeley DM, Boursnell ME, *et al.* (2001) Preclinical safety testing of DISC-hGMCSF to support phase I clinical trials in cancer patients. *J Gene Med* **3**: 458–67.
Lukonis CJ, Burkham J, Weller SK. (1997) Herpes simplex virus type 1 prereplicative sites are a heterogeneous population: only a subset are likely to be precursors to replication compartments. *J Virol* **71**: 4771–81.
Lusby E, Fife KH, Berns KI. (1980) Nucleotide sequence of the inverted terminal repetition in adeno-associated virus DNA. *J Virol* **34**: 402–9.
Lycke E, Johansson M, Svennerholm B, Lindahl U. (1991) Binding of herpes simplex virus to cellular heparan sulphate, an initial step in the adsorption process. *J Gen Virol* **72**: 1131–37.

干细胞之家www.stemcell8.cn ←点击进入

Markert JM, Parker JN, Gillespie GY, Whitley RJ. (2001) Genetically engineered human herpes simplex virus in the treatment of brain tumours. *Herpes* **8**: 17–22.

Maul GG, Ishov AM, Everett RD. (1996) Nuclear domain 10 as preexisting potential replication start sites of herpes simplex virus type-1. *Virology* **217**: 67–75.

McCarthy AM, McMahan L, Schaffer PA. (1989) Herpes simplex virus type 1 ICP27 deletion mutants exhibit altered patterns of transcription and are DNA deficient. *J Virol* **63**: 18–27.

McCarty DM, Pereira DJ, Zolotukhin I, *et al*. (1994) Identification of linear DNA sequences that specifically bind the adeno-associated virus Rep protein. *J Virol* **68**: 4988–97.

McGeoch DJ, Dalrymple MA, Davison AJ, *et al*. (1988) The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. *J Gen Virol* **69**: 1531–74.

Mettenleiter TC. (2002) Herpesvirus assembly and egress. *J Virol* **76**: 1537–47.

Montgomery RI, Warner MS, Lum BJ, Spear PG. (1996) Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. *Cell* **87**: 427–36.

Muralidhar S, Becerra SP, Rose JA. (1994) Site-directed mutagenesis of adeno-associated virus type 2 structural protein initiation codons: effects on regulation of synthesis and biological activity. *J Virol* **68**: 170–76.

Musatov S, Roberts J, Pfaff D, Kaplitt M. (2002) A cis-acting element that directs circular adeno-associated virus replication and packaging. *J Virol* **76**: 12792–802.

Muzyczka N, Berns KI. (2001) Parvoviridae: the viruses and their replication. In: *Fields Virology*, Knipe DM, Howley PM (eds.), Lippincott Williams & Wilkins, Philadelphia, vol. 2, 4th edn., pp. 2327–46.

Nada S, Trempe JP. (2002) Characterization of adeno-associated virus rep protein inhibition of adenovirus E2a gene expression. *Virology* **293**: 345–55.

Natsume A, Mata M, Wolfe D, *et al*. (2002) Bcl-2 and GDNF delivered by HSV-mediated gene transfer after spinal root avulsion provide a synergistic effect. *J Neurotrauma* **19**: 61–68.

Nony P, Chadeuf G, Tessier J, *et al*. (2003) Evidence for packaging of rep-cap sequences into adeno-associated virus (AAV) type 2 capsids in the absence of inverted terminal repeats: a model for generation of rep-positive AAV particles. *J Virol* **77**: 776–81.

Nony P, Tessier J, Chadeuf G, *et al*. (2001) Novel cis-acting replication element in the adeno-associated virus type 2 genome is involved in amplification of integrated rep-cap sequences. *J Virol* **75**: 9991–94.

Owens RA. (2002) Second generation adeno-associated virus type 2-based gene therapy systems with the potential for preferential integration into AAVS1. *Curr Gene Ther* **2**: 145–59.

Palombo F, Monciotti A, Recchia A, *et al*. (1998) Site-specific integration in mammalian cells mediated by a new hybrid baculovirus-adeno-associated virus vector. *J Virol* **72**: 5025–34.

干细胞之家www.stemcell8.cn ←点击进入

Pawlik TM, Nakamura H, Mullen JT, *et al*. (2002) Prodrug bioactivation and oncolysis of diffuse liver metastases by a herpes simplex virus 1 mutant that expresses the CYP2B1 transgene. *Cancer* **95**: 1171–81.

Pereira DJ, McCarty DM, Muzyczka N. (1997) The adeno-associated virus (AAV) Rep protein acts as both a repressor and an activator to regulate AAV transcription during a productive infection. *J Virol* **71**: 1079–88.

Perez A, Li QX, Perez-Romero P, *et al*. (2005) A new class of receptor for herpes simplex virus has heptad repeat motifs that are common to membrane fusion proteins. *J Virol* **79**: 7419–30.

Persichetti F, Trettel F, Huang CC, *et al*. (1999) Mutant huntingtin forms *in vivo* complexes with distinct context-dependent conformations of the polyglutamine segment. *Neurobiol Dis* **6**: 364–75.

Philpott NJ, Giraud-Wali C, Dupuis C, *et al*. (2002a) Efficient integration of recombinant adeno-associated virus DNA vectors requires a p5-rep sequence in cis. *J Virol* **76**: 5411–21.

Philpott NJ, Gomos J, Berns KI, Falck-Pedersen E. (2002b) A p5 integration efficiency element mediates Rep-dependent integration into AAVS1 at chromosome 19. *Proc Natl Acad Sci USA* **99**: 12381–85.

Philpott NJ, Gomos J, Falck-Pedersen E. (2004) Transgene expression after rep-mediated site-specific integration into chromosome 19. *Hum Gene Ther* **15**: 47–61.

Qing K, Mah C, Hansen J, *et al*. (1999) Human fibroblast growth factor receptor 1 is a co-receptor for infection by adeno-associated virus 2. [see comment]. *Nat Med* **5**: 71–77.

Raj K, Ogston P, Beard P. (2001) Virus-mediated killing of cells that lack p53 activity. [see comment] [erratum appears in *Nature* March 14, 2002; **416**(6877): 202]. *Nature* **412**: 914–17.

Recchia A, Parks RJ, Lamartina S, *et al*. (1999) Site-specific integration mediated by a hybrid adenovirus/adeno-associated virus vector. *Proc Natl Acad Sci USA* **96**: 2615–20.

Rees RC, McArdle S, Mian S, *et al*. (2002) Disabled infectious single cycle-herpes simplex virus (DISC-HSV) as a vector for immunogene therapy of cancer. *Curr Opin Mol Ther* **4**: 49–53.

Richardson WD, Westphal H. (1984) Requirement for either early region 1a or early region 1b adenovirus gene products in the helper effect for adeno-associated virus. *J Virol* **51**: 404–10.

Rizzuto G, Gorgoni B, Cappelletti M, *et al*. (1999) Development of animal models for adeno-associated virus site-specific integration. *J Virol* **73**: 2517–26.

Rock DL, Nesburn AB, Ghiasi H, *et al*. (1987) Detection of latency-related viral RNAs in trigeminal ganglia of rabbits latently infected with herpes simplex virus type 1. *J Virol* **61**: 3820–26.

Roizman B, Knipe DM. (2001) Herpes simplex viruses and their replication. In: *Fields Virology*, Knipe DM, Howley PM (eds.), Lippincott Williams & Wilkins, Philadelphia, Vol. 2, 4th edn., pp. 2399–459.

干细胞之家www.stemcell8.cn ←点击进入

Roizman B, Pellett PE. (2001) The family herpesviridae: a brief introduction. In: *Fields Virology*, Knipe DM, Howley PM (eds.), Lippincott Williams & Wilkins, Philadelphia, Vol. 2, 4th edn., pp. 2381–97.

Saeki Y, Fraefel C, Ichikawa T, *et al*. (2001) Improved helper virus-free packaging system for HSV amplicon vectors using an ICP27-deleted, oversized HSV-1 DNA in a bacterial artificial chromosome. *Mol Ther: J Am Soc Gene Ther* **3**: 591–601.

Saeki Y, Ichikawa T, Saeki A, *et al*. (1998) Herpes simplex virus type 1 DNA amplified as bacterial artificial chromosome in *Escherichia coli*: rescue of replication-competent virus progeny and packaging of amplicon vectors. *Hum Gene Ther* **9**: 2787–94.

Samaniego LA, Neiderhiser L, DeLuca NA. (1998) Persistence and expression of the herpes simplex virus genome in the absence of immediate-early proteins. *J Virol* **72**: 3307–20.

Samulski RJ, Berns KI, Tan M, Muzyczka N. (1982) Cloning of adeno-associated virus into pBR322: rescue of intact virus from the recombinant plasmid in human cells. *Proc Natl Acad Sci USA* **79**: 2077–81.

Samulski RJ, Srivastava A, Berns KI, Muzyczka N. (1983) Rescue of adeno-associated virus from recombinant plasmids: gene correction within the terminal repeats of AAV. *Cell* **33**: 135–43.

Samulski RJ, Zhu X, Xiao X, *et al*. (1991) Targeted integration of adeno-associated virus (AAV) into human chromosome 19. [erratum appears in *EMBO J* March 1992; **11**(3): 1228]. *EMBO J* **10**: 3941–50.

Sanlioglu S, Benson PK, Yang J, *et al*. (2000) Endocytosis and nuclear trafficking of adeno-associated virus type 2 are controlled by rac1 and phosphatidylinositol-3 kinase activation. *J Virol* **74**: 9184–96.

Saudan P, Vlach J, Beard P. (2000) Inhibition of S-phase progression by adeno-associated virus Rep78 protein is mediated by hypophosphorylated pRb. *EMBO J* **19**: 4351–61.

Saydam O, Glauser DL, Heid I, *et al*. (2005) Herpes simplex virus 1 amplicon vector-mediated siRNA targeting epidermal growth factor receptor inhibits growth of human glioma cells *in vivo*. *Mol Ther: J Am Soc Gene Ther* **12**: 803–12.

Schmidt M, Chiorini JA, Afione S, Kotin R. (2002) Adeno-associated virus type 2 Rep78 inhibition of PKA and PRKX: fine mapping and analysis of mechanism. *J Virol* **76**: 1033–42.

Severini A, Scraba DG, Tyrrell DL. (1996) Branched structures in the intracellular DNA of herpes simplex virus type 1. *J Virol* **70**: 3169–75.

Shieh MT, WuDunn D, Montgomery RI, *et al*. (1992) Cell surface receptors for herpes simplex virus are heparan sulfate proteoglycans. *J Cell Biol* **116**: 1273–81.

Shukla D, Liu J, Blaiklock P, *et al*. (1999) A novel role for 3-O-sulfated heparan sulfate in herpes simplex virus 1 entry. *Cell* **99**: 13–22.

Sodeik B, Ebersold MW, Helenius A. (1997) Microtubule-mediated transport of incoming herpes simplex virus 1 capsids to the nucleus. *J Cell Biol* **136**: 1007–21.

干细胞之家www.stemcell8.cn ←点击进入

Sourvinos G, Everett RD. (2002) Visualization of parental HSV-1 genomes and replication compartments in association with ND10 in live infected cells. *EMBO J* **21**: 4989–97.
Spaete RR, Frenkel N. (1982) The herpes simplex virus amplicon: a new eucaryotic defective-virus cloning-amplifying vector. *Cell* **30**: 295–304.
Srivastava A, Lusby EW, Berns KI. (1983) Nucleotide sequence and organization of the adeno-associated virus 2 genome. *J Virol* **45**: 555–64.
Stavropoulos TA, Strathdee CA. (1998) An enhanced packaging system for helper-dependent herpes simplex virus vectors. *J Virol* **72**: 7137–43.
Stevens JG. (1975) Latent herpes simplex virus and the nervous system. *Curr Top Microbiol Immunol* **70**: 31–50.
Stow ND. (1982) Localization of an origin of DNA replication within the TRS/IRS repeated region of the herpes simplex virus type 1 genome. *EMBO J* **1**: 863–67.
Straus SE, Sebring ED, Rose JA. (1976) Concatemers of alternating plus and minus strands are intermediates in adenovirus-associated virus DNA synthesis. *Proc Natl Acad Sci USA* **73**: 742–46.
Summerford C, Bartlett JS, Samulski RJ. (1999) AlphaVbeta5 integrin: a co-receptor for adeno-associated virus type 2 infection. [see comment]. *Nat Med* **5**: 78–82.
Summerford C, Bartlett JS, Samulski RJ. (2000) Adeno-associated viral vectors and successful gene therapy, the gap is closing. *Gene Ther Regul* **1**: 9–32.
Summerford C, Samulski RJ. (1998) Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. *J Virol* **72**: 1438–45.
Surosky RT, Urabe M, Godwin SG, *et al*. (1997) Adeno-associated virus Rep proteins target DNA sequences to a unique locus in the human genome. *J Virol* **71**: 7951–59.
Tattersall P, Ward DC. (1976) Rolling hairpin model for replication of parvovirus and linear chromosomal DNA. *Nature* **263**: 106–9.
Terry-Allison T, Montgomery RI, Whitbeck JC, *et al*. (1998) HveA (herpesvirus entry mediator A), a coreceptor for herpes simplex virus entry, also participates in virus-induced cell fusion. *J Virol* **72**: 5802–10.
Thomson BJ, Efstathiou S, Honess RW. (1991) Acquisition of the human adeno-associated virus type-2 rep gene by human herpesvirus type-6. *Nature* **351**: 78–80.
Thomson BJ, Weindler FW, Gray D, *et al*. (1994) Human herpesvirus 6 (HHV-6) is a helper virus for adeno-associated virus type 2 (AAV-2) and the AAV-2 rep gene homologue in HHV-6 can mediate AAV-2 DNA replication and regulate gene expression. *Virology* **204**: 304–11.
Thrasher AJ, Gaspar HB, Baum C, *et al*. (2006) Gene therapy: X-SCID transgene leukaemogenicity. *Nature* **443**: E5–6; discussion E6–7.
Timpe JM, Verrill KC, Trempe JP. (2006) Effects of adeno-associated virus on adenovirus replication and gene expression during coinfection. *J Virol* **80**: 7807–15.

干细胞之家www.stemcell8.cn ←点击进入

Tsunoda H, Hayakawa T, Sakuragawa N, Koyama H. (2000) Site-specific integration of adeno-associated virus-based plasmid vectors in lipofected HeLa cells. *Virology* **268**: 391–401.

Urcelay E, Ward P, Wiener SM, *et al*. (1995) Asymmetric replication *in vitro* from a human sequence element is dependent on adeno-associated virus Rep protein. *J Virol* **69**: 2038–46.

Vlazny DA, Kwong A, Frenkel N. (1982) Site-specific cleavage/packaging of herpes simplex virus DNA and the selective maturation of nucleocapsids containing full-length viral DNA. *Proc Natl Acad Sci USA* **79**: 1423–27.

Wade-Martins R, Smith ER, Tyminski E, *et al*. (2001) An infectious transfer and expression system for genomic DNA loci in human and mouse cells. *Nat Biotechnol* **19**: 1067–70.

Wagner JA, Nepomuceno IB, Messner AH, *et al*. (2002) A phase II, double-blind, randomized, placebo-controlled clinical trial of tgAAVCF using maxillary sinus delivery in patients with cystic fibrosis with antrostomies. *Hum Gene Ther* **13**: 1349–59.

Wang XS, Ponnazhagan S, Srivastava A. (1996) Rescue and replication of adeno-associated virus type 2 as well as vector DNA sequences from recombinant plasmids containing deletions in the viral inverted terminal repeats: selective encapsidation of viral genomes in progeny virions. *J Virol* **70**: 1668–77.

Wang XS, Srivastava A. (1997) A novel terminal resolution-like site in the adeno-associated virus type 2 genome. *J Virol* **71**: 1140–46.

Wang Y, Camp SM, Niwano M, *et al*. (2002a) Herpes simplex virus type 1/adeno-associated virus rep(+) hybrid amplicon vector improves the stability of transgene expression in human cells by site-specific integration. *J Virol* **76**: 7150–62.

Wang Y, Fraefel C, Protasi F, *et al*. (2000) HSV-1 amplicon vectors are a highly efficient gene delivery system for skeletal muscle myoblasts and myotubes. *Am J Physiol Cell Physiol* **278**: C619–26.

Wang Y, Mukherjee S, Fraefel C, *et al*. (2002b) Herpes simplex virus type 1 amplicon vector-mediated gene transfer to muscle. *Hum Gene Ther* **13**: 261–73.

Ward P, Berns KI. (1995) Minimum origin requirements for linear duplex AAV DNA replication *in vitro*. *Virology* **209**: 692–95.

Ward P, Elias P, Linden RM. (2003) Rescue of the adeno-associated virus genome from a plasmid vector: evidence for rescue by replication. *J Virol* **77**: 11480–90.

Ward P, Falkenberg M, Elias P, *et al*. (2001) Rep-dependent initiation of adeno-associated virus type 2 DNA replication by a herpes simplex virus type 1 replication complex in a reconstituted system. *J Virol* **75**: 10250–58.

Ward P, Urcelay E, Kotin R, *et al*. (1994) Adeno-associated virus DNA replication *in vitro*: activation by a maltose binding protein/Rep68 fusion protein. *J Virol* **68**: 6029–37.

干细胞之家www.stemcell8.cn ←点击进入

Weitzman MD, Fisher KJ, Wilson JM. (1996) Recruitment of wild-type and recombinant adeno-associated virus into adenovirus replication centers. *J Virol* **70**: 1845–54.

Weitzman MD, Kyostio SR, Kotin RM, Owens RA. (1994) Adeno-associated virus (AAV) Rep proteins mediate complex formation between AAV DNA and its integration site in human DNA. *Proc Natl Acad Sci USA* **91**: 5808–12.

Wild P, Schraner EM, Cantieni D, *et al*. (2002) The significance of the Golgi complex in envelopment of bovine herpesvirus 1 (BHV-1) as revealed by cryobased electron microscopy. *Micron* **33**: 327–37.

Xiao W, Warrington KH Jr, Hearing P, *et al*. (2002) Adenovirus-facilitated nuclear translocation of adeno-associated virus type 2. *J Virol* **76**: 11505–17.

Xiao X, Li J, Samulski RJ. (1998) Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. *J Virol* **72**: 2224–32.

Xiao X, Xiao W, Li J, Samulski RJ. (1997) A novel 165-base-pair terminal repeat sequence is the sole cis requirement for the adeno-associated virus life cycle. *J Virol* **71**: 941–48.

Young SM Jr, McCarty DM, Degtyareva N, Samulski RJ. (2000) Roles of adeno-associated virus Rep protein and human chromosome 19 in site-specific recombination. *J Virol* **74**: 3953–66.

Young SM Jr, Samulski RJ. (2001) Adeno-associated virus (AAV) site-specific recombination does not require a Rep-dependent origin of replication within the AAV terminal repeat. *Proc Natl Acad Sci USA* **98**: 13525–30.

Zhang HG, Wang YM, Xie JF, *et al*. (2001) Recombinant adenovirus expressing adeno-associated virus cap and rep proteins supports production of high-titer recombinant adeno-associated virus. *Gene Ther* **8**: 704–12.

Zhang X, Efstathiou S, Simmons A. (1994) Identification of novel herpes simplex virus replicative intermediates by field inversion gel electrophoresis: implications for viral DNA amplification strategies. *Virology* **202**: 530–39.

Zhou X, Muzyczka N. (1998) *In vitro* packaging of adeno-associated virus DNA. *J Virol* **72**: 3241–47.

Zolotukhin S, Potter M, Zolotukhin I, *et al*. (2002) Production and purification of serotype 1, 2, and 5 recombinant adeno-associated viral vectors. *Methods* **28**: 158–67.

干细胞之家www.stemcell8.cn ←点击进入

Chapter 4

Endonuclease-Boosted Gene Targeting and Cybridization for Long-Term Stem Cell Gene Therapy

Roger Bertolotti

Endonuclease-boosted gene targeting and *ex vivo* cybridization are the driving force of the long-term gene therapy arm of our universal stem cell gene therapy platform. They are ideal to tackle inherited diseases, since the mutant nuclear genome can be repaired by gene targeting and the wild-type mitochondrial genome (mtDNA) substituted for its mutant counterpart by cybridization, thereby restoring wild-type genomic homeostasis in both cases. Such an approach eliminates random-integration hazards of therapeutic transgenes, i.e., dysregulations and adverse oncogenic events that hamper current clinical gene therapy trials. Importantly enough, for other long-term stem cell gene therapy applications (mtDNA-independent acquired and degenerative/aging disorders), endonuclease-boosted gene targeting opens a promising custom site-specific integrative gene therapy era in which therapeutic transgenes can be integrated at selected hazard-free locations for optimized regulated expression. Our long-term gene therapy arm and synergistic transient regenerative gene therapy aimed at magnifying stem cell therapeutic homing/regenerative capabilities are thus discussed in terms of 1) the breakthrough development of custom site-specific endonucleases able to raise gene targeting efficiency to a level compatible with clinical protocols for both single-point mutations and targeted transgene integration, 2) of intensive investigations aimed at overcoming the current custom-endonuclease engineering bottleneck for both pioneering zinc-finger nucleases and emerging homing endonucleases, and 3) the extensive/clonal *ex vivo* growth potential of emerging adult/postnatal multipotent/pluripotent stem cells able to sustain the selective amplification of transmitochondrial stem cells (cybrids) and of engineered autologous stem cells under conditions of low gene targeting efficiencies. Low gene targeting efficiencies are incurred when the mutation track is too long or too far from the endonuclease

CNRS, Gene Therapy and Regulation Research, Faculty of Medicine, University of Nice-Sophia Antipolis, 06107 Nice, France. Roger.Bertolotti@unice.fr

干细胞之家www.stemcell8.cn ←点击进入

target site. Importantly enough, the breakthrough ability of the aforementioned adult/postnatal stem cells to contribute to all three germ layers (pluripotency) is presented in light of the universality of our autologous stem cell gene therapy platform, and in light of strategies aimed at tackling both tissue-specific and multi-systemic pathologies (including mtDNA diseases and aging disorders) through stem cell repopulation dynamics into appropriate niches (long-term engraftment) and tissues (cell turnover).

Keywords: Autologous pluripotent stem cells; cybrid stem cells; endonuclease-boosted gene targeting; gene repair/alteration; targeted transgene integration; long-term gene therapy; transient regenerative gene therapy.

1. Introduction: Pioneering Clinical Retroviral Gene Therapy is Hampered by Random-Integration Oncogenic Hazards

The most obvious approach to tackle an inherited disease is to repair the dysfunctional gene in appropriate stem cells (Bertolotti, 1996a and 2000a). Re-establishing the wild type sequence of the mutant gene before or after the onset of the disease would prevent or reverse the genetic disorder, respectively. Prevention is of course the best way since complete phenotypic reversion is not likely to be possible in many cases. Although ideal for inherited diseases, this method of gene therapy requires efficient techniques of *in situ* gene repair that were not available until recently (Bertolotti, 1996a, 1998, 2004a and 2007). This is why pioneering gene therapy clinical trials relied on retrovirus-mediated transduction of transgenes encoding the wild-type product of the disease gene (Blaese *et al.*, 1995; Kohn *et al.*, 1995). Such an approach culminated in the early 2000s with the first unequivocal successes for gene therapy (Cavazzana-Calvo *et al.*, 2000; Aiuti *et al.*, 2002; Gaspar *et al.*, 2004).

Unfortunately, this therapeutic breakthrough is plagued by adverse oncogenic events resulting from the fact that retroviral vectors integrate at random into host chromosomal DNA (Hacein-Bey-Abina *et al.*, 2003b; Baum, 2007). Random integration of transgenic DNA into host chromosomal DNA is an insertional mutagenesis event that can hit a cancer-prone gene (e.g., oncogene, tumor suppressor or DNA-repair gene), thereby promoting a true long-term carcinogenesis hazard as experienced by patients from the first unequivocal successful gene therapy trial (Cavazzana-Calvo *et al.*, 2000; Hacein-Bey-Abina *et al.*, 2003b). In this trial, in which four out of eight successfully treated severe combined immunodeficiency (SCID)-X1 patients incurred retroviral vector-mediated random-insertional T cell lymphomas, the first lymphoma case occurred almost three years after successful therapeutic gene transfer (Hacein-Bey-Abina *et al.*, 2003a) and the fourth

干细胞之家www.stemcell8.cn ←点击进入

case a few months ago (Baum, 2007). Extensive analyses showed that retroviral vector integration sites preferentially map to growth-regulating genes expressed in hematopoietic stem cell (HSC) targets, thereby highlighting that integration occurs preferentially near/in active gene loci and suggesting that insertional activation of growth-related genes mediated by retroviral long-terminal repeat (LTR) enhancers may confer an *in vivo* clonal selective advantage to the relevant transduced HSCs (Deichmann *et al.*, 2007). Such an LTR enhancing effect may be a premalignant event as shown with *LMO2* proto-oncogene dysregulations in the aforedescribed T lymphoma cases (Hacein-Bey-Abina *et al.*, 2003b). Retroviral vector improvements are thus discussed in terms of control of LTR enhancers through either postintegration inactivation or insulators (Yi *et al.*, 2005; Deichmann *et al.*, 2007).

The second successful X-SCID trial (Gaspar *et al.*, 2004) is until now free from adverse events (Thrasher *et al.*, 2006). The same holds true for the aforementioned ADA-SCID trial (Aiuti *et al.*, 2002). However, the very first successful trial (Cavazzana-Calvo *et al.*, 2000; Hacein-Bey-Abina *et al.*, 2003b) has shown that, although a theoretically low-probability risk, random insertional oncogenesis is a true safety hazard of retroviral vectors. Such a hazard is all the more puzzling as retroviral vector integration has been shown to be favored near transcriptional start sites (Wu *et al.*, 2003; Mitchell *et al.*, 2004), a key location for gene regulation. In addition, random integration into host chromosomal DNA does not provide for optimal transgene expression/regulation, a major concern when, unlike with SCID patients, we deal with a tightly regulated function (see Bertolotti, 1998 and 2000a). Site-specific integrating vectors are thus under intensive investigations, and are now emerging for both viral and nonviral gene therapy protocols (e.g., Olivares and Calos, 2003; Allen and Samulski, 2003; Wilkinson *et al.*, 2005). Although promising (see Urabe *et al.* and Fraefel *et al.*, this volume), these site-specific integrating vectors lack chromosomal target flexibility and might thus incur differentiation background troubles if one wishes to target a broad spectrum of tissue stem cells. Unlike these approaches, integration of exogenous DNA mediated by gene targeting is driven by homologous recombination with chromosomal DNA (see Capecchi, 1989) and has thus no target restriction, since the integration site is not specified by the DNA-binding domain of a protein but by the very DNA sequence of the targeting vector that is homologous to target chromosomal DNA. Gene targeting mediates flexible DNA exchanges between chromosomal DNA and transfecting/transducing DNA, thereby providing the means to modify at will the sequence of target

干细胞之家www.stemcell8.cn ←点击进入

chromosomal DNA (Capecchi, 1989). Gene targeting stands thus not only as the ultimate process for site-specific (i.e., targeted) transgene integration, but also for gene repair/alteration (Bertolotti, 1996a and 1999). Therefore, clinical gene targeting is discussed both in terms of gene repair/alteration for inherited diseases/anti-viral therapy and in terms of custom site-specific integrative gene therapy for acquired/degenerative and aging disorders (Bertolotti, 1999, 2000a and 2004a).

2. Stem Cell Gene Targeting: Custom Site-Specific Endonuclease Breakthrough

2.1. *From Conventional to Endonuclease-Boosted Gene Targeting*

Gene targeting is ideal to tackle inherited diseases, since mutated sequences can be corrected and wild-type genomic homeostasis ideally restored (Bertolotti, 1996a, 2000a and 2007). Such a gene repair approach eliminates dysregulations and oncogenic hazards that hamper random integration of therapeutic DNA into host chromosomes (Bertolotti, 1998 and 2004a). However, conventional gene targeting is a highly inefficient process (Capecchi, 1989; Yanez and Porter, 1998). In addition, it is overwhelmed by random integration (Capecchi, 1989; Merrihew *et al.*, 1996; Yanez and Porter, 1998), most likely reflecting the prevalence of the nonhomologous end joining (NHEJ) DNA repair pathway over the homologous recombination one in mammalian cells (Vasquez *et al.*, 2001). For these reasons, we devised in 1991 a new approach to gene targeting aimed at increasing its efficiency to a level compatible with gene therapy protocols (Bertolotti R., Grant application to the French Ministry of Research and Technology, 1991; Bertolotti, 1996a and 1996b).

Further to our previous use of *in vivo* homologous recombination as a shuttle processing mechanism in both mammalian cells and fission yeast (Lutfalla *et al.*, 1985; Bertolotti *et al.*, 1995) as well as to our interest in eukaryotic RecA-like recombinases (Angulo *et al.*, 1989; Borchiellini *et al.*, 1997), we first focused on presynaptic filaments, i.e., the active recombinase–DNA nucleoprotein complexes that mediate the key reaction from homologous recombination, and developed a strategy to master *in vivo* homologous recombination through transfer of premade recombinase–DNA complexes comprising either a standard single-stranded DNA (ssDNA) backbone or designed double-stranded DNA (dsDNA)-cored backbones (Bertolotti, 1996a, 1996b and 2000b). Chimeric zinc-finger nucleases (ZFNs) were then designed that create sequence-specific double-strand breaks (DSBs) in target

干细胞之家www.stemcell8.cn ←点击进入

chromosomal DNA and stimulate gene targeting as expected (Bibikova *et al.*, 2003; Porteus and Baltimore, 2003). We thus devised a synergistic zinc-finger nuclease-boosted gene targeting approach, in which random integration may be blocked by the use of premade presynaptic complexes (stoichiometric coating of transfecting DNA by recombinase RAD51 protein) and homologous recombination promoted both by premade presynaptic complexes/DNA backbones and by sequence-specific DSBs in target chromosomal DNA (Bertolotti, 2004a and 2004b). Exquisite optimization of ZFN specificity and affinity culminates now in targeting efficiencies compatible with clinical practice (Urnov *et al.*, 2005; Moehle *et al.*, 2007), thereby paving the way to personalized stem cell gene targeting (Bertolotti, 2007).

2.2. *Double-strand Break (DSB) in Target Chromosomal DNA as a Dramatic Stimulator of Gene Targeting*

Like in yeast in which homologous recombination drives the main DNA DSB repair pathway, the genesis of a DSB in target chromosomal DNA is an efficient way to dramatically increase the frequency of gene targeting in mammalian cells (Smih *et al.*, 1995; Choulika *et al.*, 1995; Donoho *et al.*, 1998). Such a stimulation was, however, initially achieved in artificial systems in which prior modification of the chromosomal target was required to introduce the 18-base pair recognition site of the rare-cutting endonuclease I-SceI. An 18-bp sequence provides a remarkable degree of specificity in a mammalian genome (3×10^9 bp) since its predicted occurrence is once in 6.9×10^{10} (i.e., 4^{18}) bp. Consistent with this calculation, expression of this yeast meganuclease is nontoxic to the mammalian cells that have been used in these experiments, presumably because there are no endogenous sites in their genomes (Rouet *et al.*, 1994; see below).

Although essential to the proof of principle, I-SceI and other well-characterized homing endonucleases, which cleave long (typically >18 bp) DNA target sites, were of course not an option for gene targeting applications in the absence of site-specificity customization (see Bertolotti, 2004a and Stoddard *et al.*, this volume). Therefore, the pioneering breakthrough has been the design of ZFNs that provide the means to cleave DNA at virtually any site of interest and stimulate gene targeting as expected (Bibikova *et al.*, 2003; Porteus and Baltimore, 2003; Urnov *et al.*, 2005). Intensive investigations aimed at homing endonuclease customization are now eventually able to match the flexibility of the ZFN design (see Arnoult *et al.*, 2007; Gimble, 2007; Stoddard *et al.*, this volume). Therefore, both ZFNs and customized homing endonucleases designed to cut the human

干细胞之家www.stemcell8.cn ←点击进入

genome at a single specific location are now driving the endonuclease-boosted gene targeting breakthrough, thereby opening the clinical era for gene repair/alteration and custom site-specific integrative gene therapy (Bertolotti, 2007; see below).

3. Chimeric Zinc-finger Nucleases as Pioneering Custom Gene Targeting Boosters

3.1. *Genesis of Sequence-specific DSBs with Designed Zinc-finger Nucleases*

Chimeric zinc-finger nucleases are artificial proteins that comprise a designed zinc-finger DNA-binding domain linked to the nonspecific cleavage domain from the restriction enzyme *Fok*I (see Fig. 1 and Kim *et al.*, 1996). Like for the original *Fok*I enzyme, the active form of the chimeric nuclease is a dimer (Smith *et al.*, 2000). Such a dimerization through the *Fok*I cleavage domain is promoted by the binding of the two zinc-finger domains to their respective DNA targets (see Fig. 1 and Smith *et al.*, 2000). There are therefore steric requirements both in the spacing of the two binding sites on target DNA and in the length of the linker peptide that joins the zinc-finger domain

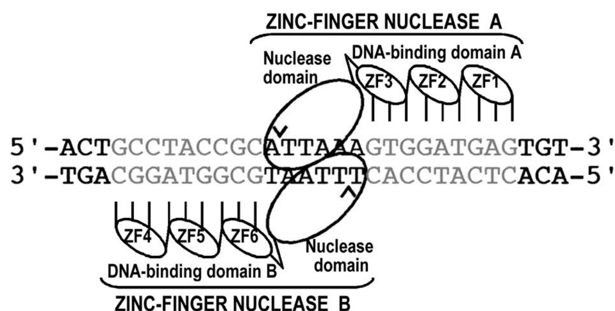


Fig. 1. Genesis of a site-specific DSB by a set of designed zinc-finger nucleases. The prototypic pair of zinc-finger nucleases *y*A and *y*B designed to cleave at a unique site the *yellow* (*y*) gene of *Drosophila* is schematized together with its target genomic sequence (modified from Fig. 1 of Bibikova *et al.*, 2002). The active form of the enzyme being a dimer, the unique 18-bp recognition site is designed to be specified by a pair of DNA-binding domains. A 9-bp specificity is achieved by the modular combination of three zinc fingers. The zinc-fingers recognize the target sequence in the major groove of dsDNA while making contacts primarily with bases in one strand, and are thus designed to provide close and anti-parallel binding to the two chimeric nucleases. DNA is cleaved as indicated by carats. Reprinted from: Bertolotti R., *Biogenic Amines*, **18**: 503–538 (2004), with permission of VSP/Brill.

干细胞之家www.stemcell8.cn ←点击进入

to the nucleasic one. Optimization of the design of ZFN prototypes has been performed by Carroll, Chandrasegaran and co-workers (Smith *et al.*, 2000; Bibikova *et al.*, 2001), and is illustrated in Fig. 1 with the *y*A-*y*B set that has been designed for a target site in the drosophila *yellow* (*y*) gene (Bibikova *et al.*, 2002). The *y*A-*y*B set has been shown to mediate efficient targeted chromosomal cleavage in *Drosophila* (Bibikova *et al.*, 2002) and to enhance gene targeting as expected (Bibikova *et al.*, 2003), thereby validating the ZFN approach in a true biological context (i.e., the target is a true endogenous gene in which the nuclease binding sites are not of transgenic origin). The same validation has been concurrently achieved in human cells in an artificial genomic context, where gene targeting is directed at an engineered green fluorescent protein (GFP) transgene stably integrated in the genome of a human cell line (Porteus and Baltimore, 2003).

3.2. *Pioneering DSB-boosted Gene Targeting with Designed Zinc-finger Nucleases in Human Cells*

Consistent with the optimization of Bibikova *et al.* (2001; see Fig. 1), the binding sites for the two ZFNs were spaced by a 6-bp insert in the target GFP transgene (Porteus and Baltimore, 2003). Gene targeting was thus performed with a set of appropriate ZFNs and transfecting dsDNA comprising a stretch of 2700 bp homologous to target GFP gene sequences. This transfecting dsDNA was the substrate of homologous recombination with the genomic target; it carried a heterologous core sequence for gene repair/modification. The reaction was initiated by the co-transfection of substrate DNA and minigenes encoding the two ZFNs, and culminated in the genesis of easily scorable GFP gene targeting events. As anticipated, a dramatic increase in gene targeting frequencies was associated to the ZFNs and fairly matched the effect of endonuclease I-SceI in concurrent control experiments (Porteus and Baltimore, 2003). However, contrary to I-SceI, the set of ZFNs used in this experiment exhibited some toxicity, and extrapolation based on control I-SceI results indicated that, upon optimization, gene repair/modification rates of 3%–5% could be achievable (Porteus and Baltimore, 2003, supporting online material).

3.3. *Zinc Finger Nuclease-boosted Gene Targeting: Pioneering Single-base Correction Achievement*

Exquisite optimization of ZFNs has indeed been shown to be the critical point for highly efficient DSB-boosted gene targeting. With such an

干细胞之家www.stemcell8.cn ←点击进入

optimized set of ZFNs, DSB-boosted gene targeting culminated in a single-base correction frequency of ≈20% on human lymphoblastoid K562 cells and of ≈5% on primary human $CD4^+$ T cells (Urnov *et al.*, 2005). Under these conditions, random integration was negligible for both therapeutic DNA and ancillary vectors encoding the ZFNs (Urnov *et al.*, 2005, supplemental online material). The experimental target was an X-linked SCID mutation hotspot, thereby opening a new avenue for the very disease that has been the subject of both the first unequivocal successful gene therapy trial and the first random-insertional oncogenic cases (Cavazzano-Calvo *et al.*, 2000; Hacein-Bey-Abina *et al.*, 2003b). Such a single-base correction approach is amenable to hematopoietic stem cell gene therapy, even though $CD34^+$ stem cells are still in the low-frequency ranges for clinical gene targeting (Holmes *et al.*, 2004). A new seminal clinical trial can thus be envisioned in which gene repair could substitute for retrovirus-mediated hazardous random integration of therapeutic transgenes.

3.4. *From Single-base Correction to Multi-base Modification*

Such a high gene targeting efficiency is compatible with the synthesis-dependent strand annealing (SDSA) mechanism of the homologous recombination DSB repair pathway (Fig. 2; see Ira *et al.*, 2006). Strand invasion mediated by presynaptic filaments resulting from recombinase RAD51 polymerization onto 3′ overhangs of resected DSB ends results in pairing of homologous strands, thereby initiating DNA synthesis on the donor plasmid DNA template (Fig. 2). The invading strand is then displaced from the template sequence and anneals to the second resected end of the DSB. The second strand is then synthesized, using the first, newly copied strand as the template (Fig. 2 and Ira *et al.*, 2006). Such a gene conversion mechanism appears to drive mitotic DSB repair in yeast (Ira *et al.*, 2006) and is thus expected to be active in mitotic mammalian cells.

The SDSA mechanism can easily explain why sequence modification efficiency is excellent when the distance between the target bases and the DSB is short (less than ~50 bp), but quickly drops when the distance increases (Elliott *et al.*, 1998; Miller *et al.*, 2005; Porteus, 2006). Such a limitation is a major hurdle for the clinical management of most multi-base mutations. In addition, it pinpoints the personalized facet of current endonuclease-boosted gene repair since most sites of a disease gene have to be targeted with specific sets of designed nucleases. Therefore, in order to reach full clinical potentialities, vectorization of therapeutic

干细胞之家www.stemcell8.cn ←点击进入

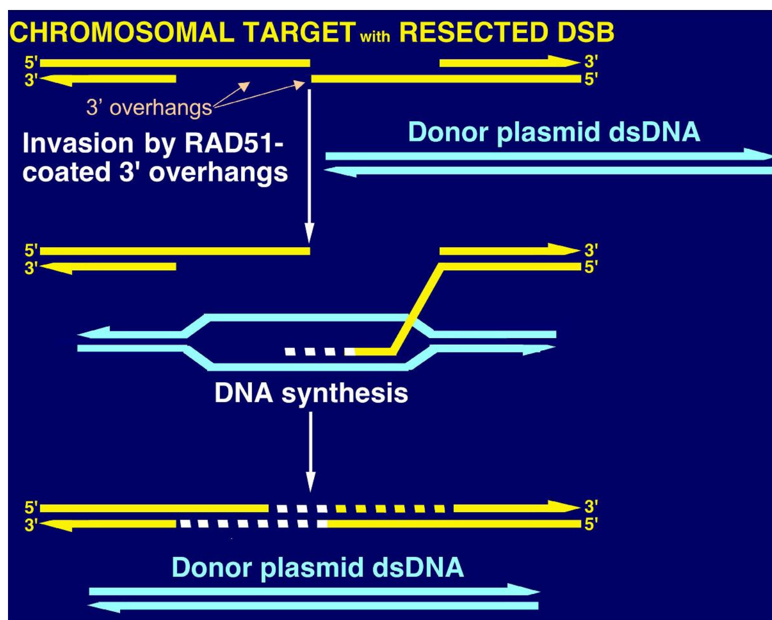


Fig. 2. Model of DSB-boosted gene targeting mediated by the synthesis-dependent strand-annealing (SDSA) mechanism. Like in the seminal DSB repair model of Szostak *et al.* (1983), the ends of a DSB are resected by a 5′-to-3′ exonuclease, producing 3′-ended ssDNA (3′ overhangs). Stoichiometric polymerization of recombinase RAD51 and ancillary proteins onto 3′ overhangs generates presynaptic filaments able to invade donor plasmid dsDNA (5497-bp circle carrying a 1.5-kb donor fragment; Urnov *et al.*, 2005) and to prime new DNA synthesis (dashed line) upon pairing with the homologous donor sequence. The invading strand is then displaced from the donor template sequence and anneals to the second resected end of the DSB. The second strand is then synthesized, using the first, newly copied strand as the template (Ira *et al.*, 2006). In this figure, the mutation is located near the DSB, on its left side.

DNA might need optimization based on our recombinase–DNA nucleoprotein complexes or DNA backbones (Bertolotti, 1996a, 2004a and 2004b) or on viral gene targeting constructs (Hendrie and Russell, 2005). Interestingly enough, an alternate approach is related to the mitochondrial DNA (mtDNA) arm of the stem cell gene therapy platform we have devised (Bertolotti, 2005 and 2006). It involves autologous multipotent/pluripotent stem cells that have an extensive *ex vivo* growth potential such as MAPCs, MIAMIs, hBMSCs or maGSCs (Jiang *et al.*, 2002; D'Ippolito *et al.*, 2004; Yoon *et al.*, 2005; Guan *et al.*, 2006), and are therefore amenable to *in vitro* post-gene targeting selective amplification in order to compensate for nonclinical gene targeting frequencies (Bertolotti, 2006 and 2007; see below).

干细胞之家www.stemcell8.cn ←点击进入

3.5. *Custom Site-specific Integrative Gene Therapy: The ZFN Breakthrough*

The aforedescribed SDSA mechanism suggests that targeted integration of some base pairs should be efficiently achieved using the previous set of ZFNs optimized for an X-linked SCID mutation hotspot (Urnov *et al.*, 2005). Unexpectedly, this set has been shown to drive high-efficiency site-specific integration of an 8-kb transgene construct in human lymphoblastoid K562 cells: targeted integration occurred at a frequency of 6% without any selection (Moehle *et al.*, 2007). Such a high frequency was slightly lower than for a 12-bp sequence (15%), but fairly matched the 5%–6% obtained with 900-bp and 1.5-kb sequences. This surprising result is however consistent with biochemical data and a SDSA mechanism in which both 3′ overhangs invade donor dsDNA and, after extensive DNA synthesis, anneal to each other and use each other as templates to complete DNA synthesis (Moehle *et al.*, 2007). The same efficiency appears to apply to two other loci and to a variety of human primary and stem cells, thereby opening a new era of site-specific integrative gene therapy in which therapeutic transgenes can be integrated in their endogenous counterparts for optimized regulated expression or at selected hazard-free locations (see below).

4. Customized Homing Endonucleases: New Impulse to Endonuclease-Boosted Gene Targeting

Unlike the aforedescribed unoptimized ZFNs, I-SceI as a prototypic yeast homing endonuclease is devoid of cytotoxicity in mammalian cells and is used as a reference in ZFN-mediated gene targeting experiments (Rouet *et al.*, 1994; Porteus and Baltimore, 2003; Urnov *et al.*, 2005; Alwin *et al.*, 2005; Porteus, 2006). Homing endonucleases have long and highly specific DNA-binding recognition sites (14–40 bp) that generate site-specific DSBs into intron- and intein-less targets, thereby initiating the transfer of the very introns or intein-DNAs that encode them (see Gimble, 2007 and Stoddard *et al.*, this volume). Such an extremely high DNA-binding specificity and the prototypic efficiency of I-SceI in mammalian cell gene targeting have been instrumental in the development of homing endonuclease engineering aimed at customizing their binding sites (see Gimble, 2007 and Stoddard *et al.*, this volume). Engineering involves enzymes that act as monomers (e.g., I-SceI) or dimers (e.g., I-CreI). Such an approach culminates now with the isolation of a prototypical set of engineered I-CreI homing endonucleases that has been shown with transgenic CHO-K1 cells to be a perfect reagent

干细胞之家www.stemcell8.cn ←点击进入

to efficiently boost gene targeting in the human *xeroderma pigmentosum complementation group C (XPC)* gene (Arnould *et al.*, 2007).

5. Customization of Site-Specific Endonucleases as the Limiting Factor of Clinical Gene Targeting

5.1. *Chimeric Zinc-finger Nucleases: Polydactyl Assembly of the DNA-binding Domain as the Critical Point of ZFN Customization*

As previously discussed, the critical point of zinc-finger nuclease design is the assembly of the Cys_2His_2 zinc-finger modules that comprise the DNA-binding domain (Bertolotti, 2004a and 2004b). Each finger module comprises about 30 amino acids that adopt a compact DNA-binding structure on chelating a zinc ion (Miller *et al.*, 1985), and primarily recognizes a specific DNA triplet sequence by forming base-specific contacts through the binding of its α helix into the major groove of dsDNA (Pavletich and Pabo, 1991). A panel of individual zinc fingers specific to each of the 64 possible DNA base triplets should theoretically cover all tandem-array assembling needs. However, straightforward modular assembly is hampered by cooperative and context-dependent contacts between neighboring fingers/neighboring DNA sites that interfere with the binding specificity of each finger module, thereby requiring sophisticated optimization strategies relying on random zinc-finger libraries (see Hirsch and Joung, 2004). New strategies are now emerging that might directly sustain simultaneous randomized selection of the zinc finger modules comprising the three-finger domains requested for ZFN site-specificity (Hurt *et al.*, 2003; Sepp and Choo, 2005), the latter involving breakthrough emulsion-based *in vitro* compartmentalization (Tawfik and Griffith, 1998; Sepp *et al.*, this volume).

Such an exquisite optimization of the DNA-binding domains is critical to the clinical use of ZFNs, since it maximizes gene targeting efficiency and minimizes/eliminates potential off-target DSB cleavages. Importantly enough, off-target DSBs are mutagenic/oncogenic hazards since they are not provided with therapeutic repair DNA (see Bibikova *et al.*, 2002) and, together with random integration of therapeutic DNA/ZFN vectors, are therefore a major long-term safety concern for the ZFN-boosted gene targeting approach. Custom assembly of predefined zinc-finger modules from various data sets stands as an attractive way to obtain active ZFNs for experimental gene targeting (Porteus and Baltimore, 2003; Bibikova *et al.*, 2003; Alwin *et al.*, 2005; Porteus, 2006). In this respect, online modular assembly

干细胞之家www.stemcell8.cn ←点击进入

is now available (Zinc Finger Targeter: Sander *et al.*, 2007), whereby pre-existing individual zinc fingers are sorted from the pooled archives of the three research groups that have designed/identified their own module sets for constructing multi-finger arrays: the Barbas laboratory at The Scripps Research Institute (Segal *et al.*, 1999; Dreier *et al.*, 2001 and 2005), Sangamo BioSciences Inc. (Zhang *et al.*, 2000; Liu *et al.*, 2001 and 2002) and ToolGen Inc. (Bae *et al.*, 2003); an alternate option is restricted to the Barbas laboratory modules (Zinc Finger Tools: Mandell and Barbas, 2006). However, even if specificity/functionality tests are used (Wright *et al.*, 2006; Carroll *et al.*, 2006), such a modular assembly approach is not currently amenable to the clinic without the aforedescribed exquisite optimization: the seminal achievement of Urnov *et al.* on the X-linked SCID model relies on proprietary optimization of the zinc-finger DNA-binding domains (Urnov *et al.*, 2005). Indeed, the final optimization step resulted both in a five-fold increase in gene targeting frequencies and in the disappearance of cytotoxic side-effects (Urnov *et al.*, 2005).

Cytotoxic side-effects are associated to unexquisite ZFN optimization and therefore appear to result from off-target DSBs (Porteus and Baltimore, 2003; Alwin *et al.*, 2005; Porteus, 2006). Hot-off-the-press data show that off-target DSBs are associated both to homodimer formation and to low affinity off-target sites, thereby validating the use of engineered variants in which nuclease domains are unable to homodimerize and have weakened dimerization affinity requiring stronger interactions between each monomer and its target DNA sequence, thereby minimizing off-target dimerization (Miller *et al.*, 2007; Szczepek *et al.*, 2007). Importantly enough, the destabilization of the dimer interface should be particularly efficient in preventing single-binding site cleavage that occurs when only one monomer of the active dimer is bound to DNA; such off-target DSBs are expected to appear at high ZFN concentrations, typically with unoptimized reagents (Mani *et al.*, 2005; Carroll and Porteus, 2005).

Another potential way to reduce off-target DSBs is to increase the length of the zinc-finger DNA-binding domain. DSB site specificity relies on a pair of matched ZFNs, i.e., a set of elements with nonunique DNA-binding specificity. The zinc-finger number of the DNA-binding domain was originally set to three in order to have an 18-bp recognition site, long enough to specify a unique genomic address in mammals (Smith *et al.*, 2000). However, each three-finger domain primarily recognizes a 9-base sequence and therefore statistically has $\approx 22,000$ binding sites in the human genome. Six-finger DNA-binding domains are currently used for designed transcription

干细胞之家www.stemcell8.cn ←点击进入

factors that have an exquisite specificity (Tan *et al.*, 2003), and could thus be amenable to ZFN design. However, the seminal achievement of Urnov *et al.* relied on four-finger domains, thereby suggesting that four-finger arrays could be a good safety compromise since gross genomic alterations were undetectable (Urnov *et al.*, 2005, supplemental online material). Shifting to a four-finger domain drastically reduces the statistical binding site number to ≈300 and, interestingly enough, may promote an attractive assembly method that uses optimized two-finger units instead of basic zinc-finger modules (Moore *et al.*, 2001). However, even with this assembly method, an ultimate exquisite optimization step is not dispensable (Urnov *et al.*, 2005).

In summary, emerging clinical ZFN design is thus discussed in terms of exquisite optimization of the zinc-finger domain, and of improved *Fok*I cleavage domains for asymmetic and low-energy dimerization.

5.2. *Emerging Customized Homing Endonucleases*

As shown above, the exquisite optimization of the zinc-finger DNA-binding domain of clinical grade ZFNs is a tedious and time-consuming process and is essential to the genesis of efficient, cytotoxic-free, hazard-free reagents (e.g., Urnov *et al.*, 2005). Therefore, modular assembly of designed polydactyl zinc-finger DNA binding domains is an illusive advantage when one deals with clinical-grade ZFNs. Such a situation is a fantastic booster for homing endonuclease engineering. As detailed by Stoddard *et al.* (this volume), the LAGLIDADG homing endonuclease family to which belongs prototypical I-SceI has many advantageous features, two of which being critical for the development of the appropriate reagents: they are the most specific of all known homing endonucleases, typically recognizing 19 bp to 22 bp DNA target sites, and employ a DNA-binding mechanism that is directly amenable to design and engineering. Intensive investigations are thus currently aimed at customizing the binding sites of prototypical LAGLIDADG homing endonuclease for both genomic and clinical gene targeting applications (see Gimble, 2007 and Stoddard *et al.*, this volume). High-throughput screening has already resulted in the isolation of customized I-CreI endonucleases that efficiently cleave two sequences from the human *XPC* gene and induce high gene targeting frequencies in transgenic CHO-K1 cells, thereby matching on a human target sequence the efficiency of prototypical I-SceI (Arnoult *et al.*, 2007). The above high-throughput technology developed by Cellectis S.A. (France) is still time-demanding, but new technical breakthroughs are arising (e.g., surface display of homing endonucleases: Volna

干细胞之家www.stemcell8.cn ←点击进入

et al., 2007) that may ease the current custom site-specific endonuclease bottleneck for clinical gene targeting (see below).

5.3. *Engineered Group II Intron Ribonucleoproteins as Potential Site-specific Endonucleases or Site-specific Integrating Vectors*

Group II introns are mobile, retroposable genetic elements that have a DNA target recognition site compatible with site specificity in a mammalian genome (Guo *et al.*, 2000). Their mobility is driven by retrohoming, whereby the excised intron RNA inserts directly into one strand of a dsDNA target site by reverse splicing and is then reverse-transcribed by the intron-encoded protein (IEP) (see Lambowitz and Zimmerly, 2004). IEP acts as a ribonucleoprotein complex with the excised intron RNA for both DNA target site recognition and retrohoming catalysis. However, most of the target specificity comes from base pairing of the intron RNA to the DNA target sequence, thereby opening simple intron RNA sequence modification to designed retrohoming vector engineering. Prototypical *Lactococcus lactis* group II intron Ll.LtrB has been subjected to a detailed analysis of its DNA target site recognition rules and is currently used for the production of designed bacterial targetrons (Guo *et al.*, 2000; Karberg *et al.*, 2001). Interestingly enough, group II introns straightforwardly convert into site-specific endonucleases when a reverse transcriptase-deficient IEP mutant is used. With such a mutant, reverse splicing in one strand of target DNA and cleavage of the opposite strand occur; however, in the absence of cDNA synthesis, the inserted intron RNA is apparently degraded, leaving a DSB at the target site that is readily amenable to gene targeting (Karberg *et al.*, 2001). Unfortunately, group II introns are restricted to bacteria and organelles, and, until now, engineered introns have been inactive on genomic DNA in mammalian cells (Jones *et al.*, 2005). Converting the IEP ribonucleoprotein complex into an active mammalian targeting vector stands as a true challenge, since promising results on episomic DNA were published in 2000 that did not yet translate into success (Guo *et al.*, 2000).

Interestingly enough, group II introns can accommodate sizable exogenous sequences; therefore, in addition to gene knock-out disruption, they drive site-specific integration of intron-embedded sequences culminating in targeted transgene integration (Guo *et al.*, 2000; Karberg *et al.*, 2001). The high targeting efficiency of engineered Ll.LtrB introns in bacteria is a strong incentive for their conversion into mammalian cell vectors; investigations

干细胞之家www.stemcell8.cn ←点击进入

are all the more intensive as these vectors are amenable to *cis*-splicing gene therapy, whereby a mutation is reversed by mRNA *cis*-splicing correction mediated by Ll.LtrB intron-embedded surrogate exon modules targeted into a disease gene intron (Jones *et al.*, 2005). However, although attractive for gene regulation and safety purposes, *cis*-splicing gene therapy does not re-establish wild-type genomic homeostasis, an ultimate feature of gene targeting.

5.4. *Custom Site-specific Endonucleases as the Ultimate Personalized Tools*

Zinc-finger nucleases, customized homing endonucleases and reverse transcriptase-deficient group II intron derivatives are designed not to tolerate a single-base variation in their recognition sequence. The corollary is that such a design is ultimately patient-specific and eventually mutation-specific. Mutation specificity is an attractive feature for improved DSB-boosted gene targeting, since inclusion of the mutated base(s) in the recognition sequence of a customized endonuclease prevents unwanted postrepair nucleasic activity and protects the wild-type allele when the target is a dominant mutation. Although dispensable in the aforedescribed pioneering achievement (Urnov *et al.*, 2005), such a desirable and ultimate personalization should improve both gene targeting efficiency (no therapeutic DNA cleavage and no postrepair cleavage) and long-term safety (postrepair cleavage increases mutagenic hazards culminating, possibly, in translocations; see Richardson and Jasin, 2000). However, it requires facilities that might accommodate site-specificity engineering relying mainly on RNA–DNA base pairing (group II ribonucleoproteins, see above) but that are incompatible with current customization technology of ZFNs or homing endonucleases. Indeed, for both ZFNs and homing endonucleases, a single base change in the recognition site requires us to start over exquisite optimization and selective isolation, i.e., time-consuming and labor-intensive processes under present methodologies. Therefore, strategic choices currently focus on highly conserved genomic sequences and on target sites located near the most common mutations of disease genes (e.g., X-linked SCID mutation hotspot chosen by Urnov *et al.*, 2005), thereby approaching all mutations from a single hotspot and nearby mutation sites with a single ZFN set or a single (single set of) custom homing endonuclease(s). However, silent sequence variations (e.g., no amino-acid change in a coding sequence) and other single nucleotide polymorphisms are likely to interfere in many cases.

干细胞之家www.stemcell8.cn ←点击进入

6. *Ex vivo* and *in vivo* Stem Cell Gene Therapy: Transfection (Plasmid dsDNA) or Transduction (AAV ssDNA and HD-Ad dsDNA)

Initiated on an easily transfectable human cell line (293) with the calcium phosphate co-precipitate method, zinc-finger nuclease-boosted gene targeting mediated by plasmid DNA culminated in gene repair/modification rates of 1%–3%, i.e., the very level of random integration frequency (Porteus and Baltimore, 2003). In their seminal achievement, Urnov *et al.* (2005) used either lipofection (LipofectAMINE 2000) or nucleofection (Amaxa Nucleofector) on the human lymphoblastoid K562 cell line and primary CD4$^+$ T cells and raised the gene repair/alteration frequency to ≈20% (K562 cells) and ≈5.3% (CD4$^+$ T cells), i.e., far above the random integration level (≈1%). The same successful conditions applied to custom site-specific integrative gene therapy too (Moehle *et al.*, 2007). Importantly enough, the approach is also working with CD34$^+$ hematopoietic stem cells, however with a fair but currently lower efficiency (≈1%–3%; Holmes *et al.*, 2004). It should therefore be soon amenable to *ex vivo* clinical gene therapy for both X-SCID and ADA-SCID, and also for a series of other monogenic diseases.

Although promising for *ex vivo* gene therapy, the plasmid DNA transfection approach may eventually be challenged by AAV transduction for *in vivo* protocols. Indeed, the gene targeting ability of recombinant AAV vectors has been recently established (Russell and Hirata, 1998) and shown to be much more efficient than conventional technology relying on dsDNA (Russell and Hirata, 1998; Inoue *et al.*, 1999; Hirata *et al.*, 2002). Importantly enough, like conventional gene targeting, AAV-mediated gene targeting has been shown to be dramatically stimulated by a designed DSB in target chromosomal DNA on two I-SceI artificial transgene models (Miller *et al.*, 2003; Porteus *et al.*, 2003). Such a stimulation reduces the critical AAV multiplicity of infection (MOI) from 10,000–400,000 to 100–1000 viral genomes/cell, i.e., to a level that might be compatible with a clinical trial (Porteus *et al.*, 2003; Miller *et al.*, 2003). Under optimal conditions, the gene targeting frequency is raised to the random-integration level, i.e., ≈1%–3% of transduced cells. Therefore, this approach that has been already used in pioneering *in vivo* gene targeting experiments (Miller *et al.*, 2006) is promising enough to be amenable to custom site-specific endonuclease-boosted protocols. The same holds true for helper-dependent adenoviral (HD-Ad) vectors that are able to transduce a wider spectrum of stem cells than AAV vectors (see Ohbayashi *et al.*, 2005; Mitani, this volume).

干细胞之家www.stemcell8.cn ←点击进入

Interestingly enough, the active substrate for the AAV-mediated gene targeting reaction appears to be the very genomic DNA from recombinant AAV, i.e., ssDNA (Hirata and Russell, 2000). Such a single-stranded genomic DNA is converted to dsDNA in a host-cell–mediated limiting step during the standard viral transducing process (see McCarty *et al.*, 2003 and Wang *et al.*, 2003). This host-cell–mediated synthesis step is apparently not involved in AAV-mediated gene targeting. The same appears to hold true for the minute virus of mouse (MVM), the second parvovirus that has been used in gene targeting experiments (Hendrie *et al.*, 2003). Such an observation suggests that, unlike standard homologous recombination DSB repair (see Fig. 2), the invading strands are not resected chromosomal DNA but AAV genomic ssDNA (see Miller *et al.*, 2003; Bertolotti, 2004a; Hendrie and Russell, 2005), thereby opening a potential new avenue for vector improvement (Bertolotti, 2004a and 2004b).

7. Endonuclease-Boosted Stem Cell Gene Targeting: From Highly Personalized Single-Base Correction to Broad Selective Gene Therapy

As discussed above, current gene targeting efficiency is restricted to a ≈50 bp distance from the site-specific DSB, thereby restricting clinical gene targeting to short-mutation correction and limiting the use of a ZFN set or a custom homing endonuclease to a short portion of a disease gene. Such a tendency to mutation-site specificity and to short DNA-track exchange is currently addressed by vectorization approaches (see above; Bertolotti, 2004a and 2004b; Hendrie and Russell, 2005), while long deletions are now the perfect targets for endonuclease-boosted integrative gene therapy (Moehle *et al.*, 2007). Importantly enough, the stem cell gene therapy platform we have devised provides another way to cope with low gene targeting frequencies incurred when the target is distant from the DSB or the mutation track too long (Bertolotti, 2006 and 2007). As illustrated below with the mitochondrial arm of our platform, multipotent/pluripotent stem cells such as the afore-described MAPCs, MIAMIs and maGSCs have an extended *in vitro* growth potential and can thus accommodate a drastic selective/amplificative step (e.g., cybrid rescue) before being returned to the patient (Bertolotti, 2005). Such a selective stem cell gene therapy strategy is also amenable to gene targeting, whereby properly engineered autologous cells are selected or sorted and amplified *in vitro* in order to increase their proportion/number to a level compatible with a clinical trial (Bertolotti, 2006 and 2007). This selective amplification strategy is less drastic than conventional gene targeting aimed

干细胞之家www.stemcell8.cn ←点击进入

at selecting discrete ES cell clones for the production of transgenic animals (see Capecchi, 1989; Abuin and Bradley, 1996; Sligh *et al.*, 2000) and is thus more appropriate for clinical applications. Such an approach is consistent with the breakthrough emergence of *ex vivo* amplification technologies for tissue-stem cells (e.g., Zhang *et al.*, 2006), and is therefore expected to extend soon to a variety of autologous stem cells including hematopoietic ones.

8. Cybridization as the Driving Force of Long-Term Stem Cell Gene Therapy for mtDNA Diseases

For inherited diseases, gene targeting provides the means to correct mutated sequences, thereby ideally restoring wild-type genomic homeostasis (Bertolotti, 1996a, 2000a and 2007). Gene targeting is, however, not amenable to mtDNA diseases (see Bertolotti, 2005). Indeed, due to the current lack of effective techniques for intramitochondrial genetic engineering, wild-type mtDNA sequence cannot be restored in mtDNA mutants (see below). Therefore, we have devised a strategy in which autologous transmitochondrial stem cells are generated *ex vivo* and then returned to the patient as in standard stem cell gene therapy (see above and Bertolotti, 2005). As detailed below, current approaches rely on nuclear transgenes for allotopic expression of mtDNA-encoded proteins or for mutant-mtDNA cleavage, culminating in the mtDNA-free mitochondria concept in which all mtDNA-encoded proteins are converted into allotopic gene products (Zullo, 2001). Unlike these approaches, stem cell cybrid gene therapy can accommodate all types of mtDNA mutations and is not hampered by random-integration hazards and transgene dysregulations associated to current long-term nuclear gene therapy (Bertolotti, 2005).

8.1. *Mitochondrial Genome (mtDNA) Mutants: From Human Diseases to Transmitochondrial Mouse Models*

With its $\approx 17,000$ base pairs comprised in a self-replicating DNA circle (Anderson *et al.*, 1981; Bibb *et al.*, 1981), the mitochondrial genome is small and can be easily handled in bacterial plasmids (see Chang *et al.*, 1975; Lutfalla *et al.*, 1985; Yoon and Koob, 2003). Yet, mtDNA is the target of mutations that lead to a variety of diseases in particular neuromuscular degenerative disorders (Wallace, 1999 and 2005; DiMauro and Schon, 2001; http://www.mitomap.org, 2007). The large number of identified mtDNA mutants is, however, restricted to humans. As emphasized by Cannon *et al.* (2005), mouse mtDNA mutants are few and difficult to generate due to high

干细胞之家www.stemcell8.cn ←点击进入

cellular mtDNA copy number and the current lack of effective techniques for intramitochondrial genetic engineering. Therefore, in order to produce mouse models of mtDNA diseases, an approach has been successfully devised in which exogenous mitochondria are used to create transmitochondrial animals (Sligh *et al*., 2000; see Cannon *et al*., 2005). Unfortunately, the human repertoire of mtDNA mutants cannot be used because the mitochondrial entity involves a series of nuclear genes in addition to mtDNA, thereby restricting functional integration of mtDNA and nuclear mitochondrial gene products to xenogeneic combinations between closely related species (e.g., human and nonhuman primates: Kenyon and Moraes, 1997). However, xenogeneic combinations between different mouse species culminate in slight dysregulations between mtDNA and nuclear mitochondrial gene products that mimic true mtDNA mutations (McKenzie *et al*., 2004; Trounce *et al*., 2004). A new approach for the production of animal models of mtDNA diseases is thus currently emerging and relies on xenomitochondrial mice (see Cannon *et al*., 2005).

8.2. *Homoplasmic Embryonic Stem (ES) Cybrids as Basic Tools for the Production of Transmitochondrial Animals*

Either with allo- or xeno-mitochondria, transmitochondrial mice are produced, like standard transgenic animals, by injecting engineered embryonic stem (ES) cells into normal mouse blastocysts and by using the resulting chimeric mice to obtain transgenic founders. In this case, the relevant ES cells are transmitochondrial cybrids obtained upon fusing a mtDNA-free female ES cell to an enucleated cell (cytoplast) derived either from a homoplasmic mtDNA mutant or from a xenogeneic cell. Initiated on somatic ρ^0 mutant cells that are devoid of mtDNA (King and Attardi, 1989) and then on rhodamine-6G–treated (ρ^0-like) cells (Trounce and Wallace, 1996; Levy *et al*., 1999), such a repopulation of mtDNA-free cells by exogenous mitochondria results in homoplasmic cybrids, i.e., cells containing a pure population of mtDNA. Homoplasmic ES cybrids have been instrumental in the derivation of the first homoplasmic mouse model for a mtDNA disease, i.e., chimeric females able to transmit the chloramphenicol-resistant (CAP^R) mtDNA in homoplasmic state to their progeny (Sligh *et al*., 2000). Unlike heteroplasmic cybrids obtained by fusing somatic cytoplasts either with ES cells (Marchington *et al*., 1999) or with single-cell embryos that generated the first heteroplasmic mouse model for a mtDNA disease (Inoue *et al*., 2000), transmitochondrial cybrids derived from rhodamine-6G–treated ES cells are thus the basic tools for the production of homoplasmic mouse models for

干细胞之家www.stemcell8.cn ←点击进入

mtDNA diseases, as illustrated by current xenomitochondrial mice (McKenzie *et al.*, 2004; Trounce *et al.*, 2004; Cannon *et al.*, 2005). Importantly enough, mtDNA being maternally inherited (Giles *et al.*, 1981), transmitochondrial mouse production relies on female ES cells.

8.3. *Cybrid Stem Cells: From Homoplasmic ES Cybrids to Autologous Adult Transmitochondrial Stem Cells*

8.3.1. *Transmitochondrial engineering does not alter the ES epigenotype*

Transmitochondrial ES cells are cybrids resulting from the fusion of a somatic cytoplast with a totipotent ES cell which has been cured from its mtDNA content by a transient growth period in the presence of rhodamine 6G (Levy *et al.*, 1999). Pioneering experiments (Sligh *et al.*, 2000; McKenzie *et al.*, 2004) have shown that neither the elimination of mtDNA mediated by rhodamine 6G nor the fusion with an enucleated somatic cell appear to interfere with the totipotentiality of the original ES cell. Such a result is consistent with basic somatic cell genetics (Ephrussi, 1972) and the chromosomal basis of epigenotype stability (Klebe *et al.*, 1970; Bertolotti, 1977): cybrids maintain the differentiated state of the nucleated parental cell even though they may incur a transient (few hours) extinction of the differentiated phenotype (Kahn *et al.*, 1981). Importantly enough, cloning experiments stand as a notable exception since cytoplast-mediated reprogramming eventually occurs at a very low frequency in oocyte cybrids, i.e., highly peculiar fusion products of somatic cells with overwhelming oocyte cytoplasts (see Wilmut *et al.*, 1997). Like ES cells, adult stem cells are thus expected to maintain their potentialities under appropriate growth conditions when fused to somatic cytoplasts, thereby opening mtDNA diseases to transmitochondrial stem cell gene therapy.

8.3.2. *Therapeutic autologous cybrid stem cells*

Therapeutic transmitochondrial stem cells should thus be obtained by fusing donor enucleated cells with rhodamine-6G–treated patient stem cells, preferentially multipotent/pluripotent ones that can be extensively grown and expanded *in vitro* (e.g., aforedescribed MAPCs, MIAMIs, hBMSCs or maGSCs). Such an extended *in vitro* expansion should be critical for the resulting cybrids in order to eliminate nuclear-encoded donor immunogens. True autologous stem cell cybrids would comprise wild-type mtDNA originating from the mother side of the patient's kindred (e.g., mother,

干细胞之家www.stemcell8.cn ←点击进入

mother's sisters and brothers, sisters and brothers, sisters' children, etc.) since mtDNA is maternally inherited (see above). Some flexibility is, however, expected for wild-type mtDNA, since the major concern of nonself mitochondrial protein immunogenicity has been successfully addressed in a bovine therapeutic cloning model (Lanza *et al.*, 2002). As schematized in Fig. 3, a clinical platform could thus be envisioned for the production of autologous stem cell cybrids, whereby a carefully selected hazard-free human ρ^0 cell line with a nuclear gene marker could be used as a mitochondria repopulating mediator like in homoplasmic ES cybrid

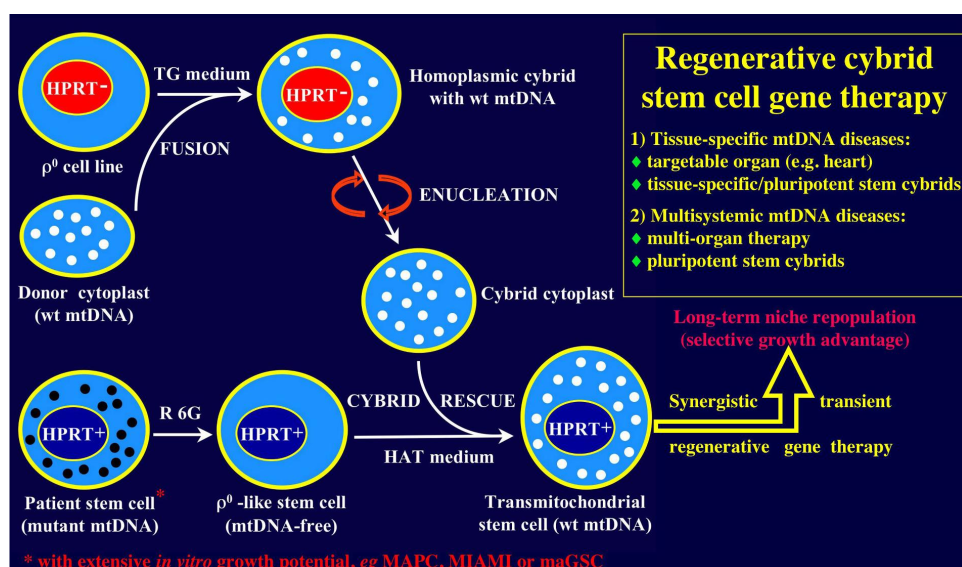


Fig. 3. Autologous transmitochondrial stem cells. *Left*: production platform. Wild-type (wt) mtDNA is amplified through cybridization of donor cytoplasts with a carefully selected hazard-free human ρ^0 cell line. The resulting homoplasmic cybrids are expanded under *in vitro* growth conditions and then enucleated before being fused to ρ^0-like patient stem cells obtained by a transient sub-lethal growth period in the presence of rhodamine 6G (R6G). While repopulating stem cells with wt mtDNA mitochondria, this second cybridization step rescues ρ^0-like cells that would otherwise die (see: Trounce and Wallace, 1996; Cannon *et al.*, 2005). In both cybridization steps, a selective marker such as the *hypoxanthine guanine phosphoribosyl transferase (HPRT)* gene is used to eliminate residual unenucleated "cytoplasts" either in thioguanine (TG, negative selection) or in HAT (positive selection; Szybalska and Szybalski, 1962; Littlefield, 1964) medium. Depending on the donor source, the ρ^0 cell line and its nuclear marker are dispensable. Adult/Postnatal stem cells (including umbilical cord derivatives) with an extensive *in vitro* growth potential should be amenable to this transmitochondrial platform. Based on Fig. 1 of: Bertolotti R., *Gene Therapy and Regulation*, **2**: 275–282 (2005), with permission of VSP/Brill. *Right*: regenerative cybrid stem cell gene therapy. See text for details.

干细胞之家www.stemcell8.cn ←点击进入

experiments (Sligh *et al.*, 2000; McKenzie *et al.*, 2004). With emerging multipotent/pluripotent adult stem cells like the aforedescribed MAPCs, MIAMIs and maGSCs that have an extensive growth potential, such a platform could culminate in a cybrid production efficiency compatible with clinical trials. It could also accommodate autologous ES-like cells that might arise from research on oocyte-free epigenotype-remodeling methods (see Bertolotti, 2001).

8.4. *Toward Transmitochondrial Stem Cell Gene Therapy for mtDNA diseases*

In vivo fusion of transplanted somatic cytoplasts with resident cells from target tissues/organs has been proposed for the treatment of mtDNA diseases (Kagawa and Hayashi, 1997; Kagawa *et al.*, 2001). However, we believe that the most promising approach is to transplant autologous cybrid stem cells, thereby relying on a true regenerative stem cell gene therapy approach that could culminate in a synergistic association with transient regenerative gene therapy (Bertolotti, 2005).

Autologous transmitochondrial stem cells are designed to avoid nonself donor protein immunogenicity (see above) and, together with their differentiating/differentiated progeny, could possibly convert their gain in energy metabolism efficiency into a selective *in vivo* growth advantage over resident mtDNA mutant cognate cells. Like the nuclear γC transgene in the seminal X-SCID trial (Cavazzana-Calvo *et al.*, 2000), wild-type mtDNA could thus drive effective therapeutic repopulation dynamics of autologous engineered stem cells and differentiated derivatives into appropriate niches (long-term engraftment) and relevant tissues/organs (cell turnover) of patients, respectively. Importantly enough, magnification of this repopulating/regenerative capability will be under the control of the transient regenerative gene therapy arm of our stem cell gene therapy platform (Bertolotti, 2005 and 2007).

Unlike current approaches that rely on nuclear transgenes for allotopic expression of mtDNA-encoded proteins (e.g., Owen *et al.*, 2000; Manfredi *et al.*, 2002; Guy *et al.*, 2002) or for mutant mtDNA cleavage/methylation (e.g., Srivastava and Moraes, 2001; Tanaka *et al.*, 2002; Minczuk *et al.*, 2006), transmitochondrial stem cell gene therapy is amenable to all types of mtDNA mutations and is not hampered by random-integration safety/dysregulation concerns. On the other hand, rhodamine-6G–treated ES cells did not display any obvious carcinogenic/teratogenic activity in the aforedescribed experiments (Levy *et al.*, 1999; Sligh *et al.*, 2000; McKenzie *et al.*, 2004; Trouce *et al.*, 2004), thereby indicating that the putative equivocal

千细胞之家www.stemcell8.cn ←点击进入

carcinogenic hazard of rhodamine 6G (National Toxicology Program, 1989) will most likely be easily cleared under transient mtDNA-curing conditions with both ES and various somatic cells. Transmitochondrial stem cell gene therapy stands thus as an attractive potential therapeutic approch for mtDNA diseases affecting targetable tissues (e.g., homoplasmic hypertrophic cardiomyopathy, Taylor *et al.*, 2003; see multisystemic/tissue-specific morbidity map of mtDNA in DiMauro and Schon, 2001) and, possibly, also for degenerative aging disorders linked to the accumulation of somatic mtDNA mutations (Wallace, 1992 and 2005; Michikawa *et al.*, 1999; Ruiz-Pesini *et al.*, 2007) when relevant autologous wild-type stem cells are not available. On the other hand, although tissue-specific mtDNA diseases are priority targets for the sake of easiness, the multisystemic prevalence of mtDNA pathologies for both inherited and aging disorders (DiMauro and Schon, 2001; Wallace, 2005) deserves strategies aimed at taking full advantage of the differentiative ability of adult/postnatal pluripotent stem cells. Such stem cells are able to contribute to all three germ layers and might thus be amenable to multi-organ transmitochondrial stem cell therapy.

9. Endonuclease-Boosted Gene Targeting and Cybridization as the Long-Term Gene Therapy Arm of our Stem Cell Gene Therapy Platform

As emphasized in our Introduction (Bertolotti, this volume), endonuclease-boosted gene targeting and *ex vivo* cybridization are the driving force of the long-term gene therapy arm of our universal stem cell gene therapy platform (Bertolotti, 2004a, 2005 and 2007). They are ideal to tackle inherited diseases, since the mutant nuclear genome can be repaired by gene targeting and the wild-type mitochondrial genome (mtDNA) substituted for its mutant counterpart by cybridization, thereby restoring a perfect wild-type genomic homeostasis in both cases (Bertolotti, 1996a, 2005 and 2007). Such an approach eliminates dysregulations and oncogenic hazards that hamper the random integration of conventional or allotopic transgenes into host chromosomes (Bertolotti, 1998 and 2005). Importantly enough, for other long-term stem cell gene therapy applications (mtDNA-independent acquired and degenerative/aging disorders), the hot-off-the-press gene targeting breakthrough achievement that culminated in high-efficiency integration of an ≈8 kb transgene (Moehle *et al.*, 2007) opens a promising custom site-specific integrative gene therapy era, in which therapeutic transgenes can be integrated at selected hazard-free locations for optimized regulated expression.

干细胞之家www.stemcell8.cn ←点击进入

For gene repair/alteration, the personalized features of site-specific endonucleases culminates with mutation specificity, whereby inclusion of the mutated base(s) in the recognition sequence of a customized endonuclease prevents unwanted postrepair nucleasic activity and protects the wild-type allele when the target is a dominant mutation (see above). Such a desirable and ultimate personalization should improve both gene targeting efficiency (no therapeutic DNA cleavage and no postrepair cleavage) and long-term safety (postrepair cleavage increases mutagenic hazards culminating, possibly, in translocations; see Richardson and Jasin, 2000). However, customization of both ZFNs and homing endonucleases for clinical applications is still a tedious and time-consuming process, thereby focusing current strategic choices on polyvalent ZFNs/homing endonucleases that target mutation hotspots and nearby mutation sites (see above). Another strategy, at least for a transient time period, is to tackle nuclear inherited diseases with the emerging custom site-specific integrative gene therapy, i.e., to design a single set of ZFNs/homing endonuclease(s) for a disease gene and to treat most of its dysfunctions with a single transgene (see Moehle *et al.*, 2007). Being targeted into a hazard-free site of its endogenous counterpart, the therapeutic transgene can be designed for optimized regulation.

The aforedescribed current strategies aimed at overcoming the custom site-specific endonuclease bottleneck are attractive. However, we believe that through vector improvement (see above and Bertolotti, 2004a and 2004b) and, most importantly, through the extensive/clonal growth properties of emerging autologous multipotent/pluripotent stem cells (e.g., aforementioned MAPCs, MIAMIs and maGSC) we will be able to overcome the current limited availability of custom site-specific endonucleases, thereby relying on *ex vivo* selective stem cell gene therapy to isolate/sort and amplify *in vitro* both autologous cybrid stem cells and stem cells incurring low-frequency gene targeting conditions. Then, like for standard selection-free processing, the homing/repopulating potential of the therapeutic stem cells will be magnified by the synergistic action of the transient regenerative gene therapy arm of our platform (Bertolotti, 2003a, 2003b, 2005 and 2007).

The breakthrough ability of the aforementioned adult/postnatal stem cells to contribute to all three germ layers (pluripotency) is an important parameter in the development of our universal autologous stem cell gene therapy platform, and opens exiting avenues to tackle both tissue-specific and multisystemic pathologies (including mtDNA diseases and aging disorders) through stem cell repopulation dynamics into appropriate niches

千细胞之家www.stemcell8.cn ←点击进入

(long-term engraftment) and tissues (cell turnover). Interestingly enough, the long-term gene therapy arm of our universal platform stands as the main personalized facet of our approach, and is expected to significantly contribute to the rise of tantalizing personalized medicine in the not-too-distant future (Bertolotti, 2007).

References

Abuin A, Bradley A. (1996) Recycling selectable markers in mouse embryonic stem cells. *Mol Cell Biol* **16**: 1851–56.

Aiuti A, Slavin S, Aker M, *et al*. (2002) Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. *Science* **296**: 2410–13.

Allen NA, Samulski RJ. (2003) The pros and cons of using the mechanism of AAV site-specific recombination in gene delivery. *Gene Ther Regul* **2**: 121–38.

Alwin S, Gere MB, Guhl E, *et al*. (2005) Custom zinc-finger nucleases for use in human cells. *Mol Ther* **12**: 610–17.

Anderson S, Bankier AT, Barrell BG, *et al*. (1981) Sequence and organization of the human mitochondrial genome. *Nature* **290**: 457–65.

Angulo J, Moreau P, Maunoury R, *et al*. (1989) Kin, a mammalian nuclear protein immunologically related to *E. coli* RecA protein. *Mutation Res* **217**: 123–34.

Arnould S, Chames P, Perez C, *et al*. (2006). Engineering of large numbers of highly specific homing endonucleases that induce recombination on novel DNA targets. *J Mol Biol* **355:** 443–58.

Arnould S, Perez C, Cabaniols JP, *et al*. (2007) Engineered I-CreI derivatives cleaving sequences from the human XPC gene can induce highly efficient gene correction in mammalian cells. *J Mol Biol* **371**: 49–65.

Bae KH, Kwon YD, Shin HC, *et al*. (2003) Human zinc fingers as building blocks in the construction of artificial transcription factors. *Nat Biotechnol* **21**: 275–80.

Baum C. (2007) Fourth case of leukaemia in the first SCID-X1 gene therapy trial, and the diversity of gene therapy. *http://www.esgct.org/upload/4th_CaseofLeukemial.pdf*

Bertolotti R. (1977) Expression of differentiated functions in hepatoma cell hybrids: selection in glucose-free media of segregated hybrid cells which reexpress gluconeogenic enzymes. *Somatic Cell Genet* **3**: 579–602.

Bertolotti R. (1996a) Recombinase-mediated gene therapy: strategies based on Lesch-Nyhan mutants for gene repair/inactivation using human RAD51 nucleoprotein filaments. *Biogenic Amines* **12**: 487–98.

Bertolotti R. (1996b) Recombinase-mediated gene therapy: targeting single stranded DNA to chromosomal DNA with human RAD51 presynaptic fibers. *Hepatology* **24**: 484A.

Bertolotti R. (1998) Gene therapy 1998: transient or stable minigene expression and gene repair/inactivation. *Biogenic Amines* **14**: 389–406.

干细胞之家www.stemcell8.cn ←点击进入

Bertolotti R. (1999) Recombinase-DNA nucleoprotein filaments as vectors for gene repair/inactivation and targeted integration of minigenes. *Biogenic Amines* **15**: 169–95.

Bertolotti R. (2000a) RNA and gene repair/alteration: from inherited diseases to acquired disorders and tantalizing applications for non-disease conditions. *Gene Ther Regul* **1**: 115–22.

Bertolotti R. (2000b) Gene therapy: dsDNA-cored presynaptic filaments as vectors for gene repair and targeted integration of transgenes. In: *Progress in Gene Therapy — Basic and Clinical Frontiers*, Bertolotti P, Parvez H, Nagatsu T (eds.), VSP, Utrecht, NL, pp. 513–49.

Bertolotti R. (2001) Adult and embryonic-like stem cells: toward a major gene therapy breakthrough relying on autologous multipotent stem cells. *Gene Ther Regul* **1**: 207–12.

Bertolotti R. (2003a) Stem cell gene therapy: breakthrough culminating in combination of *ex vivo* protocols with transient topical gene therapy. *Gene Ther Regul* **2**: 91–102.

Bertolotti R. (2003b) Stem cell gene therapy: a breakthrough combination magnified by therapeutic stem cell homing. In: *Progress in Gene Therapy — 2: Pioneering Stem Cell/Gene Therapy Trials*, Bertolotti R, Ozawa K, Hammond HK (eds.), VSP, Utrecht, NL, pp. 1–31.

Bertolotti R. (2004a) Zinc finger nuclease-boosted gene targeting and synergistic transient regenerative gene therapy for long-term stem cell gene therapy. *Biogenic Amines* **18**: 503–38.

Bertolotti R. (2004b) Zinc finger nuclease-boosted gene targeting: toward clinical gene repair/alteration and custom site-specific integrative gene therapy. *Gene Ther Regul* **2**: 177–89.

Bertolotti R. (2005) Transmitochondrial stem cells: from mouse models of mtDNA diseases to autologous cybrid stem cell gene therapy. *Gene Ther Regul* **2**: 275–82.

Bertolotti R. (2006) Stem cell gene therapy: toward a universal platform relying on custom endonuclease-boosted gene targeting, cybridization, transient regenerative/epigenetic gene therapy and synergistic combinations. *Mol Ther* **13**: S392.

Bertolotti R. (2007) Autologous stem cell gene therapy: toward a universal platform for personalized therapy. *Gene Ther Regul* **3**: 1–14.

Bertolotti R, Armbruster-Hilbert L, Okayama H. (1995) Liver fructose-1,6-bisphosphatase cDNA: trans-complementation of fission yeast and characterization of two human transcripts. *Differentiation* **59**: 51–60.

Bibb MJ, Van Etten RA, Wright CT, *et al*. (1981) Sequence and gene organization of mouse mitochondrial DNA. *Cell* **26**: 167–80.

Bibikova M, Carroll D, Segal DJ, *et al*. (2001) Stimulation of homologous recombination through targeted cleavage by chimeric nucleases. *Mol Cell Biol* **21**: 289–97.

干细胞之家www.stemcell8.cn ←点击进入

Bibikova M, Golic M, Golic KG, Carroll D. (2002) Targeted chromosomal cleavage and mutagenesis in *Drosophila* using zinc-finger nucleases. *Genetics* **161**: 1169–75.

Bibikova M, Beumer K, Trautman JK, Carroll D. (2003) Enhancing gene targeting with designed zinc finger nucleases. *Science* **300**: 764.

Blaese RM, Culver K, Miller AD, *et al*. (1995) T lymphocyte-directed gene therapy for ADA- SCID: initial trial results after 4 years. *Science* **270**: 475–80.

Borchiellini P, Angulo JF, Bertolotti R. (1997) Genes encoding mammalian recombinases: cloning approach with anti-RecA antibodies. *Biogenic Amines* **13**: 195–215.

Cannon MV, Pinkert CA, Trounce IA. (2005) Xenomitochondrial embryonic stem cells and mice: modeling human mitochondrial biology and disease. *Gene Ther Regul* **2**: 283–300.

Capecchi M. (1989) The new mouse genetics: altering the genome by gene targeting. *Trends Genet* **5**: 70–76.

Carroll D, Morton JJ, Beumer KJ, Segal DJ. (2006) Design, construction and *in vitro* testing of zinc finger nucleases. *Nat Protoc* **1**: 1329–41.

Castanotto D, Tommasi S, Li M, *et al*. (2005) Short hairpin RNA-directed cytosine (CpG) methylation of the RASSF1A gene promoter in HeLa cells. *Mol Ther* **12**: 179–83.

Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, *et al*. (2000) Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science* **288**: 669–72.

Chames P, Epinat JC, Guillier S, *et al*. (2005) *In vivo* selection of engineered homing endonucleases using double-strand break induced homologous recombination. *Nucl Acids Res* **33**: e178.

Chang AC, Lansman RA, Clayton DA, Cohen SN. (1975) Studies of mouse mitochondrial DNA in *Escherichia coli*: structure and function of the eukaryotic–prokaryotic chimeric plasmids. *Cell* **6**: 231–44.

Choulika A, Perrin A, Dujon B, Nicolas JF. (1995) Induction of homologous recombination in mammalian chromosomes by using the I-SceI system of *Saccharomyces cerevisiae*. *Mol Cell Biol* **15**: 1968–73.

D'Ippolito G, Diabira S, Howard GA, *et al*. (2004) Marrow-isolated adult multilineage inducible (MIAMI) cells, a unique population of postnatal young and old human cells with extensive expansion and differentiation potential. *J Cell Sci* **117**: 2971–81.

Deichmann A, Hacein-Bey-Abina S, Schmidt M, *et al*. (2007) Vector integration is nonrandom and clustered and influences the fate of lymphopoiesis in SCID-X1 gene therapy. *J Clin Invest* **117**: 2225–32.

DiMauro S, Schon EA. (2001) Mitochondrial DNA mutations in human disease. *Am J Med Genet* **106**: 18–26.

Donoho G, Jasin M, Berg P. (1998) Analysis of gene targeting and intrachromosomal homologous recombination stimulated by genomic double-strand breaks in mouse embryonic stem cells. *Mol Cell Biol* **18**: 4070–78.

干细胞之家www.stemcell8.cn ←点击进入

Dreier B, Beerli RR, Segal DJ, *et al*. (2001) Development of zinc finger domains for recognition of the 5′-ANN-3′ family of DNA sequences and their use in the construction of artificial transcription factors. *J Biol Chem* **276**: 29466–78.

Dreier B, Fuller RP, Segal DJ, *et al*. (2005) Development of zinc finger domains for recognition of the 50-CNN-30 family DNA sequences and their use in the construction of artificial transcription factors. *J Biol Chem* **280**: 35588–97.

Elliott B, Richardson C, Winderbaum J, *et al*. (1998) Gene conversion tracts from double-strand break repair in mammalian cells. *Mol Cell Biol* **18**: 93–101.

Ephrussi B. (1972) *Hybridization of Somatic Cells*. Princeton University Press, NJ.

Gaspar HB, Parsley KL, Howe S, *et al*. (2004) Gene therapy of X-linked severe combined immunodeficiency by use of a pseudotyped gammaretroviral vector. *Lancet* **364**: 2181–87.

Giles RE, Blanc H, Cann HM, Wallace DC. (1981) Maternal inheritance of human mitochondrial DNA. *Proc Natl Acad Sci USA* **77**: 6715–19.

Gimble FS. (2007) Engineering homing endonucleases to modify complex genomes. *Gene Ther Regul* **3**: 33–50.

Guan K, Nayernia K, Maier LS, *et al*. (2006) Pluripotency of spermatogonial stem cells from adult mouse testis. *Nature* **440**: 1199–203.

Guo H, Karberg M, Long M, *et al*. (2000) Group II introns designed to insert into therapeutically relevant DNA target sites in human cells. *Science* **289**: 452–57.

Guy J, Qi X, Pallotti F, *et al*. (2002) Rescue of a mitochondrial deficiency causing Leber Hereditary Optic Neuropathy. *Ann Neurol* **52**: 534–42.

Hacein-Bey-Abina S, von Kalle C, Schmidt M, *et al*. (2003a) A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *N Engl J Med* **348**: 255–56.

Hacein-Bey-Abina S, Von Kalle C, Schmidt M, *et al*. (2003b) LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* **302**: 415–19.

Hendrie PC, Russell DW. (2005) Gene targeting with viral vectors. *Mol Ther* **12**: 9–17.

Hendrie PC, Hirata RK, Russell DW, (2003) Chromosomal integration and homologous gene targeting by replication-incompetent vectors based on the autonomous parvovirus minute virus of mice. *J Virol* **77**: 13136–45.

Hirata RK, Russell DW, (2000) Design and packaging of adeno-associated virus gene targeting vectors. *J Virol* **74**: 4612–20.

Hirata R, Chamberlain J, Dong R, Russell DW. (2002) Targeted transgene insertion into human chromosomes by adeno-associated virus vectors. *Nat Biotechnol* **20**: 735–38.

Hirsh AS, Joung JK. (2004) Engineered Cys2His2 zinc finger DNA-binding domains. *Gene Ther Regul* **2**: 191–206.

Holmes MC, Urnov FD, Lee YL, *et al*. (2004) Gene correction therapy using designed zinc finger-based endonucleases. *Mol Ther* **9**: S272.

干细胞之家www.stemcell8.cn ←点击进入

Hurt JA, Thibodeau SA, Hirsh AS, *et al*. (2003) Highly specific zinc finger proteins obtained by directed domain shuffling and cell-based selection. *Proc Natl Acad Sci USA* **100**: 12271–76.

Imamura T, Yamamoto S, Ohgane J, *et al*. (2004) Non-coding RNA directed DNA demethylation of Sphk1 CpG island. *Biochem Biophys Res Commun* **322**: 593–600.

Inoue N, Hirata RK, Russell DW. (1999) High-fidelity correction of mutations at multiple chromosomal positions by adeno-associated virus vectors. *J Virol* **73**: 7376–80.

Inoue K, Nakada K, Ogura A, *et al*. (2000) Generation of mice with mitochondrial dysfunction by introducing mouse mtDNA carrying a deletion into zygotes. *Nat Genet* **26**: 176–81.

Ira G, Satory D, Haber JE. (2006) Conservative inheritance of newly synthesized DNA in double-strand break-induced gene conversion. *Mol Cell Biol* **26**: 9424–29.

Jiang Y, Jahagirdar BN, Reinhardt RL, *et al*. (2002) Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* **418**: 41–49.

Jones JP, 3rd, Kierlin MN, Coon RG, *et al*. (2005) Retargeting mobile group II introns to repair mutant genes. *Mol Ther* **11**: 687–94.

Kagawa Y, Hayashi JI. (1997) Gene therapy of mitochondrial diseases using human cytoplasts. *Gene Ther* **4**: 6–10.

Kagawa Y, Inoki Y, Endo H. (2001) Gene therapy by mitochondrial transfer. *Adv Drug Deliv Rev* **49**: 107–19.

Kahn CR, Bertolotti R, Ninio M, Weiss MC. (1981) Short-lived cytoplasmic regulators of gene expression in cell cybrids. *Nature* **290**: 717–20.

Karberg M, Guo H, Zhong J, *et al*. (2001) Group II introns as controllable gene targeting vectors for genetic manipulation of bacteria. *Nat Biotechnol* **19**: 1162–67.

Kenyon L, Moraes CT. (1997) Expanding the functional human mitochondrial DNA database by the establishment of primate xenomitochondrial cybrids. *Proc Natl Acad Sci USA* **94**: 9131–35.

Kim YG, Cha J, Chandrasegaran S. (1996) Hybrid restriction enzymes: zinc finger fusions to Fok I cleavage domain. *Proc Natl Acad Sci USA* **93**: 1156–60.

King MP, Attardi G. (1989) Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation. *Science* **246**: 500–503.

Klebe RJ, Chen T, Ruddle RH. (1970) Mapping of a human genetic regulator element by somatic cell genetic analysis. *Proc Natl Acad Sci USA* **66**: 1220–27.

Kohn DB, Weinberg KI, Nolta JA, *et al*. (1995) Engraftment of gene-modified cells from umbilical cord blood in neonates with adenosine deaminase deficiency. *Nat Med* **1**: 1017–26.

Lambowitz AM, Zimmerly S. (2004) Mobile group II introns. *Ann Rev Genet* **38**, 1–35.

干细胞之家www.stemcell8.cn ←点击进入

Lanza RP, Chung HY, Yoo JJ, *et al*. (2002) Generation of histocompatible tissues using nuclear transplantation. *Nat Biotechnol* **20**: 689–96.

Leamon JH, Braverman MS, Rothberg JM. (2006) High-throughput, Massively Parallel DNA Sequencing Technology for the era of personalized medicine. *Gene Ther Regul* **3**: in press.

Levy SE, Waymire KG, Kim YL, *et al*. (1999) Transfer of chloramphenicol-resistant mitochondrial DNA into the chimeric mouse. *Transgenic Res* **8**: 137–45.

Littlefied J. (1964) Selection of hybrids from mating of fibroblasts *in vitro* and their presumed recombinants. *Science* **145**: 709–10.

Liu PQ, Rebar EJ, Zhang L, *et al*. (2001) Regulation of an endogenous locus using a panel of designed zinc finger proteins targeted to accessible chromatin regions. Activation of vascular endothelial growth factor A. *J Biol Chem* **276**: 11323–34.

Liu Q, Xia Z, Zhong X, Case CC. (2002) Validated zinc finger protein designs for all 16 GNN DNA triplet targets. *J Biol Chem* **277**: 3850–56.

Lutfalla G, Blanc H, Bertolotti R. (1985) Shuttling of integrated vectors from mammalian cells to *E. coli* is mediated by head-to-tail multimeric inserts. *Somat Cell Mol Genet* **11**: 223–38.

Mandell JG, Barbas CF, 3rd. (2006) Zinc finger tools: custom DNA-binding domains for transcription factors and nucleases. *Nucl Acids Res* **34** (Web Server issue): W516–23.

Manfredi G, Fu J, Ojaimi J, *et al*. (2002) Rescue of a deficiency in ATP synthesis by transfer of MTATP6, a mitochondrial DNA-encoded gene, to the nucleus. *Nat Genet* **30**: 394–99.

Mani M, Smith J, Kandavelou K, *et al*. (2005) Binding of two zinc finger nuclease monomers to two specific sites is required for effective double-strand DNA cleavage. *Biochem Biophys Res Commun*. **334**: 1191–97.

Marchington DR, Barlow D, Poulton J. (1999) Transmitochondrial mice carrying resistance to chloramphenicol on mitochondrial DNA: developing the first mouse model of mitochondrial DNA disease. *Nat Med* **5**: 957–60.

McCarty DM, Fu H, Monahan PE, *et al*. (2003) Adeno-associated virus terminal repeat (TR) mutant generates self-complementary vectors to overcome the rate-limiting step to transduction *in vivo*. *Gene Ther* **10**: 2112–18.

McKenzie M, Trounce IA, Cassar CA, Pinkert CA. (2004) Production of homoplasmic xenomitochondrial mice. *Proc Natl Acad Sci USA* **101**: 1685–90.

Merrihew R, Marburger K, Pennington S, *et al*. (1996) High-frequency illegitimate integration of transfected DNA at preintegrated target sites in a mammalian genome. *Mol Cell Biol* **16**: 10–18.

Michikawa Y, Mazzucchelli F, Bresolin N, *et al*. (1999) Aging-dependent large accumulation of point mutations in the human mtDNA control region for replication. *Science* **286**: 774–79.

Miller DG, Petek LM, Russell DW. (2003) Human gene targeting by adeno-associated virus vectors is enhanced by DNA double-strand breaks. *Mol Cell Biol* **23**: 3550–57.

干细胞之家www.stemcell8.cn ←点击进入

Miller DG, Wang PR, Petek L, *et al.* (2006) Gene targeting *in vivo* by adeno-associated virus vectors. *Nat Biotechnol* **24**: 1022–26.

Miller J, McLachlan AD, Klug A. (1985) Repetitive zinc-binding domains in the protein transcription factor IIIA from Xenopus oocytes. *EMBO J* **4**: 1609–14.

Miller JC, Urnov FD, Lee YL, *et al.* (2005) Development of Zinc finger nucleases for therapeutic gene correction of sickle cell anemia. *Mol Ther* **11**: S35.

Miller JC, Holmes MC, Wang J, *et al.* (2007) An improved zinc-finger nuclease architecture for highly specific genome editing. *Nat Biotechnol* **25**: 778–85.

Minczuk M, Papworth MA, Kolasinska P, *et al.* (2006) Sequence-specific modification of mitochondrial DNA using a chimeric zinc finger methylase. *Proc Natl Acad Sci USA* **103**: 19689–94.

Mitchell RS, Beitzel BF, Schroder AR, *et al.* (2004) Retroviral DNA integration: ASLV, HIV, and MLV show distinct target site preferences. *PLoS Biol* **2**: E234.

Moehle EA, Rock JM, Lee YL, *et al.* (2007) Targeted gene addition into a specified location in the human genome using designed zinc finger nucleases. *Proc Natl Acad Sci USA* **104**: 3055–60.

Moore M, Klug A, Choo Y. (2001) Improved DNA binding specificity from polyzinc finger peptides by using strings of two-finger units. *Proc Natl Acad Sci USA* **98**: 1437–41.

Morris KV, Chan SW, Jacobsen SE, Looney DJ. (2004) Small interfering RNA-induced transcriptional gene silencing in human cells. *Science* **305**: 1289–92.

National Toxicology Program. (1989) NTP Toxicology and Carcinogenesis Studies of Rhodamine 6G (C.I. Basic Red 1) (CAS No. 989-38-8) in F344/N Rats and B6C3F1 Mice (Feed Studies) *Natl Toxicol Program Tech Rep Ser* **364**: 1–192.

Ohbayashi F, Balamotis MA, Kishimoto A, *et al.* (2005) Correction of chromosomal mutation and random integration in embryonic stem cells with helper-dependent adenoviral vectors. *Proc Natl Acad Sci USA* **102**: 13628–33.

Olivares EC, Calos MP. (2003) Phage ϕC31 integrase-mediated site-specific integration for gene therapy. *Gene Ther Regul* **2**: 103–20.

Owen R, IV, Lewin AP, Peel A, *et al.* (2000) Recombinant adeno-associated virus vector-based gene transfer for defects in oxidative metabolism. *Hum Gene Ther* **11**: 2067–78.

Pavletich NP, Pabo CO. (1991) Zinc finger-DNA recognition: crystal structure of a Zif268-DNA complex at 2.1 A. *Science* **252**: 809–17.

Porteus MH. (2006) Mammalian gene targeting with designed zinc finger nucleases. *Mol Ther* **13**: 438–46.

Porteus MH, Baltimore D. (2003) Chimeric nucleases stimulate gene targeting in human cells. *Science* **300**: 763.

Porteus MH, Carroll D. (2005) Gene targeting using zinc finger nucleases. *Nat Biotechnol* **23**: 967–73.

Porteus MH, Cathomen T, Weitzman MD, Baltimore D. (2003) Efficient gene targeting mediated by adeno-associated virus and DNA double-strand breaks. *Mol Cell Biol* **23**: 3558–65.

干细胞之家www.stemcell8.cn ←点击进入

Richardson C, Jasin M. (2000) Frequent chromosomal translocations induced by DNA double-strand breaks. *Nature* **405**: 697–700.

Rouet P, Smih F, Jasin M. (1994) Expression of a site-specific endonuclease stimulates homologous recombination in mammalian cells. *Proc Natl Acad Sci USA* **91**: 6064–68.

Ruiz-Pesini E, Lott MT, Procaccio V, *et al*. (2007) An enhanced MITOMAP with a global mtDNA mutational phylogeny. *Nucl Acids Res* **35** (Database issue): D823–28.

Russell DW, Hirata RK. (1998) Human gene targeting by viral vectors. *Nat Genet* **18**: 325–30.

Sander JD, Zaback P, Joung JK, *et al*. (2007) Zinc Finger Targeter (ZiFiT): an engineered zinc finger/target site design tool. *Nucl Acids Res* **35** (Web Server issue): W599–605.

Segal DJ, Dreier B, Beerli RR, Barbas CF, 3rd. (1999) Toward controlling gene expression at will: selection and design of zinc finger domains recognizing each of the 50-GNN-30 DNA target sequences. *Proc Natl Acad Sci USA* **96**: 2758–63.

Sepp A, Choo Y. (2005) Cell-free selection of zinc finger DNA-binding proteins using *in vitro* compartmentalization. *J Mol Biol* **354**: 212–19.

Sepp A, Ghadessy F, Choo Y. (2006) Cell-free selection of DNA-binding proteins for future gene therapy applications. *Gene Ther Regul* **3**: in press.

Sligh JE, Levy SE, Waymire KG, *et al*. (2000) Maternal germ-line transmission of mutant mtDNAs from embryonic stem cell-derived chimeric mice. *Proc Natl Acad Sci USA* **97**: 14461–66.

Smih F, Rouet P, Romanienko PJ, Jasin M. (1995) Double-strand breaks at the target locus stimulate gene targeting in embryonic stem cells. *Nucl Acids Res* **23**: 5012–19.

Smith J, Bibikova M, Whitby FG, *et al*. (2000) Requirements for double-strand cleavage by chimeric restriction enzymes with zinc finger DNA–recognition domains. *Nucl Acids Res* **28**: 3361–69.

Srivastava S, Moraes CT. (2001) Manipulating mitochondrial DNA heteroplasmy by a mitochondrially targeted restriction endonuclease. *Hum Mol Genet* **10**: 3093–99.

Stoddard BL. (2005) Homing endonuclease structure and function. *Q Rev Biophys* **38**: 49–95.

Suzuki K, Shijuuku T, Fukamachi T, *et al*. (2005) Prolonged transcriptional silencing and CpG methylation induced by siRNAs targeted to the HIV-1 promoter region. *J RNAi Gene Silencing* **1**: 66–78.

Szczepek M, Brondani V, Buchel J, *et al*. (2007) Structure-based redesign of the dimerization interface reduces the toxicity of zinc-finger nucleases. *Nat Biotechnol*. **25**(7): 786–93.

Szostak JW, Orr-Weaver TL, Rothstein RJ, Stahl FW. (1983) The double-strand-break repair model for recombination. *Cell* **33**: 25–35.

干细胞之家www.stemcell8.cn ←点击进入

Szybalska E, Szybalski W. (1962) Genetics of human cell lines, IV. DNA-mediated heritable transformation of a biochemical trait. *Proc Natl Acad Sci USA* **48**: 2026–34.

Tan S, Guschin D, Davalos A, *et al*. (2003) Zinc-finger protein-targeted gene regulation: genomewide single-gene specificity. *Proc Natl Acad Sci USA* **100**: 11997–2002.

Tanaka M, Borgeld HJ, Zhang J, *et al*. (2002) Gene therapy for mitochondrial disease by delivering restriction endonuclease SmaI into mitochondria. *J Biomed Sci* **9**: 534–41.

Tawfik DS, Griffiths AD. (1998) Man-made cell-like compartments for molecular evolution. *Nat Biotechnol* **16**: 652–56.

Taylor RW, Giordano C, Davidson MM, *et al*. (2003) A homoplasmic mitochondrial transfer ribonucleic acid mutation as a cause of maternally inherited hypertrophic cardiomyopathy. *J Am Coll Cardiol* **41**: 1786–96.

Ting AH, Schuebel KE, Herman JG, Baylin SB. (2005) Short double-stranded RNA induces transcriptional gene silencing in human cancer cells in the absence of DNA methylation. *Nat Genet* **37**: 906–10.

Trounce I, Wallace DC. (1996) Production of transmitochondrial mouse cell lines by cybrid rescue of rhodamine-6G pre-treated L-cells. *Somat Cell Mol Genet* **22**: 81–85.

Trounce IA, McKenzie M, Cassar CA, *et al*. (2004) Development and initial characterization of xenomitochondrial mice. *J Bioenerg Biomembr* **36**: 421–27.

Urnov FD, Miller JC, Lee YL, *et al*. (2005) Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature* **435**: 646–51.

Vasquez KM, Marburger K, Intody Z, Wilson JH. (2001) Manipulating the mammalian genome by homologous recombination. *Proc Natl Acad Sci USA* **98**: 8403–10.

Volna P, Jarjour J, Baxter S, *et al*. (2007) Flow cytometric analysis of DNA binding and cleavage by cell surface-displayed homing endonucleases. *Nucl Acids Res* **35**: 2748–58.

Wallace DC. (1992) Mitochondrial genetics: a paradigm for aging and degenerative diseases? *Science* **256**: 628–32.

Wallace DC. (1999) Mitochondrial diseases in man and mouse. *Science* **283**: 1482–88.

Wallace DC. (2005) A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. *Ann Rev Genet* **39**: 359–407.

Wang Z, Ma HI, Li J, *et al*. (2003) Rapid and highly efficient transduction by double-stranded adeno-associated virus vectors *in vitro* and *in vivo*. *Gene Ther* **10**: 2105–11.

Weinberg MS, Villeneuve LM, Ehsani A, *et al*. (2006) The antisense strand of small interfering RNAs directs histone methylation and transcriptional gene silencing in human cells. *RNA* **12**: 256–62.

Wilkinson TA, Tan W, Chow SA. (2005) Safe delivery of therapeutic genes into specific chromosomal sites using engineered retroviral integrase. *Gene Ther Regul* **2**: 369–85.

干细胞之家www.stemcell8.cn ←点击进入

Wilmut I, Schnieke AE, McWhir J, *et al*. (1997) Viable offspring derived from fetal and adult mammalian cells. *Nature* **385**: 810–13.

Wright DA, Thibodeau-Beganny S, Sander JD, *et al*. (2006) Standardized reagents and protocols for engineering zinc finger nucleases by modular assembly. *Nat Protoc* **1**: 1637–52.

Wu X, Li Y, Crise B, Burgess SM. (2003) Transcription start regions in the human genome are favored targets for MLV integration. *Science* **300**: 1749–51.

Yanez RJ, Porter AC. (1998) Therapeutic gene targeting. *Gene Ther* **5**: 149–59.

Yi Y, Hahm SH, Lee KH. (2005) Retroviral gene therapy: safety issues and possible solutions. *Curr Gene Ther* **5**: 25–35.

Yoon YG, Koob MD. (2003) Efficient cloning and engineering of entire mitochondrial genomes in *Escherichia coli* and transfer into transcriptionally active mitochondria. *Nucl Acids Res* **31**: 1407–15.

Yoon YS, Wecker A, Heyd L, *et al*. (2005) Clonally expanded novel multipotent stem cells from human bone marrow regenerate myocardium after myocardial infarction. *J Clin Invest* **115**: 326–38.

Zhang CC, Kaba M, Ge G, *et al*. (2006) Angiopoietin-like proteins stimulate *ex vivo* expansion of hematopoietic stem cells. *Nat Med* **12**: 240–45.

Zhang L, Spratt SK, Liu Q, *et al*. (2000) Synthetic zinc finger transcription factor action at an endogenous chromosomal site. Activation of the human erythropoietin gene. *J Biol Chem* **275**: 33850–60.

Zullo SJ. (2001) Gene therapy of mitochondrial DNA mutations: a brief, biased history of allotopic expression in mammalian cells. *Semin Neurol* **21**: 327–35.

干细胞之家www.stemcell8.cn ←点击进入

Chapter 5

Cell-Free Protein-Evolution Systems for Engineering of Novel Sequence-Specific DNA-Binding and -Modifying Activities

Armin Sepp[†], Farid Ghadessy[‡] and Yen Choo[*,§]

Engineered DNA-binding proteins, in particular zinc finger proteins (ZFPs), comprise a promising new class of therapeutic proteins for applications in gene therapy that range from regulation of gene expression to *in vivo* mutation correction. All these approaches depend on the availability of highly specific DNA-binding proteins for genome-wide accurate targeting of therapeutic constructs. Until now, the design of novel ZFPs has mainly been achieved by selection using phage display. This is a labor-intensive approach which allows working with only moderately-sized combinatorial libraries. We overcame these limitations by adapting a convenient cell-free selection method known as *in vitro* compartmentalization (IVC) for the selection of ZFPs. In IVC, the expression libraries are assembled by PCR and up to 10^{10} independent clones can be selected conveniently in a single reaction. In addition to ZFPs, IVC has previously been used to engineer DNA-modifying enzymes, e.g., polymerase and methyltransferase activities. In this chapter, we describe the IVC procedure and review the progress made.

Keywords: *In vitro*; selection; expression; emulsion; zinc finger; methyltransferase.

*Corresponding author. yen@plasticell.co.uk
†43 Bullen Close, Cambridge CB1 8YU, UK
‡Institute of Molecular and Cell Biology, 61 Biopolis Drive, Singapore 138673
§Plasticell Ltd, Imperial Bioincubator, Bessemer Building (RSM), Prince Consort Road, South Kensington, London SW7 2BP, UK

干细胞之家www.stemcell8.cn ←点击进入

1. Introduction

1.1. *DNA-Binding Proteins: From Gene Regulation to Gene Therapy*

Gene therapy may be achieved by the introduction, deletion, modification or sequence editing of specific genes, and/or the exogenous regulation of their expression. In nature, these processes are frequently mediated by DNA-binding proteins and the ability to manipulate their activity is thus of key importance to future biotechnology in general and gene therapy in particular.

There are an estimated 25,000 genes (International Human Genome Sequencing Consortium, 2004) in the 3 billion bp human genome (Ventes *et al.*, 2001; Lander *et al.*, 2001), many of them encoding DNA-binding proteins from several structurally distinct protein families directly involved in cell growth, differentiation and homeostasis. Although thousands of different DNA-binding proteins are known, it is unlikely that the naturally-occurring ones can be used to achieve therapeutic goals, e.g., the repression of oncogenes and other disease-related genes, disruption of viral infection and reproductive cycles, as well as for the control of the expression of therapeutic transgenes delivered in gene therapy. The action of wild-type transcription factors on genes is synergistic, hence no one protein may be exploited to control a given gene of interest. In order to achieve this, it is necessary to engineer a novel DNA-binding protein that can bind specifically to a defined gene-associated DNA sequence typically 15–18 bp in length.

1.2. *Designed Zinc Finger Proteins*

Such novel DNA-binding proteins can be engineered using zinc finger proteins (ZFPs), small, Zn^{2+}-coordinated, modular DNA-binding motifs discovered in *Xenopus* oocyte transcription factor IIIA (Miller *et al.*, 1985). It has been demonstrated that the DNA-binding specificity of ZFPs may be altered by mutating key DNA-contacting amino acids present on the helix of the motif (Nardelli *et al.*, 1991). Although initial results suggested the existence of a simple base recognition code for ZFPs (Choo and Klug, 1997), it soon became evident that combinatorial phage display methods, modeled on those established for antibody engineering (Osbourn *et al.*, 2003), were far superior engineering strategies (Isalan and Choo, 2001; Isalan *et al.*, 2001; Choo and Klug, 1995; Jamieson *et al.*, 2003). These selected zinc fingers could be assembled into strings of up to six or seven units, thus recognizing long sequence motifs of at least 18 bp in length (Moore *et al.*, 2001a;

干细胞之家www.stemcell8.cn ←点击进入

Kim and Pabo, 1998; Moore *et al.*, 2001b) — theoretically specific enough to deliver a fused protein domain precisely to any desired location of the human genome. Engineered ZFPs have been used for the up- and down-regulation of plasmid-borne and chromosomally-embedded genes (Zhang *et al.*, 2000; Choo *et al.*, 1997; Beerli *et al.*, 2000; Choo *et al.*, 1994; Reynolds *et al.*, 2003; Papworth *et al.*, 2003) and even for the correction of a X-SCID gene point mutation through targeted recombination induced by ZFP-*Fok* I chimeric nuclease (Urnov *et al.*, 2005). At least one engineered ZFP transcription factor, which targets the VEGF-A gene, is already in gene therapy clinical trials for the treatment of diabetic neuropathy and peripheral artery disease (Yu *et al.*, 2006; Rebar *et al.*, 2002; Dai *et al.*, 2004), while several more, also based on zinc finger scaffolds, are at the preclinical development stage (Graslund *et al.*, 2005; Kim *et al.*, 2005; Manwani *et al.*, 2007).

1.3. *Methods for Combinatorial Selections of Proteins*

1.3.1. *Phage display*

In phage display, which is the most common method for combinatorial selections of proteins, a ZFP library is expressed in fusion to the N-terminus of phage p3 protein which is displayed on the viral capsid and is essential for bacterial infection (Smith, 1985; McCafferty *et al.*, 1990). Although phage display has been used successfully for the selection of novel ZFPs (Choo and Klug, 1994, 1995 and 1997; Jamieson *et al.*, 1994; Rebar and Pabo, 1994), and of course for selection of antibodies from very large libraries (Ling, 2003), the method has potential shortcomings related to library bias resulting from cytotoxicity, secretion or folding problems of individual clones. Additionally, since the phage particles that carry the library are produced in bacteria, these need to be transformed with plasmid DNA, an inefficient process for a plasmid construct the size of bacteriophage (about 9 kb). The transformation efficiency is thus limited to between 10^6–10^8 clones per μg of DNA, allowing full randomization of only 4–6 amino acids or only one zinc finger at a time in libraries of rather modest size. Since zinc fingers recognize DNA synergistically (Isalan *et al.*, 2001), it would be beneficial to simultaneously randomize more zinc fingers using a larger library.

Furthermore, the selected library members must also be rescued and amplified *in vivo*, and although this procedure is generally more efficient than bacterial transformation (and therefore does not further limit library

干细胞之家www.stemcell8.cn ←点击进入

size), the *in vivo* steps nevertheless further disadvantage the technique by making the entire process rather slow and cumbersome.

1.3.2. *In vitro mRNA-based libraries: ribosome- and RNA-displays*

There are several systems which can be used for the selection of proteins from large combinatorial libraries using completely *in vitro* conditions, thus bypassing the cell-based expression problems. Ribosome-display (Mattheakis *et al.*, 1994) and mRNA-display (Nemoto *et al.*, 1997; Roberts and Szostak, 1997) systems utilize RNA as the carrier of genetic information, while CIS-display (FitzGerald, 2000), CAD-display (Reiersen *et al.*, 2005) and *in vitro* compartmentalization (IVC) (Tawfik and Griffiths, 1998) use DNA. These methods produce relatively large library sizes (about 10^{10}–10^{12} independent clones per reaction) and allow convenient *in vitro* assembly and selection without the use of cells.

The most extensively used method is ribosome display (Mattheakis *et al.*, 1994). In this approach, the mRNA-based library is translated in a cell extract (i.e. *in vitro*) from a construct that lacks a stop codon, resulting in stalling of the ribosome and the formation of a ternary complex between the nascent polypeptide, coding mRNA and ribosome. These complexes, further stabilized by low temperature and Mg^{2+} ions, can be subsequently selected on the basis of the binding characteristics of the nascent polypeptide. Single-chain antibodies have been selected successfully by ribosome display both from naive and affinity maturation libraries using bacterial S30 extract (Hanes and Pluckthun, 1997; Hanes *et al.*, 1998 and 2000; Groves and Osbourn, 2005) or rabbit reticulocyte extract (He and Taussig, 1997). Recently, this technique has been successfully applied to the first selection of three-finger ZFPs from a naive Zif268-based library (Ihara *et al.*, 2006).

In mRNA display (*in vitro* virus), an alternative RNA-based system, the nascent polypeptides are covalently coupled to the encoding mRNA molecules via a puromycin linker attached to the 3-end of the encoding nucleotide molecule. These libraries can be expected to be even larger than those made by ribosome display, since they are not limited by the number of ribosomes available in the extract (Nemoto *et al.*, 1997; Roberts and Szostak, 1997). Again, although the pioneering applications were in the field of protein-protein interactions, including fibronectin domain-based combinatorial libraries (Lipovsek and Plückthun, 2004; Parker *et al.*, 2005), the method has now been applied to studying protein-DNA interactions in the form of genome-wide mapping of AP-1 class transcription factor regulatory networks (Tateyama *et al.*, 2006).

干细胞之家www.stemcell8.cn ←点击进入

1.3.3. *In vitro DNA-based libraries: CIS/CAD displays and IVC*

Both ribosome- and mRNA-display methods use RNA as the carrier of genetic information which needs to be transcribed *in vitro* from DNA at the start of each round of selection, then reverse transcribed back into DNA at the end of each round of selection for PCR amplification of selected genes to occur. DNA-based display systems eliminate these “Heath Robinson” manipulations as well as the problem of nucleic acid instability, resulting in a more robust and convenient selection system. DNA-based selection systems are also a simple means of carrying out DNA-binding protein engineering, since the expressed proteins can bind directly to the encoding DNA molecule if one or more target binding sites are incorporated into it, eliminating the need for bridging domains/ligands. They are also ideally suited for the selection and engineering of DNA-modifying enzymes which act on their encoding DNA. Alternatively, high-affinity DNA-binding proteins can be used as simple tools in DNA-based display systems, for linking peptides and other protein domains to their encoding molecules.

2. *In Vitro* Compartmentalization

A highly robust and convenient cell-free selection method for engineering ZFPs is the *in vitro* compartmentalization (IVC) procedure (Tawfik and Griffiths, 1998). IVC is based on the observation that *E. coli* coupled S30 *in vitro* transcription-translation system retains its activity upon emulsification in mineral oil, as can be demonstrated by compartmentalized expression of the green fluorescent protein (GFP) from a PCR-fragment based gene (Fig. 1, Panels A and B). The proteins expressed within the compartments can interact with their encoding genes, enabling *in vitro* selection of proteins (Fig. 2), Recent comprehensive reviews of the method give details of experimental protocols (Miller *et al.*, 2006) and applications (Griffiths and Tawfik, 2006).

Dispersion of the coupled *in vitro* transcription-translation mix containing the PCR fragment-based gene library occurs through high-speed stirring in mineral oil with a magnetic bar or a homogenizer. In the presence of detergents which act as surfactants, the resulting water-in-oil emulsion is stable for extended periods of time. Owing to the small size of the droplets formed — 2.5 μm median diameter (Tawfik and Griffiths, 1998) — 50 μl of extract can be effectively dispersed into 10^{10} compartments per 1 ml of emulsion or even more if the size of the compartments is further reduced to 1 μm through more vigorous stirring (Bertschinger and Neri, 2004). For practical purposes, these droplets can be thought of as artificial cells fully

干细胞之家www.stemcell8.cn ←点击进入

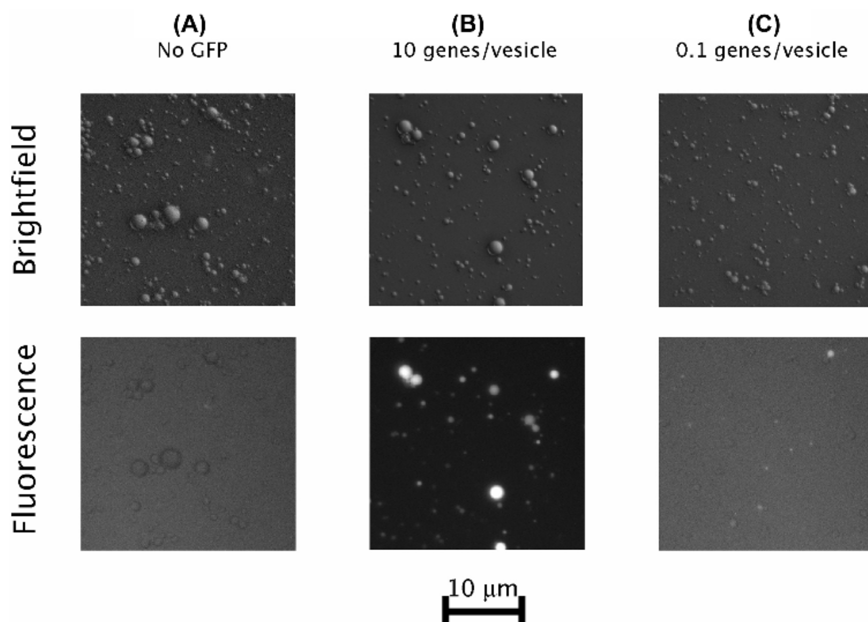


Fig. 1. Brightfield and fluorescent microphotographs of *in vitro* translation mix in water-in-oil emulsion with and without *in situ* expressed thermally stabilized GFP. The gene encoding the thermostable folding mutant GFP5 (Siemering *et al.*, 1996) was a gift from Dr. J. Haseloff. **(A)** No DNA added to the emulsified translation mix. **(B)** The gene encoding the T7-controlled expression cassette was applied at 10 genes per droplet. **(C)** The same as B but at 0.1 genes/droplet. (Sepp *et al.*, unpublished results).

equipped with ribosomes, tRNAs, amino acids, sources of energy and all the required enzymes to carry out gene expression from suitably configured DNA constructs. The average number of molecules expressed per droplet is related to the expression efficiency of the construct, the size of the droplet and the number of encapsulated genes. Nevertheless, even expression of a single gene in a droplet is possible, as can be readily seen by expressing a single copy of the GFP gene in an IVC reaction [Fig. 1, panel (C)]. For a 40-amino acid long peptide, the average yield per droplet was estimated to be about 350 molecules per droplet from a single DNA molecule (Sepp *et al.*, 2002). Owing to the small size and volume of the droplets, even a single molecule will have an effective concentration of 200 pM in 2.6 μm droplet and 3 nM in a 1 μm drop. A few hundred molecules in a 2.6 μm droplet will therefore result in a concentration of about 50 nM.

IVC selection of DNA-binding proteins requires a ZFP expression library to be emulsified so that genes end up dispersed into separate droplets wherein they are expressed. Proteins and DNA do not cross into the

干细胞之家www.stemcell8.cn ←点击进入

hydrophobic oil phase, remaining co-segregated within the aqueous interior of the droplets where they may interact. If genes that encode desired proteins bind or are modified by their products, then they may be isolated using a suitable selection strategy. The gene pool enriched for those encoding proteins with desired properties can then be re-amplified, and re-submitted for selection and eventually subcloned for further characterization. The sequence of expression, selection and amplification constitutes the basic selection cycle common to most *in vitro* selection4 systems (Fig. 2).

In order for selection of ZFPs to take place, some form of genotype-phenotype linkage between the expressed protein and the gene must occur within the IVC compartment. This may be the formation of a protein-DNA complex as discussed above (Sepp and Choo, 2005) or enzymatic modification of the encoding DNA (Lee *et al.*, 2002; Cohen *et al.*, 2004 and 2002;

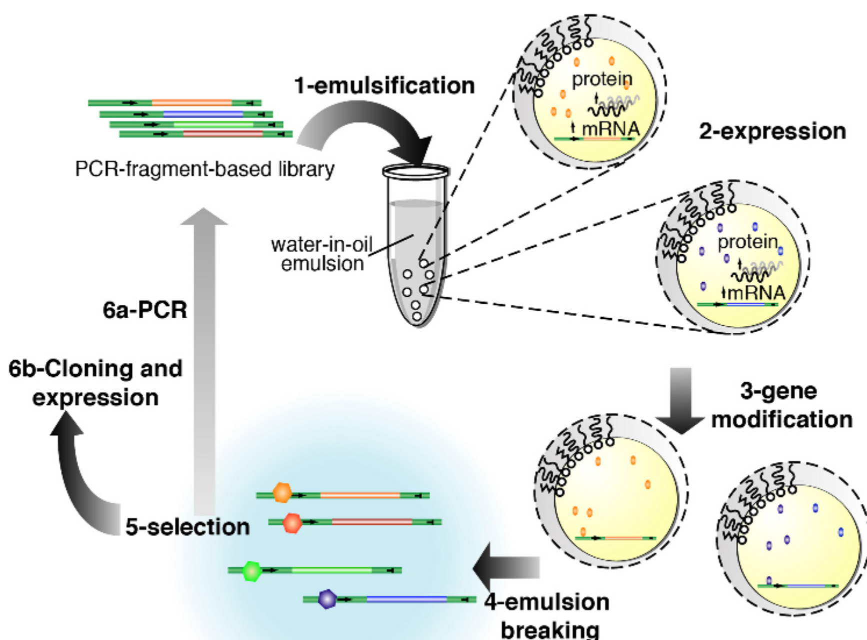


Fig. 2. IVC is based on the coupled transcription-translation occurring in an emulsified *in vitro* expression mix. The selection cycle starts with the emulsification of an *in vitro* expression mix containing the PCR fragment-based library (1). The genes are expressed within the aqueous droplets (2). The expression products interact with or modify their co-segregated genes (3). The emulsion is broken (4) and the genes modified in the desired way are isolated (5) and then amplified for the next round of selection (6a) or cloned for further characterization (6b). (Based on Fig. 1 of: Sepp *et al.*, *Gene Ther Regul*, **3**: 51–63 (2007), with permission from World Scientific Publishing.)

干细胞之家www.stemcell8.cn ←点击进入

Ghadessy and Holliger, 2007; Ghadessy *et al.*, 2004; Ghadessy and Holliger, 2004; Ghadessy *et al.*, 2001). Efficient protein evolution depends on the number of variant molecules that can be subjected to selection and it is to be expected that the higher this number is the greater the chance of isolating improved function. In an ideal case, all the emulsified droplets of the *in vitro* expression extract should contain one gene: this is to avoid any wasted extract in droplets with no DNA and conversely the loss of genotype-phenotype fidelity in droplets containing genes. However, the statistical distribution of genes between the droplets makes this unlikely to happen in practice. Assuming all droplets are of the same size (the emulsions formed by stirring are actually quite heterogeneous in size; however, the size distribution is sufficiently narrow that for practical purposes they can be treated as being homogeneous), the distribution of genes between them is described by Poisson distribution whereby the largest number of individually segregated genes occurs when the number of genes is equal to the number of compartments. However, even under these conditions, about 38% of the compartments are still expected to be empty, 38% to contain one gene and the rest two or more (Sepp *et al.*, 2007). The fraction of individually segregated genes can be increased but only at the cost of reduction in their absolute number (Fig. 3; Sepp *et al.*, 2007).

3. Selection of Zinc Finger Proteins and Zinc Finger Fusion Proteins by IVC

Cell-free selections are increasingly seen as advantageous for combinatorial protein design, as they allow large libraries to be assembled and selected rapidly. IVC which relies on DNA (the relatively stable nucleic acid) as the carrier of genetic information, is conveniently carried out at room temperature and allows flexibility in choosing the valency of protein display. There are no *in vivo* steps involved, i.e., bacterial transformation, neither during the assembly nor selection of the library. Furthermore, IVC libraries are ideally suited for the selection of DNA-binding proteins, as the encoding gene fragments can include a non-coding binding site recognized by the gene product. Any sufficiently stable protein-DNA complexes formed in the droplets can be recovered by biopanning using a universal peptide tag fused to the protein.

Using such a strategy we recently demonstrated in a model selection experiment that ZFPs can be selected by IVC (Sepp and Choo, 2005). The ZFPs were HA-tagged at the C-terminus, cloned in pET23a (Merck) *E. coli* expression vector between the T7 promoter and terminator sequences. The

干细胞之家www.stemcell8.cn ←点击进入

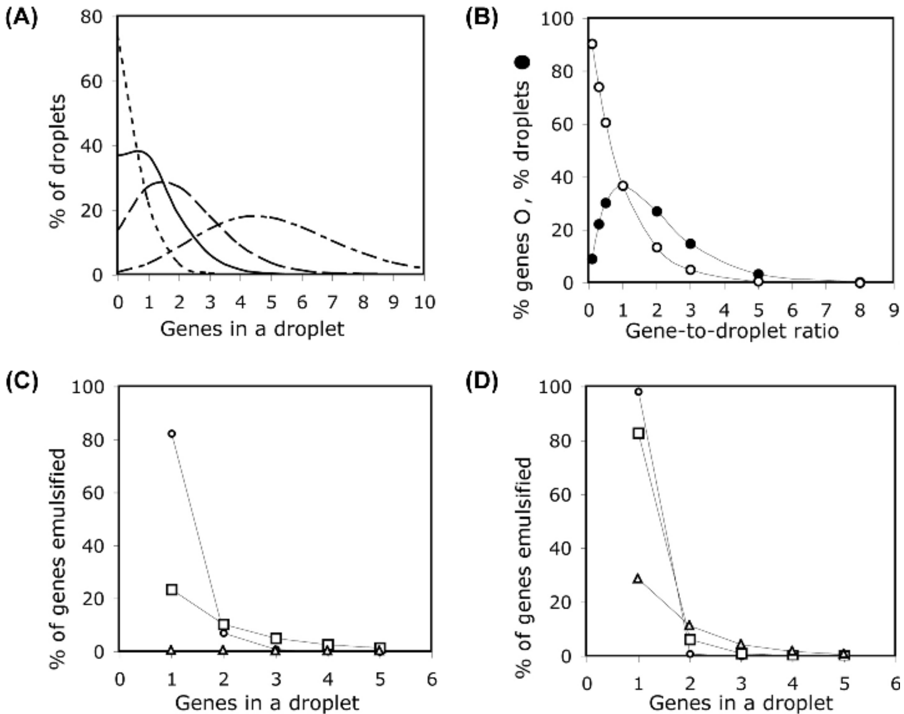


Fig. 3. The segregation of genes into droplets depends on the ratio of genes to droplets. **(A)** The predicted distribution of genes in droplets in a monodisperse emulsion according to Poisson statistics at the total gene/droplet ratios of 0.3 (dotted line), 1 (solid line), 2 (dashed line) and 5 (dotted/dashed line). **(B)** In a monodisperse emulsion, the percentage of droplets containing a single gene (filled circles) and the percentage of genes segregated individually (empty circles) depend on the ratio of total number of genes to droplets (gene-to-droplet ratio). **(C)** The predicted distribution of genes in a polydisperse emulsion containing 7×10^9 droplets with median diameter of 2.5 μm (Tawfik and Griffiths, 1998). Circles — 5×10^8 genes, rectangles — 7×10^9 genes, triangles — 1.2×10^{11} genes. **(D)** The predicted distribution of genes in a polydisperse emulsion containing 1.2×10^{11} droplets with median diameter of 1 μm (Bertschinger and Neri, 2004). Circles — 5×10^8 genes, rectangles — 7×10^9 genes, triangles — 1.2×10^{11} genes. (Based on Fig. 2 of: Sepp *et al.*, *Gene Ther Regul*, **3**: 51–63 (2007), with permission from World Scientific Publishing.)

HA tag was chosen because of commercial availability of a very high affinity anti-HA rat monoclonal antibody 3F10 (Roche). Two constructs were prepared, both carrying multiple copies of a target DNA site upstream of the T7 promoter. Upon compartmentalized expression, those ZFPs which recognize the DNA binding sites form complexes with their gene, while the rest remain unbound in solution. After breaking of the emulsion in the presence of competitor DNA, the *in vitro* expressed ZFPs are captured by immobilized anti-HA monoclonal antibody, co-precipitating a population of

干细胞之家www.stemcell8.cn ←点击进入

DNA molecules which is enriched with those encoding high affinity specific binders. This captured fraction of the library is then PCR-amplified for the next round of selection (Fig. 4).

We used two different ZFPs in the model selection. A target construct comprised the first four zinc fingers of *Xenopus* TFIIIA fused to the three zinc fingers of mouse Zif268 — the resulting chimeric protein has very high (Kd = 5 pM) affinity towards its chimeric 18 bp cognate binding site (Moore *et al.*, 2001a). A competitor construct comprised two head-to-tail fusions of engineered versions of mouse Zif268, previously designed to bind sequences in the promoter region of human immunodeficiency virus type 1 (Reynolds *et al.*, 2003). In order to simulate a realistic selection in our model, we used target and competitor proteins whose C-terminal portions of the constructs have almost identical sequence specificity.

To test the display system we carried out a series of model selections where the target gene construct was diluted 10,000-fold by the competitor gene construct and then selected by IVC at 5×10^9 genes per reaction. At this target: competitor ratio, it took only two rounds of selection for the target ZFP genes to become overwhelmingly dominant in the population, thus demonstrating the feasibility of IVC for the selection of high affinity ZFPs based on DNA-binding selectivity of a three-finger domain in a polydactyl protein (Fig. 5). We therefore conclude that selection of three-finger units attached to a three-finger fetter is possible, by analogy to previously discussed selection strategies (Isalan *et al.*, 2001).

4. Selection of DNA-Modifying Enzymes

The defining feature of IVC is its suitability for the selection of enzymes on the basis of catalytic turnover. DNA-modifying enzymes, in particular, are good selection candidates because the encoding gene can simultaneously function as a substrate. Indeed, the principle of IVC was demonstrated with M. *Hae* III DNA methyltransferase, which protected its encoding DNA fragment against cleavage by *Hae* III restriction endonuclease (Tawfik and Griffiths, 1998). A similar experimental setup was used to study the reaction mechanism and specificity of M. *Hae* III and M. *Hha* I methyltransferases, both of which catalyze the transfer of a methyl group from S-adenosylmethionine to the internal cytosine of their respective recognition sequences at the C5 position (Cohen *et al.*, 2004; Lee *et al.*, 2002). In this experiment, a two-step mutagenesis strategy was employed, involving initial randomization of DNA-contacting residues followed by randomization of the loop that lies behind these residues. This yielded a mutant with a 670-fold

干细胞之家www.stemcell8.cn ←点击进入

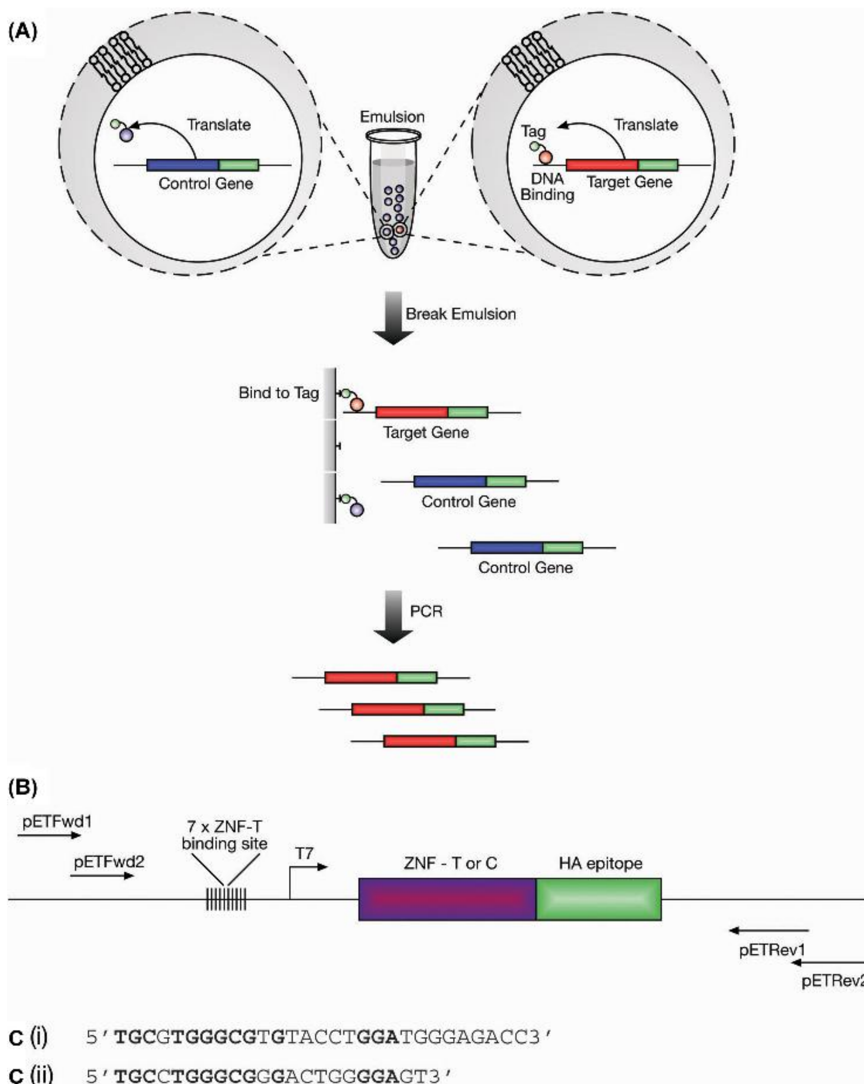


Fig. 4. ZFP selection by IVC. **(A)** Detailed scheme: DNA-binding protein molecules expressed within the vesicles form complexes with the PCR fragments carrying the encoding genes if specific binding sequences upstream of the coding sequence are present; after breaking of the emulsion, protein-gene complexes are captured using the HA epitope tag appended to all ZFPs; selected gene expression cassettes are amplified by PCR. **(B)** Expression cassettes used in the selection of ZFPs: two constructs were prepared in which either the seven finger protein ZNF-T-HA (TFIIIA-Zif268) or the six finger protein ZNF-C-HA (Zif268′-Zif268) was tagged with C-terminal HA peptide epitope; both constructs contain seven copies of the binding sequence for ZNF-T upstream of the T7 promoter; binding sites of nested PCR primers used in gene amplification are indicated. **(C)** sequences of the DNA binding sites for ZNF-T-HA and ZNF-C-HA; the regions sharing substantial sequence homology are in bold. (Based on Fig. 1 of: Sepp and Choo, *J Mol Biol* **354**: 212–219 (2005), with permission from Elsevier.)

干细胞之家www.stemcell8.cn ←点击进入

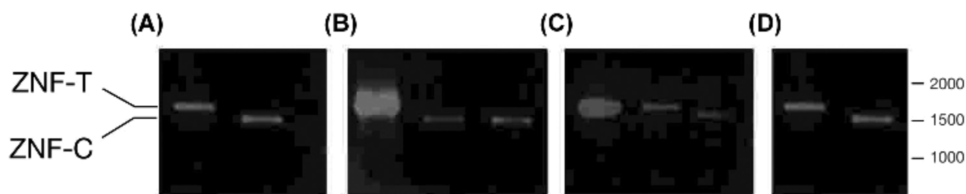


Fig. 5. Isolation of target ZNF-T-HA gene. **(A)** Agarose gel electrophoresis showing the respective sizes of ZNF-T-HA (lane 1) and ZNF-C (lane 2) genes used in the model selections. **(B)** PCR amplification of DNA fragments recovered from the first round of selection. Lane 1: positive control ZNF-T-HA; Lane 2: model library; Lane 3: negative control ZNF-C-HA. **(C)** PCR amplification of DNA fragments recovered from the second round of selection. Lane 1: positive control ZNF-T-HA; Lane 2: model librar; Lane 3: negative control ZNF-C-HA. Note the predominant band in the library after two rounds of selection is the ZNF-T-HA target gene which was originally present at a frequency of 10^{-4}. **(D)** PCR amplification of DNA fragments recovered from a mock round of selection in which T7 polymerase was omitted from the IVC reaction. Lane 1: ZNF-T-HA; Lane 2: ZNF-C-HA. (Based on Fig. 2 of: Sepp and Choo, *J Mol Biol* **354**: 212–219 (2005), with permission from Elsevier.)

improvement in catalytic efficiency (k_{cat}/K_m DNA) using non-cognate AGCC and a preference for AGCC over the wild-type GGCC. The mutant methylates three of the sites efficiently (AGCC, CGCC and GGCC) but discriminates against other noncanonical sites, including TGCC, as effectively as the wildtype enzyme. This proves that catalytic efficiency of a designed protein can surpass that of the wild-type enzyme with the principal substrate.

Should it prove possible to evolve methyltransferases with even higher specificity, it may be possible to use DNA methylation for specific repression or activation of genes through localized chromatin remodelling (Ballestar and Esteller, 2002). Indeed, this targeting approach has been explored with M. *Hha I* and M. *Hpa II* methyltransferases which were expressed and studied (McNamara *et al.*, 2002) in fusion with a man-made ZFP originally designed to bind specifically to a unique nine-base-pair region of a BCR-ABL fusion oncogene in preference to the parent genomic sequences (Choo *et al.*, 1994). Of these two enzymes, M. *Hpa II* proved to be more suitable for expression in fusion with the ZFP, as both domains of the fusion protein retained their affinity and specificity towards the DNA. Targeted *in vivo* methylation of genomic sequences can be expected to be a valuable research tool for studying the spread of methylation patterns within eukaryotes and are potentially of therapeutic value in suppressing the transcriptional activity of targeted genes via methylation of the promoter region CpG island. Unfortunately, both wild-type methyltransferase domains displayed a high level of non-specific methylation activity which had to be countered through introduction of higher affinity and specificity ZFPs. Recently, adaptation of

干细胞之家www.stemcell8.cn ←点击进入

the catalytic domain such that it is functionally dominated by the fused zinc-finger component, produced more site-specific enzymes with significantly reduced background methylation activity in the absence of target sites (Smith and Ford, 2007). Gene silencing in mammalian cell lines and interference with viral propagation has also been demonstrated in the case of mouse Dnmt3a and Dnmt3b DNA methyltransferase fusions to an engineered zinc finger DNA-binding domain, which binds a single site in the promoter region of HSV-1 gene IE175k, embedded in the cellular chromatin (Li *et al.*, 2006).

IVC may also be exceptionally suitable for the engineering of endonucleases with novel specificities because novel restriction sites can be incorporated into the DNA fragments used as genes. This has been demonstrated using *Fok* I type IIS restriction enzyme, where three residues of the catalytic site were randomized at once. Although no catalytically active variants to the wild type enzyme were isolated, selection for the desired activity did occur, emphasizing the high level of adaptation of these enzymes in nature (Doi *et al.*, 2004).

5. New Developments and Perspectives

The steady progress made in the development and adaptation of *in vitro* selection systems for antibody engineering suggests that similar advances will eventually occur in the field of transcription factor design. A number of publications describing the first applications of *in vitro* systems to transcription factor design or selection have appeared, and it remains for "consensus" to develop regarding the optimal expression media, conditions and selection strategies.

Cell-free expression systems also have come a long way in recent years and now many different formulations are available. For instance, many formulations containing RNA polymerase for coupled transcription-translation from DNA template are available. The most widely used *in vitro* expression medium is perhaps the *E. coli* S30 extract, but rabbit reticulocyte and wheat germ based (Yonezawa *et al.*, 2003 and 2004) systems are also available to provide the means for optimal expression of particular proteins such as human telomerase which is a multi-subunit ribonucleoprotein (Ghadessy *et al.*, 2004) and *Fok* I endonuclease (Doi *et al.*, 2004) in IVC.

Until recently, "cell-free" transcription-translation extracts were rather crude mixtures of cellular proteins (some of which are wholly undesirable,

干细胞之家www.stemcell8.cn ←点击进入

e.g., RNases) and ribosomes supplemented with energy regeneration systems in reducing buffer. However, Puresystem (PostGenome Institute Ltd., Co., Japan) is unique in being a reconstituted cell-free protein synthesis system which consists of purified components necessary for transcription, translation and energy regeneration (Shimizu *et al.*, 2001) and consequently very low nuclease activity. Puresystem is also available in an oxidizing formulation, as is the RTS Disulfide kit (Roche), which is a whole cell extract. Thus by careful optimization, both eukaryotic and bacterial *in vitro* expression extracts retain their activity even when supplemented with 500 μM $ZnCl_2$ (required for the activity of ZFPs) (Sepp and Choo, 2005; Moore *et al.*, 2001b), or oxidized glutathione (Ryabova *et al.*, 1997; Coia *et al.*, 2001; Irving *et al.*, 2001).

ZFPs are not the only DNA-binding proteins compatible with IVC. Both single-chain Arc, an engineered version of *Salmonella* phage 22 *Arc* repressor, and *E. coli Tus* replication factor have been successfully expressed and used for the linking of fused single domain antibody fragments to their encoding genes (Sepp and Griffiths, 2003; Sepp and Stoop, 2004). This is a process which requires very high affinity binding of the fusion protein to the encoding DNA for the duration of the experiment so that biopanning on immobilized antigen can be carried out. Even more stable complex for linking arbitrary protein domains to DNA has been achieved by using genetically engineered M. *Hae* III methyltranseferase to link protein fragments covalently to their encoding genes (Bertschinger and Neri, 2004).

Finally, while protein and nucleic acid molecules remain co-segregated within the droplets in IVC because highly charged macromolecules do not partition into hydrophobic mineral oil phase, the same is not true for low molecular weight uncharged substrates or ligands, which can be delivered through the oil directly to droplets (Bernath *et al.*, 2005). If the compound delivered is a fluorogenic substrate, the droplets that express an active enzyme capable of turning-over substrate become fluorescent and can be isolated from the rest of the population by flow cytometry after conversion into a water-in-oil-in-water emulsion (Mastrobattista *et al.*, 2005; Aharoni *et al.*, 2005; Bernath *et al.*, 2004).

6. Conclusion

In summary, a number of advanced *in vitro* selection methods have been validated that allow the formation of stable protein-DNA complexes and may be used for the selection of novel DNA-binding and modifying proteins. The expressed protein may be conjugated either to its encoding mRNA, as in

干细胞之家www.stemcell8.cn ←点击进入

ribosome or mRNA display, or to the cDNA, as in IVC. In the case of conventional display methods, the formation of genotype-linkage and the selection step are two discrete events occurring via different mechanisms and carried out separately. In IVC of DNA-binding proteins, these steps are unified, as DNA binding is a means of discriminating between active/inactive genes and also of forming a genotype-phenotype linkage. Different techniques and strategies of engineering DNA-binding proteins, each with various advantages and disadvantages, are suggested by this possibility.

References

Aharoni A, Amitai G, Bernath K, *et al*. (2005) High-throughput screening of enzyme libraries: thiolactonases evolved by fluorescence-activated sorting of single cells in emulsion compartments. *Chem Biol* **12**: 1281–89.

Ballestar E, Esteller M. (2002) The impact of chromatin in human cancer: linking DNA methylation to gene silencing. *Carcinogenesis* **23**: 1103–109.

Beerli RR, Dreier B, Barbas CF. 3rd. (2000) Positive and negative regulation of endogenous genes by designed transcription factors. *Proc Natl Acad Sci USA* **97**: 1495–500.

Bernath K, Hai M, Mastrobattista E. *et al*. (2004) *In vitro* compartmentalization by double emulsions: sorting and gene enrichment by fluorescence activated cell sorting. *Anal Biochem* **325**: 151–57.

Bertschinger J, Neri D. (2004) Covalent DNA display as a novel tool for directed evolution of proteins *in vitro*. *Protein Eng Des Sel* **17**: 699–707.

Choo Y, Castellanos A, Garcia-Hernandez B, *et al*. (1997) Promoter-specific activation of gene expression directed by bacteriophage-selected zinc fingers. *J Mol Biol* **273**: 525–32.

Choo Y, Klug A. (1994) Selection of DNA binding sites for zinc fingers using rationally randomized DNA reveals coded interactions. *Proc Natl Acad Sci USA* **91**: 11168–72.

Choo Y, Klug A. (1995) Designing DNA-binding proteins on the surface of filamentous phage. *Curr Opin Biotechnol* **6**: 431–36.

Choo Y, Klug A. (1997) Physical basis of a protein-DNA recognition code. *Curr Opin Struct Biol* **7**: 117–25.

Choo Y, Sanchez-Garcia I, Klug A. (1994) *In vivo* repression by a site-specific DNA-binding protein designed against an oncogenic sequence. *Nature* **372**: 642–45.

Cohen HM, Tawfik DS, Griffiths AD. (2002) Promiscuous methylation of non-canonical DNA sites by HaeIII methyltransferase. *Nucl Acids Res* **30**: 3880–85.

Cohen HM, Tawfik DS, Griffiths AD. (2004) Altering the sequence specificity of HaeIII methyltransferase by directed evolution using *in vitro* compartmentalization. *Protein Eng Des Sel* **17**: 3–11.

干细胞之家www.stemcell8.cn ←点击进入

Coia G, Pontes-Braz L, Nuttall SD, *et al*. (2001) Panning and selection of proteins using ribosome display. display. *J Immunol Meth* **254**: 191–97.

Dai Q, Huang J, Klitzman B, *et al*. (2004) Engineered zinc finger-activating vascular endothelial growth factor transcription factor plasmid DNA induces therapeutic angiogenesis in rabbits with hindlimb ischemia. *Circulation* **110**: 2467–75.

Doi N, Kumadaki S, Oishi Y, *et al*. (2004) *In vitro* selection of restriction endonucleases by *in vitro* compartmentalization. *Nucl Acids Res* **32**: e95.

FitzGerald K. (2000) *In vitro* display technologies — new tools for drug discovery. *Drug Discov Today* **5**: 253–58.

Ghadessy FJ, Holliger P. (2004) A novel emulsion mixture for *in vitro* compartmentalization of transcription and translation in the rabbit reticulocyte system. *Protein Eng Des Sel* **17**: 201–204.

Ghadessy FJ, Holliger P. (2007) Compartmentalized self-replication: a novel method for the directed evolution of polymerases and other enzymes. *Meth Mol Biol* **352**: 237–48.

Ghadessy FJ, Ong JL, Holliger P. (2001) Directed evolution of polymerase function by compartmentalized self-replication. *Proc Natl Acad Sci USA* **98**: 4552–57.

Ghadessy F J, Ramsay N, Boudsocq F, *et al*. (2004) Generic expansion of the substrate spectrum of a DNA polymerase by directed evolution. *Nat Biotechnol* **22**: 755–59.

Graslund T, Li X, Magnenat L, *et al*. (2005) Exploring strategies for the design of artificial transcription factors: targeting sites proximal to known regulatory regions for the induction of gamma-globin expression and the treatment of sickle cell disease. *J Biol Chem* **280**: 3707–14.

Griffiths AD, Tawfik DS. (2006) Miniaturising the laboratory in emulsion droplets. *Trends Biotechnol* **24**: 395–402.

Groves MA, Osbourn JK. (2005) Applications of ribosome display to antibody drug discovery. *Expert Opin Biol Ther* **5**: 125–35.

Hanes J, Jermutus L, Weber-Bornhauser S, *et al*. (1998) Ribosome display efficiently selects and evolves high-affinity antibodies *in vitro* from immune libraries. *Proc Natl Acad Sci USA* **95**: 14130–35.

Hanes J, Plückthun A. (1997) *In vitro* selection and evolution of functional proteins by using ribosome display. *Proc Natl Acad Sci USA* **94**: 4937–42.

Hanes J, Schaffitzel C, Knappik A, Plückthun A. (2000) Picomolar affinity antibodies from a fully synthetic naive library selected and evolved by ribosome display. *Nat Biotechnol* **18**: 1287–92.

He M, Taussig MJ. (1997) Antibody-ribosome-mRNA (ARM) complexes as efficient selection particles for *in vitro* display and evolution of antibody combining sites. *Nucl Acids Res* **25**: 5132–34.

Ihara H, Mie M, Funabashi H, *et al*. (2006) *In vitro* selection of zinc finger DNA-binding proteins through ribosome display. *Biochem Biophys Res Commun* **345**: 1149–54.

International Human Genome Sequencing Consortium. (2004) Finishing the euchromatic sequence of the human genome. *Nature* **431**: 931–45.

干细胞之家www.stemcell8.cn ←点击进入

Irving RA, Coia G, Roberts A, *et al*. (2001) Ribosome display and affinity maturation: from antibodies to single V-domains and steps towards cancer therapeutics. *J Immunol Meth* **248**: 31–45.

Isalan M, Choo Y. (2001) Rapid, high-throughput engineering of sequence-specific zinc finger DNA-binding proteins. *Meth Enzymol* **340**: 593–609.

Isalan M, Klug A, Choo Y. (2001) A rapid, generally applicable method to engineer zinc fingers illustrated by targeting the HIV-1 promoter. *Nat Biotechnol* **19**: 656–60.

Jamieson AC, Kim SH, Wells JA. (1994) *In vitro* selection of zinc fingers with altered DNA-binding specificity. *Biochemistry* **33**: 5689–95.

Jamieson AC, Miller JC, Pabo CO. (2003) Drug discovery with engineered zinc-finger proteins. *Nat Rev Drug Discov* **2**: 361–68.

Kim JS, Pabo CO. (1998) Getting a handhold on DNA: design of poly-zinc finger proteins with femtomolar dissociation constants. *Proc Natl Acad Sci USA* **95**: 2812–17.

Kim YS, Kim JM, Jung DL, *et al*. (2005) Artificial zinc finger fusions targeting Sp1-binding sites and the trans-activator-responsive element potently repress transcription and replication of HIV-1. *J Biol Chem* **280**: 21545–52.

Lander ES, Linton LM, Birren B, *et al*. (2001) Initial sequencing and analysis of the human genome. *Nature* **409**: 860–921.

Lee YF, Tawfik DS, Griffiths AD. (2002) Investigating the target recognition of DNA cytosine-5 methyltransferase HhaI by library selection using *in vitro* compartmentalisation. *Nucl Acids Res* **30**: 4937–44.

Li F, Papworth M, Minczuk M, *et al*. (2006) Inhibition of herpes simplex virus 1 gene expression by designer zinc-finger transcription factors. *Nucl Acids Res*.

Ling MM. (2003) Large antibody display libraries for isolation of high-affinity antibodies. *Comb Chem High Throughput Screen* **6**: 421–32.

Lipovsek D, Pluckthun A. (2004) *In vitro* protein evolution by ribosome display and mRNA display. *J Immunol Meth* **290**: 51–67.

Manwani D, Galdass M, Bieker JJ. (2007) Altered regulation of beta-like globin genes by a redesigned erythroid transcription factor. *Exp Hematol* **35**: 39–47.

Mastrobattista E, Taly V, Chanudet E, *et al*. (2005) High-throughput screening of enzyme libraries: *in vitro* evolution of a beta-galactosidase by fluorescence-activated sorting of double emulsions. *Chem Biol* **12**: 1291–300.

Mattheakis LC, Bhatt RR, Dower WJ. (1994) An *in vitro* polysome display system for identifying ligands from very large peptide libraries. *Proc Natl Acad Sci USA* **91**: 9022–26.

McCafferty J, Griffiths AD, Winter G, Chiswell DJ. (1990) Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* **348**: 552–54.

McNamara AR, Hurd PJ, Smith AE, Ford KG. (2002) Characterisation of site-biased DNA methyltransferases: specificity, affinity and subsite relationships. *Nucl Acids Res* **30**: 3818–30.

Miller J, McLachlan AD, Klug A. (1985) Repetitive zinc-binding domains in the protein transcription factor IIIA from Xenopus oocytes. *EMBO J* **4**: 1609–14.

干细胞之家www.stemcell8.cn ←点击进入

Miller OJ, Bernath K, Agresti JJ, *et al*. (2006) Directed evolution by *in vitro* compartmentalization. *Nat Meth* **3**: 561–70.

Moore M, Choo Y, Klug A. (2001a) Design of polyzinc finger peptides with structured linkers. *Proc Natl Acad Sci USA* **98**: 1432–36.

Moore M, Klug A, Choo Y. (2001b) Improved DNA binding specificity from polyzinc finger peptides by using strings of two-finger units. *Proc Natl Acad Sci USA* **98**: 1437–41.

Nardelli J, Gibson TJ, Vesque C, Charnay P. (1991) Base sequence discrimination by zinc-finger DNA-binding domains. *Nature* **349**: 175–78.

Nemoto N, Miyamoto-Sato E, Husimi Y, Yanagawa H. (1997) *In vitro* virus: bonding of mRNA bearing puromycin at the 3′-terminal end to the C-terminal end of its encoded protein on the ribosome *in vitro*. *FEBS Lett* **414**: 405–408.

Osbourn J, Jermutus L, Duncan A. (2003) Current methods for the generation of human antibodies for the treatment of autoimmune diseases. *Drug Discov Today* **8**: 845–51.

Papworth M, Moore M, Isalan M, *et al*. (2003) Inhibition of herpes simplex virus 1 gene expression by designer zinc-finger transcription factors. *Proc Natl Acad Sci USA* **100**: 1621–26.

Parker MH, Chen Y, Danehy F, *et al*. (2005) Antibody mimics based on human fibronectin type three domain engineered for thermostability and high-affinity binding to vascular endothelial growth factor receptor two. *Protein Eng Des Sel* **18**: 435–44.

Rebar EJ, Huang Y, Hickey R, *et al*. (2002) Induction of angiogenesis in a mouse model using engineered transcription factors. *Nat Med* **8** : 1427–32.

Rebar EJ, Pabo CO. (1994) Zinc finger phage: affinity selection of fingers with new DNA-binding specificities. *Science* **263**: 671–73.

Reiersen H, Lobersli I, Loset GA, *et al*. (2005) Covalent antibody display — an *in vitro* antibody-DNA library selection system. *Nucl Acids Res* **33**: e10.

Reynolds L, Ullman C, Moore M, *et al*. (2003) Repression of the HIV-1 5′ LTR promoter and inhibition of HIV-1 replication by using engineered zinc-finger transcription factors. *Proc Natl Acad Sci USA* **100**: 1615–20.

Roberts RW, Szostak JW. (1997) RNA-peptide fusions for the *in vitro* selection of peptides and proteins. *Proc Natl Acad Sci USA* **94**: 12297–302.

Ryabova LA, Desplancq D, Spirin AS, Pluckthun A. (1997) Functional antibody production using cell-free translation: effects of protein disulfide isomerase and chaperones. *Nat Biotechnol* **15**: 79–84.

Sepp A, Choo Y. (2005) Cell-free selection of zinc finger DNA-binding proteins using *in vitro* compartmentalization. *J Mol Biol* **354**: 212–19.

Sepp A, Ghadessy F, Choo Y. (2007) Cell-free selection of DNA-binding proteins for future gene therapy applications. *Gene Ther Regul* **3**: 51–63.

Sepp A, Griffiths AD. (2003) Method of *in vitro* polypeptide selection using the Arc DNA binding domain fused to the polypeptide selected. *PCT/GB2005/003243* (WO 2006/018650).

干细胞之家www.stemcell8.cn ←点击进入

Sepp A, Stoop A. (2004) Method of selecting polypeptides. *PCT/GB2005/004148* (WO/2006/046042).

Sepp A, Tawfik DS, Griffiths AD. (2002) Microbead display by *in vitro* compartmentalisation: selection for binding using flow cytometry. *FEBS Lett* **532**: 455–58.

Shimizu Y, Inoue A, Tomari Y, *et al*. (2001) Cell-free translation reconstituted with purified components. *Nat Biotechnol* **19**: 751–55.

Siemering KR, Golbik R, Sever R, Haseloff J. (1996) Mutations that suppress the thermosensitivity of green fluorescent protein. *Curr Biol* **6**: 1653–63.

Smith AE, Ford KG. (2007) Specific targeting of cytosine methylation to DNA sequences *in vivo*. *Nucl Acids Res* **35**: 740–54.

Smith GP. (1985) Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science* **228**: 1315–17.

Tateyama S, Horisawa K, Takashima H, *et al*. (2006) Affinity selection of DNA-binding protein complexes using mRNA display. *Nucl Acids Res* **34**: e27.

Tawfik DS, Griffiths AD. (1998) Man-made cell-like compartments for molecular evolution. *Nat Biotechnol* **16**: 652–56.

Urnov FD, Miller JC, Lee YL, *et al*. (2005) Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature* **435**: 646–51.

Venter JC, Adams MD, Myers EW, *et al.* (2001) The sequence of the human genome. *Science* **291**: 1304–51.

Yonezawa M, Doi N, Higashinakagawa T, Yanagawa H. (2004) DNA display of biologically active proteins for *in vitro* protein selection. *J Biochem (Tokyo)* **135**: 285–88.

Yonezawa M, Doi N, Kawahashi Y, *et al*. (2003) DNA display for *in vitro* selection of diverse peptide libraries. *Nucl Acids Res* **31**: E118.

Yu J, Lei L, Liang Y, *et al*. (2006) An engineered VEGF-activating zinc finger protein transcription factor improves blood flow and limb salvage in advanced-age mice. *FASEB J* **20**: 479–81.

Zhang L, Spratt SK, Liu Q, *et al*. (2000) Synthetic zinc finger transcription factor action at an endogenous chromosomal site. Activation of the human erythropoietin gene. *J Biol Chem* **275**: 33850–60.

干细胞之家www.stemcell8.cn ←点击进入

This page intentionally left blank

干细胞之家www.stemcell8.cn ←点击进入

Chapter 6

Advances in Engineering Homing Endonucleases for Gene Targeting: Ten Years After Structures

Barry L Stoddard*,†, Andrew M Scharenberg‡
and Raymond J Monnat, Jr§

Homing endonucleases (HEs) are highly site-specific endonucleases that induce homologous recombination or gene conversion *in vivo* by cleaving long (typically >18 bp) DNA target sites. Homing endonucleases are under development as tools for targeted genetic engineering applications, ranging from therapeutic gene correction to metabolic and population engineering. The first structures of homing endonucleases were reported 10 years ago. Since that time, representative structures from each of the known families of homing endonucleases have been determined, and the corresponding details of their mechanisms of DNA recognition and cleavage have been elucidated. Using this information, the LAGLIDADG homing endonuclease family has been identified as the most tractable for further modification by structure-based selection and/or engineering approaches. Most recently, successful redesign of the I-CreI endonuclease has led to the development of reagents that recognize and act on genes associated with monogenic diseases, including the human *RAG1* and *XPC* genes. These studies demonstrate the feasibility of using engineered homing endonucleases to promote efficient and target site-specific modification of chromosomal loci. Current studies are rapidly improving the throughput and efficiency of homing endonuclease design and selection, and aim to optimize the specificity and activity of the resulting endonucleases for targeted genomic applications in medicine and biotechnology.

Keywords: Homing endonuclease; LAGLIDADG; recombination; gene therapy; gene correction; protein engineering; genome engineering; DNA recognition.

*Corresponding author. bstoddar@fhcrc.org
†Division of Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle WA 981089, USA
‡Department of Pediatrics, University of Washington Seattle WA 98195 and Children's Hospital and Regional Medical Center, Seattle WA 98109, USA
§Departments of Pathology and Genome Sciences, University of Washington, Seattle WA 98195, USA

干细胞之家www.stemcell8.cn ←点击进入

1. Genomic Targeting: From Monogenic Diseases to Broad Genome Engineering

1.1. *Monogenic Disorders: A Case for Targeted Gene Repair*

Over the last decade, analyses of US and global public health issues have highlighted the increasing significance of monogenic diseases to the total health care burden, particularly for children (Baird *et al*., 1988; Christianson *et al*., 2006; McCandless *et al*., 2004). The individual and societal costs imposed by medical interventions and treatment of monogenic disorders have compelled a search for curative therapeutic options for these diseases, focusing on correcting the underlying genetic defect in a patients' cells. A variety of therapies based on either of two broad approaches have been developed: (1) allogeneic stem cell transplantation, a strategy aimed at replacing a patient's defective hematopoietic stem cells with those of a normal donor; and (2) "traditional" gene therapy. In the latter case, a wild-type version of a defective gene is integrated into the genomes of a patient's cells in order to replace the function of a defective gene. In the case of diseases affecting lymphohematopoietic development and function, patient-derived hematopoietic stem cell populations can be transfected *in vitro* with the wild-type gene and then reintroduced.

The current practice of gene replacement therapy has several attendant issues. First, gene therapy involves the random insertion of foreign DNA into the genomes of stem cells, potentially resulting in the inactivation or activation of endogenous genes. Widely publicized examples of this type of problem include the development of T-cell lymphomas in four of 10 patients treated in France for X-SCID, and the development of oligoclonal proliferation of neutrophils in two of three patients treated in Germany for chronic granulomatous disease (CGD) (Abbott, 2006; Baum, 2007; Hacein-Bey-Abina *et al*., 2003). In each of these cases, cellular proliferation appears to have been driven by retroviral insertion near a growth-related gene (LMO2 for the T-cell lymphomas in X-SCID, and MDS1/EV1 for clonal proliferations in CGD patients). In addition, liver tumors have developed following lentiviral vector-mediated factor IX hepatic gene transfer in mice (Themis *et al*., 2005). While these results are troubling, it is still important to note that four of the eight patients who have been successfully cured in the French X-SCID trial are still doing well, and all the patients from a second X-SCID trial performed in London are well and free from adverse events (Baum, 2007; Thrasher *et al*., 2006). In addition, new lentiviral vectors avoid the use of highly active LTR-based promoters, and thus may improve safety profiles

干细胞之家www.stemcell8.cn ←点击进入

(Griesenbach *et al.*, 2006); however, others remain more pessimistic (Porteus, 2006).

A second issue is that it is desirable in all cases, and required in many, to use lineage-specific transcriptional control elements. However, defining such control elements is non-trivial, and may require years of experimentation. For the treatment of hemogoblinopathies such as thalassemias, native hemoglobin regulatory elements are too large to be efficiently delivered together with the relevant coding regions to hematopoietic stem cells. It has taken an extraordinary effort, by many laboratories, to define smaller transcriptional regulatory elements and to devise ways to deliver these with the current generation viral vectors. One recent successful development made use of a knowledge of β-globin transcriptional regulation together with lentiviral delivery technology to express artificial hemoglobin genes with apparently strict transcriptional control in murine hematopoietic stem cells (Malik and Arumugam, 2005; Malik *et al.*, 2005; Puthenveetil *et al.*, 2004). The extension of these approaches to large animal models remains to be demonstrated.

A final issue with gene replacement therapy is that it is best suited to treat diseases due to the complete deficiency or absence of a gene product. Gene replacement therapy is less well-suited or may not be applicable to diseases caused by the presence of an aberrantly functioning protein that may interfere with the function of the replacement normal protein. These diseases may require a more complicated approach in which simultaneous gene replacement and the suppression or knockdown of the defective gene product may be required. One example of a disease where this combined approach may be required is sickle cell disease (Sadelain, 2006; Samakoglu *et al.*, 2006). However, gene knockdown strategies involve highly conserved cellular RNAi mechanisms whose function is required for normal hematopoietic development. The long-term safety of this type of combined approach is an open question.

1.2. *Genome Engineering: A Targeted Approach*

A potential solution to some of the drawbacks and hurdles facing traditional gene replacement therapy is the possibility of "genome engineering," which describes an emerging discipline in which genomes of target organisms or cells are manipulated *in vivo*, using site-specific recombination to alter or add genetic information. The concept of genome engineering is not new, and dates back to experiments in the late 1970s in which ectopic DNA could be incorporated into the genome of the budding yeast *Saccharomyces*

干细胞之家www.stemcell8.cn ←点击进入

cerevisiae. The success of this approach depends on endogenous homologous recombination pathways (Hinnen *et al.*, 1978; Orr-Weaver *et al.*, 1981). Depending on the exact methodology, individual yeast genes can be efficiently incorporated, deleted, mutated or corrected. However, while homologous recombination is extremely efficient in yeast, in mammalian cells it occurs at a very low frequency, often in the range of 10^{-5} to 10^{-7} per transformed cells (Doetschman *et al.*, 1987; Koller and Smithies, 1989). As we describe below, this limitation can in part be overcome by using a highly site-specific endonuclease to cleave the donor or recipient locus to stimulate targeted homologous recombination. The development of these reagents has allowed the field of genome engineering to progress dramatically over the past five years, together with the pursuit of several specific genome engineering applications (Bullard and Weaver, 2002; Glaser *et al.*, 2005; Gouble *et al.*, 2006; Tzfira and White, 2005; Vasquez *et al.*, 2001):

- *Synthetic biology.* DNA synthesis technology now allows the assembly of large artificial DNA fragments — up to several tens of thousands of base pairs — which are of sufficient size to encode entire metabolic pathways. Embedding these new pathways in a "minimal" genome or other model organism for the purpose of creating new types of synthetic organisms is the goal of the emerging science of synthetic biology (Ghatge *et al.*, 2006; Posfai *et al.*, 1999). The use of site-specific nucleases or recombinases to embed synthetic genes at specific desired target sites in model organisms represents a crucial enabling technology for synthetic biologists to create, manipulate, and control artificial genomes.
- *Pest control.* Synthetic genes encoding artificial nucleases may be used to create "selfish" genetic elements with the ability to integrate into and alter target genes while promoting their own transmission. This type of genetic drive system can strongly bias Mendelian inheritance to favor the generation of progeny that contain both the selfish genetic element and the altered target gene(s) (Burt, 2003). In modeling experiments, complete penetration of such a "hyper-dominant" allele through a population can occur in fewer than 20 generations. This strategy has been proposed as a novel means for genetic control of *Anopheles*-mediated malaria transmission by dominant transmission and inheritance of traits corresponding to resistance against *Plasmodium* infection, or by reducing the lifespan or reproductive fitness of the insect host (Chase, 2006). Practical evaluation and implementation of such a strategy are presently the subjects of a

干细胞之家www.stemcell8.cn ←点击进入

Gates Grand Challenges project focused on the control of malaria and the malaria vector *Anopheles gambiae* (Burt, 2005).

- *Gene repair.* By using standard transfection methods to introduce a site-specific nuclease and a modifying genomic template into primary lymphocytes, it has been possible to modify up to ≈5% of the target locus in the transfected cell population (Urnov *et al.*, 2005). This approach, when used to modify a mutant gene so as to restore normal function, is often termed "gene repair" or "gene correction." While gene repair has the same goal as traditional gene therapy approaches — restoration of the expression of a normally functioning protein — it has many advantages (Bertolotti, 1996). Since the endogenous gene's function is restored, the protein is expressed under the control of its natural regulatory elements, thus eliminating potential problems with inappropriate or inadequate expression of a transgene or transgene silencing. By targeting the repair with high efficiency to a single mutant locus, gene repair may also be able to dramatically reduce mutagenesis due to random insertions at other genomic locations.

2. Double Strand Break-Induced Gene Conversion and Gene-Specific Nucleases

Several different technologies have been developed to promote efficient targeted gene correction in mammalian cells. These include gene-targeted triplex forming oligonucleotides and hybrid RNA-DNA oligonucleotides (Kolb *et al.*, 2005) and the use of highly site-specific recombinases and transposases (Coates *et al.*, 2005). Each of these approaches has limitations related to the range of sequences that can be targeted (e.g., triplex-forming oligonucleotides), or the requirement for prior introduction of a target site (e.g., for site-specific recombinase-mediated targeting). Potentially the most versatile of all genome engineering technologies are those that make use of DNA double strand break-targeted homologous recombination for gene modification. Recent achievements (Urnov *et al.*, 2005; Arnould *et al.*, 2007) indicate that this method allows a desired genomic sequence to be altered in a precise manner, without the requirement for a selection marker or the introduction of additional exogenous DNA sequence(s).

Double strand break-targeted recombination requires the introduction or expression of a site-specific endonuclease in cells to generate a DNA double strand break at or near the desired modification site, together with the presence of a DNA repair template. Repair templates typically flank

干细胞之家www.stemcell8.cn ←点击进入

the DNA double strand break site and include sequence modifications to be incorporated upon repair. Homologous recombination is a normal DNA repair pathway that makes use of homologous donor DNA to repair strand breaks. This pathway is highly conserved, and is used in many cells and organisms to repair DNA double strand breaks with high efficiency and fidelity or accuracy. The machinery of homologous recombination makes use of the DNA double strand break ends, together with the repair template, to incorporate DNA sequence modification(s) in the repair template into the chromosome as part of the process of DNA break repair.

A significant practical barrier to a widespread application of this accurate and efficient gene repair mechanism in genome engineering has been the requirement for endonucleases that are able to induce DNA double strand breaks at specific chromosomal target sites. Until very recently the technology to accomplish this has been lacking. However, over the past several years, two different approaches to creating enzymes capable of inducing highly site-specific DNA double strand breaks have been developed: *zinc finger nucleases* (ZFNs) and *homing endonucleases* (HEs) (Fig. 1).

ZFN site-specific nuclease technology involves the creation of artificial nucleases by appending a non-specific nuclease domain (such as the catalytic domain of the FokI restriction endonuclease) to a DNA-recognition and binding construct consisting of a tandem array of zinc fingers (Bibikova *et al.*, 2001; Porteus, 2006; Smith *et al.*, 2000). As individual zinc fingers recognize DNA triplets within the context of long cognate target sites (Beerli and Barbas, 2002; Bulyk *et al.*, 2001; Segal *et al.*, 1999), the concatenation of a series of zinc fingers of defined triplet specificity provides the possibility to create ZFNs able to bind and cleave at rare DNA targets. ZFNs have been demonstrated to induce gene correction/modification in both Drosophila and mammalian cells (Bibikova *et al.*, 2003; Porteus and Baltimore, 2003), and the highly efficient correction of disease-associated mutations in the human *IL2Rγ* gene (Urnov *et al.*, 2005).

Zinc finger nucleases have the important advantage of some capacity for modular design, and therefore ZFN technology has been the subject of intensive study over the past ten years (Porteus, 2006). While ZFN technology is clearly useful in designing nucleases able to cleave at diverse target sites, it also has several important limitations (Segal *et al.*, 2003). The main one results from the fact that DNA-recognition by individual zinc fingers is context-dependent: the identity of neighboring zinc fingers and target DNA sequences strongly influences their specificity and affinity towards

干细胞之家www.stemcell8.cn ←点击进入

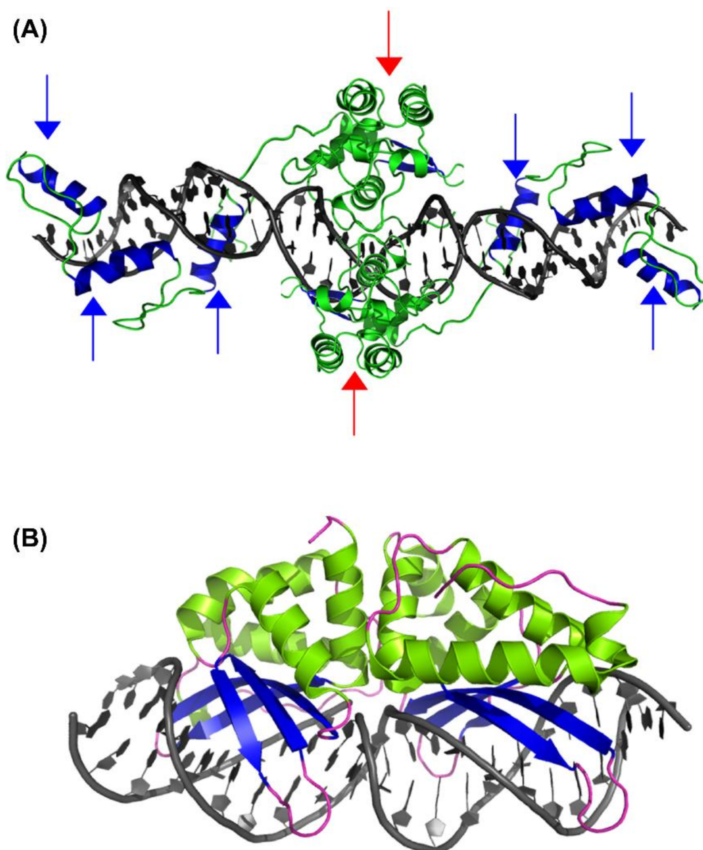


Fig. 1. **(A)** A hypothetical model of a zinc finger nuclease dimer bound to a DNA target site. Each subunit consists of a tandem series of three zinc fingers (blue arrows) tethered to the nuclease domain of the FokI restriction enzyme (red arrows). The model shown is based on the crystal structure of the Zif 268 zinc finger-DNA cocrystal structure (PDB code 1AAY) and the crystal structure of the FokI endonuclease bound to its DNA target (PDB code 1FOK). As structure of a zinc finger nuclease chimera has not yet been solved, the model is for illustrative purposes only. In the active complex, it is known that the nuclease domains form a closely associated transient dimer interface during double strand cleavage. The individual zinc fingers are each associated with three basepairs of the DNA target site (18 bases total). The two cognate binding sites for these modules are separated by a nonspecific sequence of DNA that harbors the sites of cleavage by the nuclease domains. **(B)** The crystal structure of a monomeric LAGLIDADG homing endonuclease (I-AniI) bound to its 19 basepair cognate target site. The active sites and cleaved phosphates are located at the center of the endonuclease-DNA complex; the target site is 19 basepairs in length.

"cognate" DNA codons. Therefore, engineering is not a simple modular process of appending zinc fingers with the appropriate triplet recognition specificity — multiple steps of selection and optimization are required. Indeed, the exquisite specificity and affinity of clinical grade reagents is

干细胞之家www.stemcell8.cn ←点击进入

a tedious and time-consuming process and is essential for the genesis of efficient toxic-free zinc finger nucleases (e.g., Urnov *et al*., 2005). Therefore, modular assemby of designed polydactyl zinc-finger DNA binding domains is an illusive advantage when compared to emerging customization of clinical grade homing endonucleases.

Homing endonucleases are naturally occurring, highly site-specific DNA endonucleases that are encoded as open reading frames embedded within introns or inteins (Fig. 2) (Belfort and Roberts, 1997; Stoddard, 2005). First discovered in the 1980s as part of the mobile genetic elements in yeast (Jacquier and Dujon, 1985; Kostriken *et al*., 1983), homing endonucleases promote the mobility of introns or inteins that contain the endonuclease open reading frame by generating a site-specific DNA double strand break in a homologous gene or allele that lacks the intron or intein (Colleaux *et al*., 1986). DNA break repair leads to lateral transfer of the element via homologous recombination to the cleaved allele, using the intron- or intein-containing allele as a repair template. Thus, homing endonuclease genes (HEGs) are selfish DNA sequences that are inherited in a dominant, non-Mendelian manner, and represent nature's application of double strand break-induced gene modification. Since their discovery, one of the first well characterized homing endonucleases (I-SceI protein from budding yeast) has been widely used to promote DNA double strand break-induced recombination in a wide variety of genomes. This work demonstrates the general utility of using homing endonucleases to induce site-specific recombination for gene repair, though with wide variations in the efficiency of repair and length of the repair template (commonly referred to as the "conversion tract") that is incorporated into the repaired allele (reviewed in Pâques and Duchateau, 2007).

Homing endonucleases are widespread and are found encoded within introns and inteins in all biological super-Kingdoms. Based on primary sequence homology, five homing enzyme families have been identified (Fig. 3), each primarily associated with a unique location in the host genomes: the LAGLIDADG endonucleases (found in archaea and fungal and algal organellar genomes), His-Cys Box family (found in protist nuclear genomes), the HNH and GIY-YIG endonucleases (found in bacteriophage) and the PD-(D/E)XK family, similar to archael Holliday junction resolvases and type II restriction endonucleases, as typified by I-SspI (found in bacterial genomes) (Stoddard, 2005). The His-Cys Box and HNH family appear to be descended from a common ancestral origin (which also gave rise to bacterial colicins and many additional DNA modifying enzymes), while the

干细胞之家www.stemcell8.cn ←点击进入

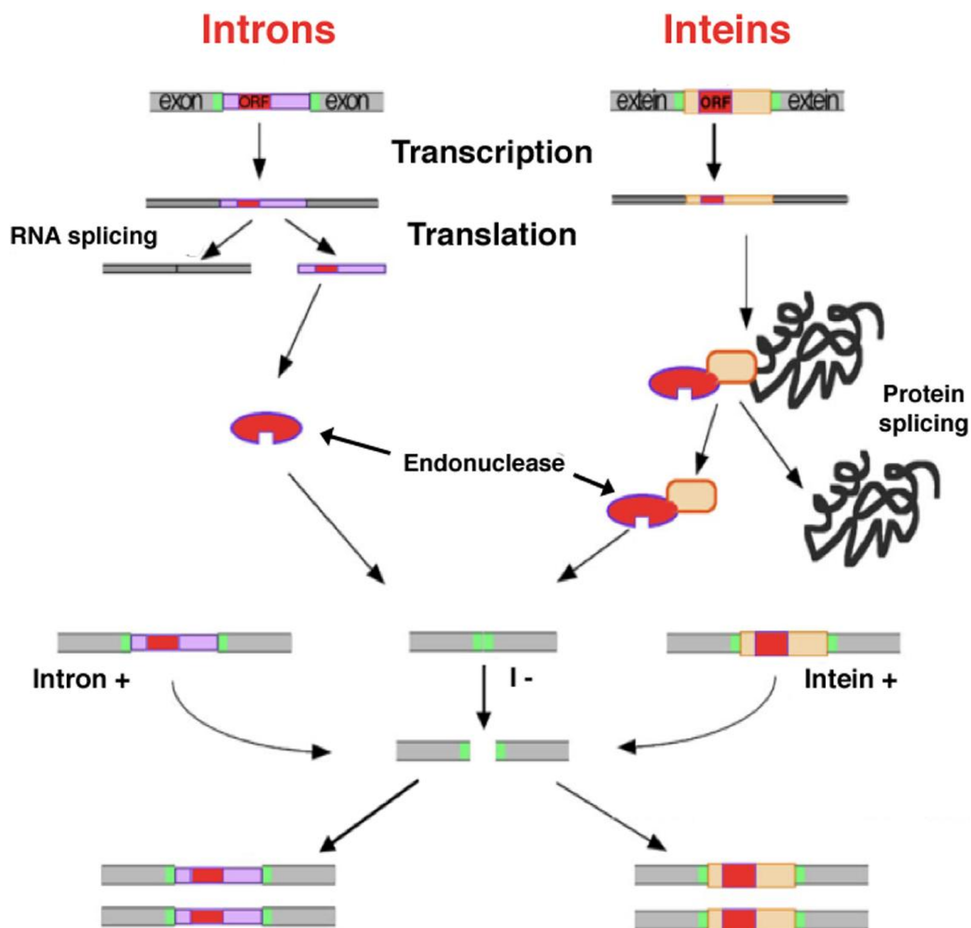


Fig. 2. Homing endonucleases and intron/intein mobility. Homing is the transfer of an intervening sequence (either an intron or intein) to a homologous allele that lacks the sequence, leading to gene conversion and dominant transmission and inheritance of the mobile element. Invasion of ectopic sites, including transfer across biological kingdoms and between different genomic compartments, has also been documented. Homing is initiated by an endonuclease, that is encoded within the mobile intervening sequence, that recognizes a DNA target site and generates a single or double strand break. Transfer of group I introns and inteins is initiated solely by the endonuclease activity, and is completed by cellular mechanisms that repair the strand breaks via homologous recombination, using the intron- or intein-containing allele as a template. (Based on Fig. 1 of: Stoddard BL, *Q Rev Biophys* **38**: 49–95 (2005), with permission of Cambridge University Press.)

remaining three families each contain a unique catalytic scaffold and appear to have arisen independently of one another. The structure and mechanisms of representatives from each of these families have been extensively characterized over the past ten years.

干细胞之家www.stemcell8.cn ←点击进入

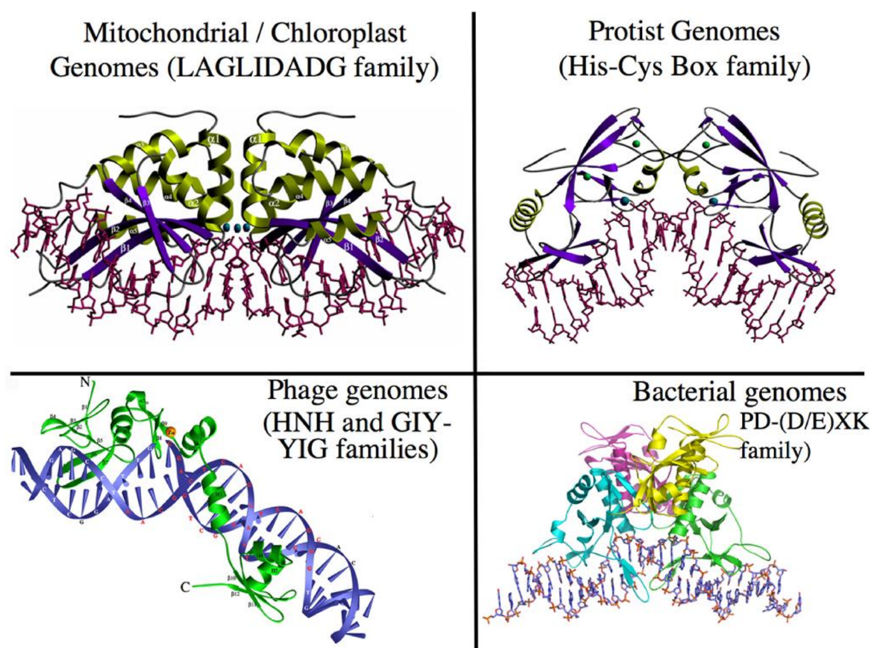


Fig. 3. Representative members of the known group I homing endonuclease families. Each particular family of homing endonuclease is generally constrained to mobility within their corresponding family of host organisms and genomes, presumably due to barriers imposed by the constraints of host toxicity and/or poor mobility when transferred into a foreign genome. Of these homing endonuclease families, the LAGLIDADG endonucleases have become increasingly useful for engineering.

3. LAGLIDADG Homing Endonuclease Proteins are Well-Suited for Protein Engineering and Genomic Targeting Applications

The LAGLIDADG homing endonucleases ("LHEs") are the largest family of such enzymes, which includes the first identified and biochemically characterized intron-encoded proteins (Dujon, 1980; Jacquier and Dujon, 1985; Kostriken *et al.*, 1983; Lazowska *et al.*, 1980). It has also been variously termed the "DOD," "dodecapeptide," "dodecamer," and "decapeptide" endonuclease family, based on the conservation of a 10-residue sequence motif (Belfort *et al.*, 1995; Belfort and Roberts, 1997; Chevalier and Stoddard, 2001; Dalgaard *et al.*, 1997; Dujon, 1989; Dujon *et al.*, 1989). LHEs are segregated into groups that possess either one or two copies of the conserved LAGLIDADG motif. Enzymes that contain a single copy of this motif act as homodimers on DNA targets sites that are palindromic or near-palindromic. In contrast,

干细胞之家www.stemcell8.cn ←点击进入

LHEs that have two copies of the motif are monomers that possess a pair of structurally similar nuclease domains on a single peptide chain. These endonucleases are not constrained to symmetric DNA target sites. The two domains are linked in these monomeric proteins by flexible linker peptides that range from three residues to over 100 residues (Dalgaard *et al.*, 1997). LHEs that are not covalently linked to additional functional domains recognize DNA sites that range from 18 to 22 base pairs (Agaard *et al.*, 1997; Dalgaard *et al.*, 1994; Durrenberger and Rochaix, 1993; Perrin *et al.*, 1993). They cleave both DNA strands to generate cohesive four-base 3′-overhangs (Colleaux *et al.*, 1988; Durrenberger and Rochaix, 1993; Thompson *et al.*, 1992). Like most nucleases, LHEs require divalent cations for activity.

As reviewed below and in Stoddard (2005), the LAGLIDADG family appear to be homing endonucleases of choice for engineering and gene-specific applications:

(i) They represent the largest collection of known and characterized HEs, with a diverse biological host range that spans the genomes of plant and algal chloroplasts, fungal and protozoan mitochondria, bacteria and *Archaea* (Dalgaard *et al.*, 1997). One reason for the wide phylogenetic distribution of LAGLIDADG genes appears to be their remarkable ability to invade unrelated types of intervening sequences, including group I introns, archaeal introns and inteins (Belfort and Roberts, 1997; Chevalier and Stoddard, 2001). Descendents of LAGLIDADG homing endonucleases also include the yeast HO mating type switch endonuclease (Jin *et al.*, 1997), and "maturases" that assist in group I intron splicing (Delahodde *et al.*, 1989; Geese and Waring, 2001; Lazowska *et al.*, 1989; Schafer *et al.*, 1994).

(ii) They are the most specific of all known homing endonucleases, typically recognizing 19 to 22 base-pair DNA target sites. Thus, LHEs target DNA sites that are sufficiently long to ensure that they cleave one or a small number of target sites, even within complex genomes. They display low- to sub-nanomolar dissocation constants against their cognate target sites. The specificity of cognate DNA recognition and cleavage, and the structural basis for this behavior, has been extensively described both for a homodimeric LHE (I-CreI: Chevalier *et al.*, 2003) and for monomeric LHEs that are prototypes for genome engineering and correction applications (I-SceI: Gimble *et al.*, 2003; and I-AniI: Scalley-Kim *et al.*, 2007); under physiological conditions, the measured specificity of DNA recognition for that latter enzyme is at least 1 in 10^9 random sequences.

干细胞之家www.stemcell8.cn ←点击进入

(iii) They possess a well-defined, relatively small and highly modularized structure, and employ a DNA-binding mechanism, described in detail below, that is amenable to design and engineering. Monomeric LHEs (those containing both active sites and DNA recognition regions on a single peptide chain) are surprisingly small (ranging from ~200 to 250 amino acids total) relative to their long DNA target sites, thus simplifying the task of delivering a functional nuclease or its gene to target cells (Dalgaard *et al.*, 1997).

(iv) LHE's have been shown to be highly effective in inducing markerless modification of genes without toxicity in both prokaryotic and eukaryotic organisms. Of note, the prototypical LHE I-SceI has been used as the "gold standard" against which the activity and specificity of engineered ZFN's have been measured (Pâques and Duchateau, 2007; Porteus and Baltimore, 2003).

(v) LHE cleavage activity is tightly linked to site-specific binding of the cognate DNA target, significantly minimizing off-site cleavage activity (Chevalier *et al.*, 2004). Extensive experimentation by a wide variety of laboratories with I-SceI and other LHEs, in which transfected eukaryotic cells overexpress active endonuclease scaffolds, indicate little or no toxicity or off-site activity in a variety of experimental contexts, together with the ability to promote efficient, DNA double strand break-dependent gene conversion (Pâques and Duchateau, 2007).

(vi) LHE proteins have the potential for biochemical diversification, as they can be converted to site-specific nicking enzymes (McConnell-Smith and Stoddard, unpublished data) and can be fused to additional function protein domains at their N- and C-termini while retaining target site-specific binding affinity and activity.

3.1. *The Structure, Specificity and Catalytic Activity of LAGLIDADG Endonucleases*

The structures of several LHEs bound to their DNA targets have been determined [Fig. 4(A) and 4(B)]. These include several homodimers [I-CreI (Chevalier *et al.*, 2003 and 2001; Heath *et al.*, 1997; Jurica *et al.*, 1998), I-MsoI (Chevalier *et al.*, 2003) and I-CeuI (Spiegel *et al.*, 2006)], pseudosymmetric monomers [I-AniI (Bolduc *et al.*, 2003) and I-SceI (Moure *et al.*, 2003)], an artificially engineered chimeric enzyme [H-DreI (Chevalier *et al.*, 2002)] and an intein-associated endonuclease from yeast [PI-SceI (Moure *et al.*, 2002)]. Structures have also been determined of additional LHE's in the absence

千细胞之家www.stemcell8.cn ←点击进入

Fig. 4. Structural features of LAGLIDADG homing endonucleases. **(A)** DNA bound complex of the I-CreI homodimer. **(B)** DNA bound complex of the I-AniI monomer. The peptide linker connecting the two related domains is outlined in yellow. **(C)** The LAGLIDADG motifs form the helices at the domain interface of the I-CreI structure and serve a similar role in all known LAGLIDADG enzymes. **(D)** Structure and orientation of the DNA-binding anti-parallel β-sheets from I-CreI. **(E)** Summary of direct and water-mediated contacts between the I-CreI enzyme and the bases of its DNA target site. Direct bonds are shown as solid arrows (red to bases, green to backbone atoms); water-mediated contacts are shown as blue dashed and solid lines. The scissile phosphates are indicated with black closed circles. **(F)** Proposed catalytic mechanism for I-CreI, as described in the text. Nucleophilic water is red; surrounding ordered water molecules are blue. (Based on Fig. 3 of: Stoddard BL, *Q Rev Biophys* **38**: 49–95 (2005), with permission of Cambridge University Press.)

干细胞之家www.stemcell8.cn ←点击进入

of DNA. LHE domains form an elongated protein fold that consists of a core fold with mixed α/β topology (α-β-β-α-β-β-α: Heath *et al.*, 1997). The overall shape of this domain is a half-cylindrical "saddle" that averages approximately 25 Å × 25 Å × 35 Å. The surface of the saddle is formed by an anti-parallel, three- or four-stranded β-sheet that presents a large number of exposed basic and polar residues for DNA contacts and binding. The complete DNA-binding surfaces of the full-length enzymes, generated by two-fold symmetry or pseudo-symmetry, are 70 to 85 Å long and thus can accommodate DNA target sites of up to 24 base pairs.

The LAGLIDADG motifs [Fig. 4(C)] form the last two turns of the N-terminal helices in each folded domain or monomer and are packed against one another. They also contribute N-terminal, conserved acidic residues to two active sites where they help coordinate divalent cations that are essential for catalytic activity. The structure and packing of the parallel, two-helix bundle in the domain interface of the LAGLIDADG enzymes is strongly conserved among the otherwise highly diverged members of this enzyme family.

Two structurally independent, antiparallel β-sheets (one from each protein domain) are used to contact nucleotide bases within the major groove, at positions flanking the central four base pairs [Fig. 4(D)]. Despite little primary sequence homology among the LHEs outside of the motif itself, the topologies of the endonuclease domains of the enzymes visualized to date, and the shape of their DNA-bound β-sheets, are remarkably similar. A structural alignment of several endonuclease domains and subunits in their DNA-bound conformation indicates that the structure of the central core of the β-sheets is well conserved (Bolduc *et al.*, 2003). The conformations of the more distant ends of the β-strands and connecting turns are more degenerate. Base pairs ±3 to ±7 in each DNA half-site are typically recognized with higher specificity than base pairs in the less conserved, distant flanks of the DNA target. In rare cases, the core fold of LHEs can be tethered to additional functional domains involved in DNA binding (Sitbon and Pietrokovski, 2003). For example, a single copy of a canonical helix-turn-helix domain is found downstream (C-terminal) from the LAGLIDADG core of the intron-associated gene product of ORF Q0255 in yeast. This motif is similar to a conserved region of the bacterial sigma54-activator DNA-binding protein (Wintjens and Rooman, 1996).

LHEs typically make contact with 65 to 75% of possible hydrogen-bond donors and acceptors of the base pairs in the major groove. They make few or no additional contacts in the minor groove, and contact approximately one-third of the backbone phosphate groups across the homing site

干细胞之家www.stemcell8.cn ←点击进入

sequence [Fig. 4(E)]. These contacts are split evenly between direct and water-mediated contacts. Target site DNA is progressively and gradually bent around the interface formed by antiparallel β-sheets to give an overall curvature across the entire length of the site of ~45°. The information content (specificity) of recognition and cleavage by these enzymes, at each base pair of their DNA target site, is correlated with the number and type of intermolecular contacts made by the enzyme to each base pair. In addition, specificity is increased at the individual base pairs, particularly near the center of the cleavage site, by the additional indirect contribution of sequence-specific conformational preferences of the DNA itself.

The structures of several LHEs have been determined at relatively high resolution in complex with DNA (2.4 to 1.5 Å). These high resolution structures demonstrate the presence of three bound divalent metal ions distributed across a pair of overlapping active sites, with one central metal shared between the active sites. These enzymes appear to employ a canonical two-metal mechanism for phosphodiester hydrolysis [Fig. 4(F); Chevalier *et al.*, 2001]. The active site metal ions are coordinated by conserved acidic residues from each LAGLIDA**D**G motif, and by oxygen atoms from scissile phosphates on each DNA strand. Individual details of the structural mechanism of nucleophilic activation appear to differ between enzyme subfamilies. With the exception of the direct metal-binding residues from the LAGLIDADG motifs, the active site residues are only moderately conserved within the LAGLIDADG enzymes (Chevalier *et al.*, 2001).

3.2. *Engineering and Selection of LAGLIDADG Homing Endonucleases with Altered Structures and Specificities*

The past 20 years of homing endonuclease research, including recent analyses of their evolution and divergence that have been facilitated by high-throughput sequencing programs, have conclusively documented the dynamic evolution of these gene and protein families. Protein open reading frames have undergone rapid divergence, structural shuffling and recombination, continuous adaptation to and invasion of ectopic target insertion sites, rapid expansion throughout novel target lineages, and cyclical acquisition and loss. As a result, it is widely believed that the actual — and potential — site recognition repertoires of homing endonucleases are extremely broad. These data argue that if the primary mechanisms by which evolution has driven specificity changes in homing endonucleases could be duplicated in the laboratory, it should be possible to generate a wide variety

干细胞之家www.stemcell8.cn ←点击进入

of HEs with diverse DNA target site specificities for genome engineering applications.

The structures of several hundred protein-DNA complexes have been determined and analyzed at high resolution, and there have been many corresponding attempts to survey and catalogue the identity of contacts made in those complexes (Pabo and Nekludova, 2000). These studies have repeatedly indicated that while certain DNA nucleotides display preferences for specific residue contact patterns (such as the use of arginine side chains to make direct contacts to guanine bases, or the use of the amides of glutamine and asparagine to make direct contacts to adenine bases), there is no simple one-to-one "code" describing and predicting such interactions, even for the most modular and simplest DNA recognition modules such as zinc-fingers (Wolfe *et al.*, 2000a and 2000b). This is due to the fact that side-chain contacts to DNA bases, and their individual contribution to specificity, are exquisitely sensitive to the surrounding structural and chemical context of the protein-DNA interface. This context is determined by the local backbone conformation and the structure of both the protein and the corresponding DNA target site, as well as the conformational changes that occur during complex formation and the interdependence of neighboring contact networks.

In addition to the context-dependent role of direct protein contacts, binding specificity is also driven by the sequence-dependent conformational preferences of potential target sites, which can greatly influence and increase specificity at the individual nucleotide positions (Lavery, 2005). This property is usually termed "indirect" readout of specificity, as contrasted to the specificity created through direct contacts as described above. For example, the homing endonuclease I-CreI clearly displays very strong sequence preferences in the DNA target at individual base pairs that are not directly contacted by the enzyme, due to significant DNA bending induced across the center of the target site (Chevalier *et al.*, 2003).

The implication of these studies for the engineering of novel protein-DNA specificities is clear: that structure-based redesign of direct protein-DNA contact points, together with the generation or selection of equally critical variants within the protein scaffold, will be required to produce proteins with the requisite target site specificity for genome engineering applications.

干细胞之家www.stemcell8.cn ←点击进入

3.3. *Redesign of Homing Endonuclease Domain Architecture and Oligomery*

Several independent studies have demonstrated that domains or subunits from unrelated LHEs can be fused to create fully active, chimeric homing endonucleases that recognize corresponding chimeric DNA target sites (Chevalier *et al.*, 2002; Epinat *et al.*, 2003; Steuer *et al.*, 2004). This technology requires extensive repacking of the domain interface, and allows the creation of new protein scaffolds with novel specificities. These studies reinforce the idea that LAGLIDADG HEs display modularity of form and function, with individual domains and subunits being largely or entirely reponsible for the recognition and binding of individual DNA target half-sites.

Of particular note, two separate groups have generated an artificial highly specific endonuclease by fusing domains of homing endonucleases I-DmoI and I-CreI (Fig. 5); the novel proteins were termed "E-DreI" (now renamed H-DreI for **H**ybrid-**D**mo/C**reI**) and "DmoCre," respectively (Chevalier *et al.*, 2002; Epinat *et al.*, 2003). In the former study leading to the creation of H-DreI, structure-based protein engineering was accomplished by combining computational redesign and an *in vivo* protein folding and solubility screen (Chevalier *et al.*, 2002). The resulting enzyme binds a long chimeric DNA target site with nanomolar affinity, cleaving it precisely at the same phosphate groups with a rate equivalent to its natural parents. The structure of the engineered protein in complex with its DNA target demonstrated the accuracy of the protein interface redesign algorithm, and revealed how catalytic function was maintained by the creation of new, chimeric active sites. Most importantly, the mechanism of DNA recognition displayed by the chimeric endonuclease, and the identity of its residues used to make contacts to individual nucleotides, appeared to be indistinguishable from the original parental enzymes. Thus, the individual domains of LAGLIDADG endonucleases appear to be highly modular, and thus can be shuffled and recombined in order to make large numbers of different DNA-binding specificities.

Additional engineering experiments have reinforced this concept. Domains isolated from homing endonucleases and from inteins (self-splicing protein ligases, that are often associated with HEs) can be fused and shuffled into artificial, bifunctional inteins with novel DNA-binding specificities and/or activities. One such construct was assembled by inserting a gene that expresses one of the two I-CreI subunits into the *Mycobacterium xenopi* GyrA mini-intein (Fitzsimons-Hall *et al.*, 2002). This engineered intein displayed appropriate protein splicing, and produced a homodimeric

干细胞之家www.stemcell8.cn ←点击进入

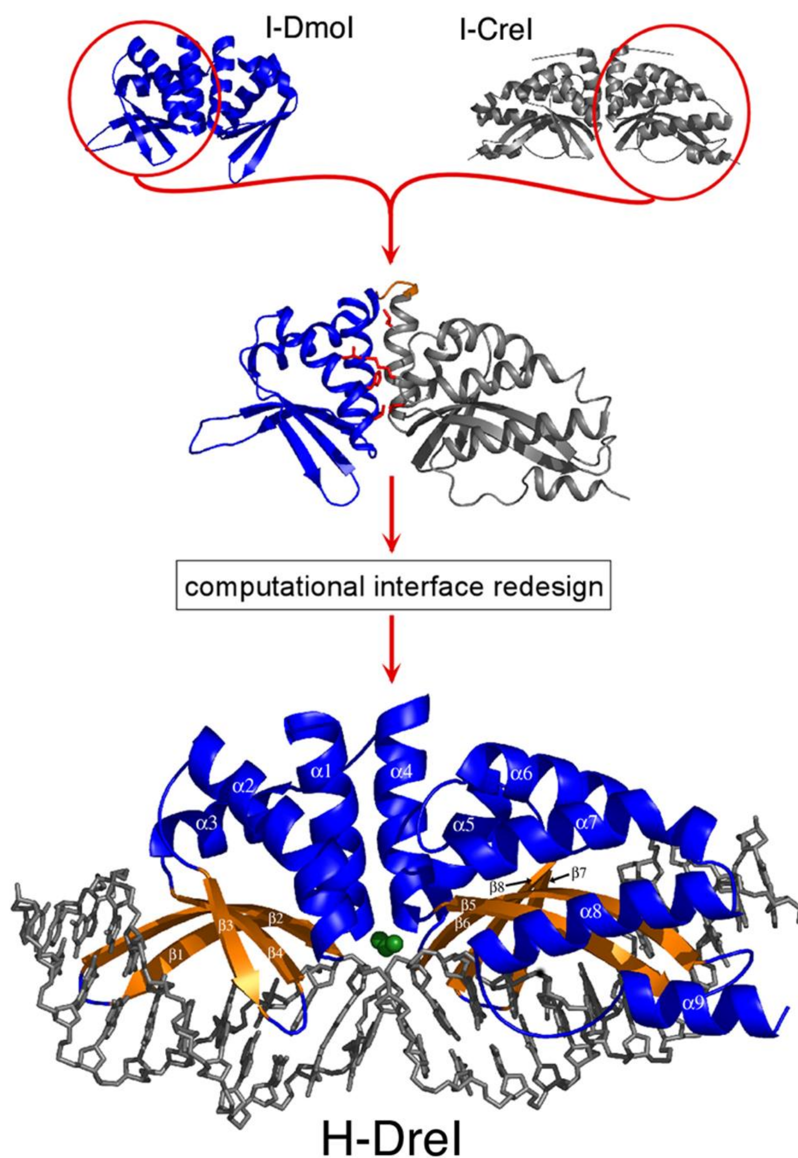


Fig. 5. Generation of an artificial, chimeric homing endonuclease. Individual domains of the I-DmoI and I-CreI parental enzymes (a monomeric LHE from an archaea host and a homodimeric LHE from a green algae, respectively) were fused into a single peptide chain, and computational protein engineering was employed to redesign and stabilize the novel domain interface. The resulting protein retains the overall thermal stability, specificity and cleavage rate of the parental enzymes, while acting at a chimeric DNA target site. (Based on Fig. 12 of: Stoddard BL, *Q Rev Biophys* **38**: 49–95 (2005), with permission of Cambridge University Press.)

干细胞之家www.stemcell8.cn ←点击进入

site-specific endonuclease activity identical to naturally occurring I-CreI. Separate experiments have also demonstrated that the PI-SceI protein splicing domain can be used as a site-specific DNA binding module in chimeric protein constructs: domain swapping between the PI-SceI and a homologue from *Candida tropicalis* (PI-CtrIP) was conducted to design altered specificity proteins (Steuer *et al.*, 2004).

A related experiment has demonstrated that a single chain, monomeric endonuclease can be generated from a homodimer predecessor, I-CreI (Epinat *et al.*, 2003). This construct was shown to initiate homologous recombination in both yeast and mammalian cells. Finally, the role and mutability of LAGLIDADG interface residues has been examined by grafting side chains from the homodimeric I-CreI into the corresponding positions in the monomeric I-DmoI enzyme, resulting in enzymes with novel nicking activities and oligomeric properties (Silva and Belfort, 2004). Subsequent experiments with this same enzyme have demonstrated that individual domains from the monomeric wild-type protein can be engineered to form stable and functional homodimers, again illustrating the modularity of LHE scaffolds (Silva *et al.*, 2006).

3.4. *Redesign of Homing Endonuclease DNA Contacts and Specificity*

Several methods have been used to alter the DNA target site specificity of LHE's (Figs. 6 and 7). These have focused in large part on the mutation of individual DNA-protein interface side chains that contact specific DNA target site base pairs. These strategies can be broadly divided into:

(i) Protocols that rely on a selection for high affinity DNA binding activity.
(ii) Protocols that rely on a selection for efficient cleavage activity. Examples have been described (summarized below) of methods that rely on the elimination of a gene (usually by destruction of a plasmid and a resident protein coding sequence) or on complementation of a gene (by cleavage-induced homologous recombination of two dysfunctional enzyme alleles into a wild-type, functional reading frame). This latter method is the basis of the most successful "high-throughput" endonuclease redesigns to date, which have progressed to targeting of physiologically relevant gene sequences. Those experiments are summarized at the end of this section.
(iii) Protocols that rely on structure-based computational redesign of DNA-protein interface contact surfaces and residues. It is important to note

干细胞之家www.stemcell8.cn ←点击进入

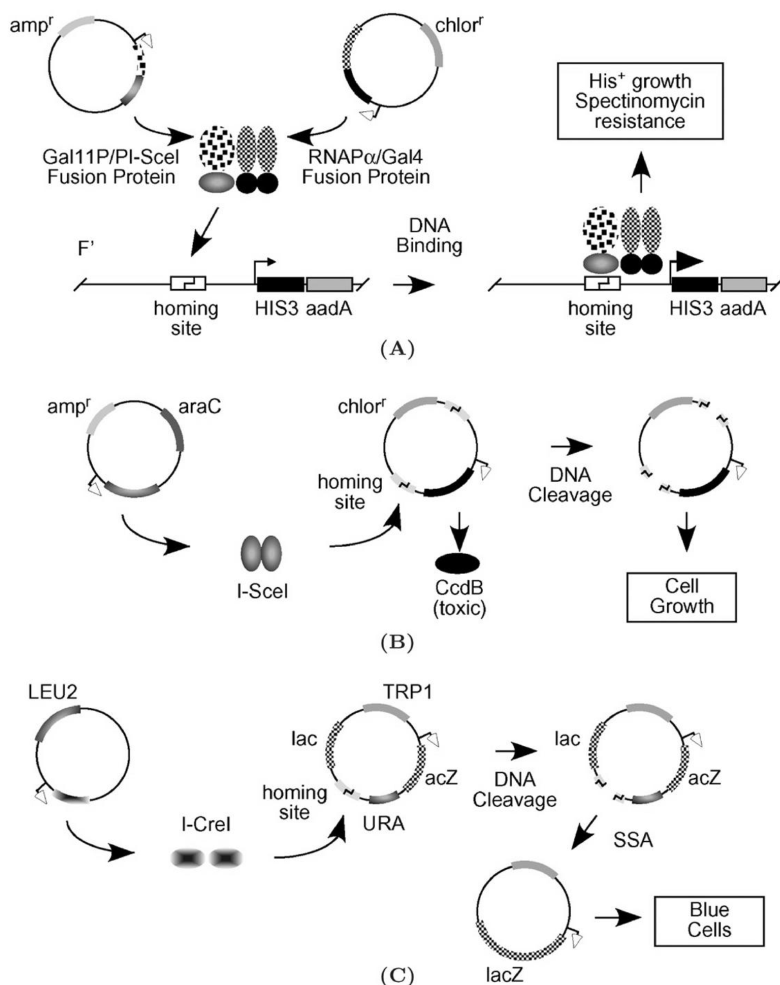


Fig. 6. Selections and screens for altered specificity homing endonucleases. **(A)** Selection for DNA binding activity using a two-hybrid system (Gimble *et al.*, 2003). Gal11P/PI-SceI variants expressed from a plasmid library that bind to a homing site increase the expression of *HIS3* and *aadA* by recruiting the RNAPα/Gal4 fusion protein to the weak P_{lac} promoter on the F′. Thus, cells that express a PI-SceI protein capable of binding a chosen DNA target sequence grow on histidine-selective and spectinomycin-selective media. **(B)** Selection for DNA cleavage activity (Chen and Zhao, 2005; Doyon and Liu, 2006). Co-existence of two plasmids kills bacterial cells because one expresses the toxic CcdB gene product. Selection can be made for I-SceI enzyme variants that cleave I-SceI target sites on the CcdB plasmid because they eliminate it and allow the cells to survive. **(C)** Screen for homologous recombination activity (Arnould *et al.*, 2006). Expression of I-CreI from a plasmid library cleaves a target site within an interrupted *lacZ* gene located on a reporter plasmid. This DSB stimulates single strand annealing (SSA) of two direct repeats that flank the site, leading to restoration of a functional *lacZ* gene and the appearance of a blue colony. (Figure and caption reproduced from: Gimble FS, *Gene Ther Regul* **3**: 33–50 (2007), with permission of the author and of World Scientific Publishing.)

干细胞之家www.stemcell8.cn ←点击进入

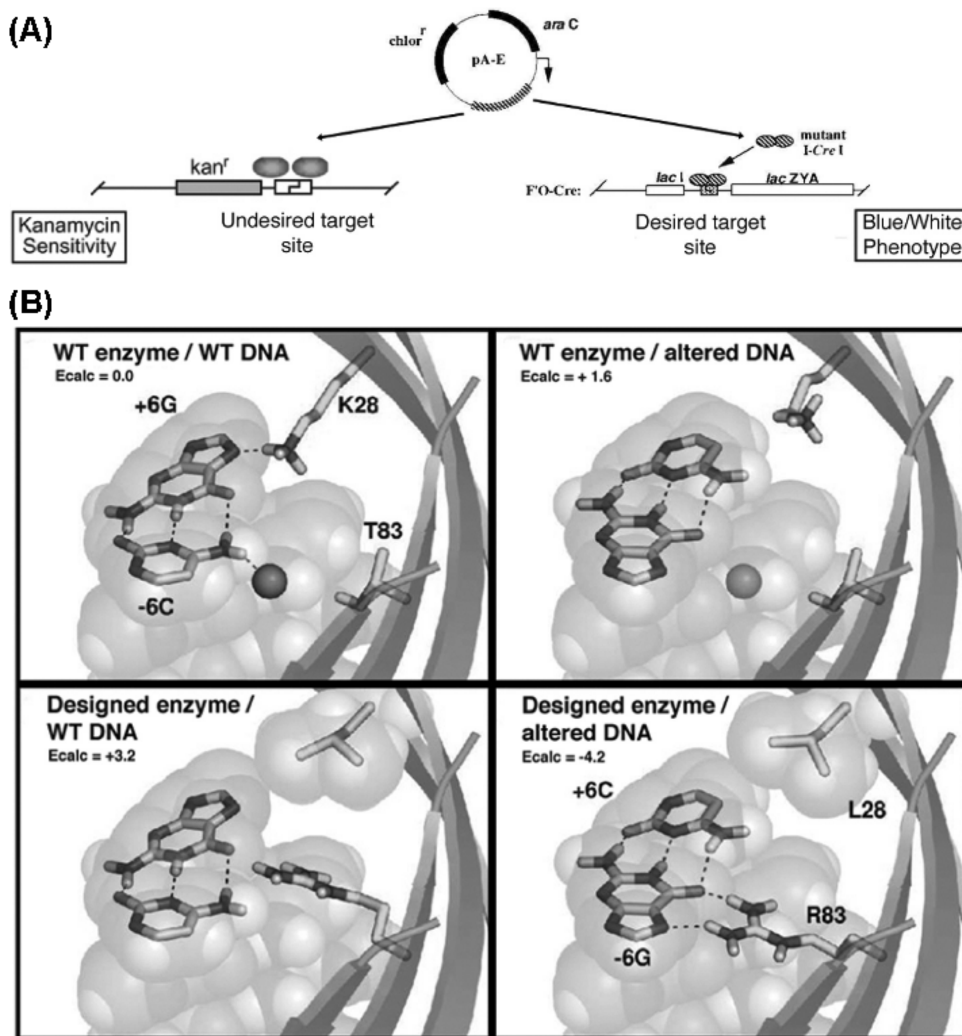


Fig. 7. From *in vivo* selection to *in silico* screening. **(A)** Screen for homing endonuclease based on cleavage and gene elimination (Seligman *et al.*, 2002 and 2004). Plasmid-encoded I-CreI derivatives that bind and cleave an I-CreI homing site located on an F′ lead to its elimination. Concomitant loss of an adjacent kanamycin antibiotic marker yields kanamycin-sensitive cells. **(B)** Alteration of specificity of the I-MsoI homing endonuclease by computational structure-based redesign (Ashworth *et al.*, 2006). The four panels provide a comparison of the experimentally determined interactions in wild-type and redesigned cognate complexes (*upper left* and *lower right*) and predicted interactions in non-cognate complexes, as well as predicted relative energies of the complexes.

干细胞之家www.stemcell8.cn ←点击进入

that high resolution crystal structures of the wild-type protein-DNA complexes have been used in various contexts, either to enable the targeted redesign of homing endonuclease-DNA contacts at individual residues (thus bypassing selection approaches altogether), or to facilitate more efficient mutational screening of enzyme libraries (by identifying, and thus greatly reducing, the number of protein residues to be randomized). The use of such crystal structures is a prominent feature of the most successful LHE redesigns described to date.

3.4.1. *Redesign of specificity at individual DNA base pairs*

An early attempt to reprogram the DNA recognition specificity of a homing endonuclease involved an adaptation of a bacterial two-hybrid screening strategy [Gimble *et al.*, 2003; Fig. 6(A)]. In this experiment, variants of the intein-encoded PI-SceI homing endonuclease with altered binding specificities were selected, and then characterized for their ability to discriminate between the corresponding substrates in DNA cleavage reactions. The specificities of the selected endonuclease variants ranged from being relaxed (i.e., able to cleave the wild-type and mutant targets equally) to being shifted to preferring the selection targets. However, none of the variants displayed the same discrimination as wild-type PI-SceI.

Two alternative strategies have used bacterial selection strategies based on the cleavage and elimination of a reporter gene to isolate homing endonuclease derivatives with altered specificities. In the first, cleavage of the target site results in cells being converted from lac^+ to lac^-, allowing selection of desired activities based on a simple blue-white colony phenotype screen [Rosen *et al.*, 2006; Seligman *et al.*, 2002; Sussman *et al.*, 2004; Fig. 7(A)]. Undesirable activity (e.g., cleavage of the original wild-type site) can be suppressed through a secondary "negative" screen for elimination of an essential reporter (such as an antibiotic resistance marker). Using this method, endonuclease mutants with single or double amino-acid substitutions, at positions predicted to make base-specific DNA contacts, were assayed against appropriate DNA target site mutants. Enzyme variants with shifted specificities, but with reduced ability to discriminate between cognate and miscognate sites, were typically obtained. Crystallographic analyses of several of these altered mutants in complex with their new cognate targets demonstrated that the "modularity" of protein-DNA recognition, previously described for entire protein domains, extends to the level of individual amino-acid side chains (Rosen *et al.*, 2006; Sussman *et al.*, 2004).

干细胞之家www.stemcell8.cn ←点击进入

A similar bacterial selection strategy has been described in which a toxic gene product results in cell death, unless a homing endonuclease variant that cleaves a homing site within the toxin expression vector is also present [Fig. 6(B)]. Two versions of this screen have been described: the first utilizing the nonspecific ribonuclease barnase (Gruen *et al.*, 2002), and the other describing a more easily controlled system based on the "control of cell death B" (CcdB) protein (Doyon *et al.*, 2006). In the latter screening strategy, CcdB expression led to very low rates of background survival, without requiring additional gene expression control elements such as those required to use the more toxic barnase protein. As with the bacterial selection described in the preceding paragraph, the CcdB system can be tailored as a positive selection for cleavage of a desired target, or as a negative selection, to strongly disfavor the recognition of a non-cognate DNA target site sequence. This system, when optimized, can lead to nearly 100% survival of cells expressing an active homing endonuclease, against a background survival of <1 in 5×10^4 with an inactive enzyme.

In separate experiments that avoided all use of combinatorial mutagenesis and screening methodologies, a successful computational redesign of the I-*Mso*I homing endonuclease was described using a physically realistic atomic level forcefield [Ashworth *et al.*, 2006; Fig. 7(B)]. Using an *in silico* screen, investigators identified single base pair substitutions predicted to disrupt binding by the wild type enzyme, and then optimized the identities and conformations of clusters of amino acids around each of these unfavorable substitutions by using Monte Carlo sampling. A redesigned enzyme predicted to display altered target site specificity was identified that maintained wild-type binding affinity. The redesigned enzyme was found to bind and cleave the redesigned recognition site more effectively than does the wild type enzyme, with a level of target discrimination comparable to the original endonuclease. Determination of the structure of the redesigned nuclease-recognition site complex by X-ray crystallography confirmed the accuracy of the computationally-predicted interface.

3.4.2. *High-throughput screening and targeting of genomic loci*

Recently, a commercial research and development group in France (Cellectis, Inc.) has developed a powerful eukaryotic assay system that reports on the generation of double-strand break-induced homologous recombination, rather than only DNA cleavage [Arnould *et al.*, 2006; Chames *et al.*, 2005; Fig. 6(C)]. In this assay, the function of a gene required for growth or for another easily scoreable phenotype is restored through the action

干细胞之家www.stemcell8.cn ←点击进入

of a homing endonuclease. Prior to expression and action of the HE, the gene sequence is interrupted with an insert containing a desired HE cut site flanked by two direct repeats. In the most recently described version of this screen, the endonuclease expression construct and the "reporter" construct are located in separate yeast strains, allowing the investigator to introduce the HE (or a library of HE variants) to a target site by mating. The mating of the two required yeast strains can be automated, and thus done as a high-throughput assay. Thus, the same library of endonuclease variants can be efficiently screened against multiple DNA target site variants, in parallel or rapid sequential experiments.

Using this method, the DNA recognition specificity of I-CreI LHE has been thoroughly analyzed. Small endonuclease mutant libraries resulting from the randomization of two to four amino acids (corresponding to the "nearest protein neighbors" of individual DNA basepairs) were individually screened against all potential variant cognate sequences. This analysis identified individual mutations in the I-CreI protein scaffold that were associated with shifts in specificity at individual target site base pair positions. From these studies, hundreds of endonuclease mutants with altered specificies were identified, catalogued and archived (Arnould *et al.*, 2006). Many of these variants displayed cleavage activities and levels of site discrimination that were at least equivalent to the wild-type endonuclease, thus providing a starting point for more ambitious endonuclease redesign to physiological targets in eukaryotic, mammalian and even human genomes.

Using the approach summarized above, derivatives of the I-CreI LHE have been generated that display sequence-specific cleavage and recombination activity against the human *RAG1* gene (Smith *et al.*, 2006) at the site of mutations, giving rise to a rare subset of severe combined immunodeficiency disease (or SCID) phenotypes. I-CreI variants directed at the human *XPC* (xeroderma pigmentosum complementation group C) gene (Arnould *et al.*, 2007) were also generated. XPC, when a mutant, confers an extreme UV or sun-sensitivity phenotype together with a predisposition to sunlight-induced skin cancer. Both of these human disease genes are candidates for corrective genetic therapies. Furthermore, in the latter experiments, the modified I-CreI derivatives cleaving sequences from the XPC gene were found to induce a high level of gene targeting, similar to levels observed with the wild-type I-CreI or I-SceI scaffolds. This is the first time an engineered homing endonuclease has been used in mammalian cells to target and modify a chromosomal target locus.

干细胞之家www.stemcell8.cn ←点击进入

3.4.3. *Surface display of homing endonucleases: avoiding bottlenecks in mutagenesis and screening?*

While the methodologies summarized above have shown great promise in delivering tailor-made gene-specific targeting reagents, they are limited by the requirement to first generate and then screen libraries of endonuclease mutants. Moreover, the selections and screens discussed above require the generation of unique intracellular reporter constructs that must be redesigned and constructed for each target sequence of interest. In order to address these issues, investigators have recently described a novel system where LHE proteins might be rapidly mutated *in vivo* and then screened to identify and isolate endonuclease variants with a new DNA target specificity (Fig. 8; Volna *et al.*, 2007). This work has built on a recent study that describes the evolution and selection of fluorescent protein spectral properties using the somatic hypermutation machinery of lymphocytes (Wang *et al.*, 2004).

Investigators demonstrated that LHEs can be expressed on the plasma membrane of a lymphocyte cell line by targeting the expression of an LHE-CD80 transmembrane fusion protein to the secretory pathway. Surface-expressed LHEs faithfully recapitulate the properties of the native enzymes in solution, as assessed by flow cytometric analysis of both the binding and the cleavage of fluorescently conjugated double-stranded DNA target site oligonucleotides. Identification of endonuclease expression clones with the desired DNA recognition properties was highly specific, allowing discrimination of endonucleases with binding preferences differing by only a single base pair. Furthermore, target sequence-specific LHE interactions with these DNA target site probes under conditions that limit substrate cleavage allow both the identification and sorting or enrichment of clones expressing LHE variants with the highest site specificity and affinity for further characterization. This coupled rapid analysis of LHE-DNA interactions on the cell surface, together with concurrent sorting or enrichment, should substantially accelerate the generation and isolation of novel endonuclease variants with unique DNA target specificities.

4. Concluding Remarks

The first X-ray structures of homing endonuclease (I-CreI and PI-SceI) were reported within one month of each other in 1997 (Duan *et al.*, 1997; Heath *et al.*, 1997). These were followed a year later by the first DNA-bound cocrystal structure (again I-CreI) (Jurica *et al.*, 1998). The subsequent 10 years have

干细胞之家www.stemcell8.cn ←点击进入

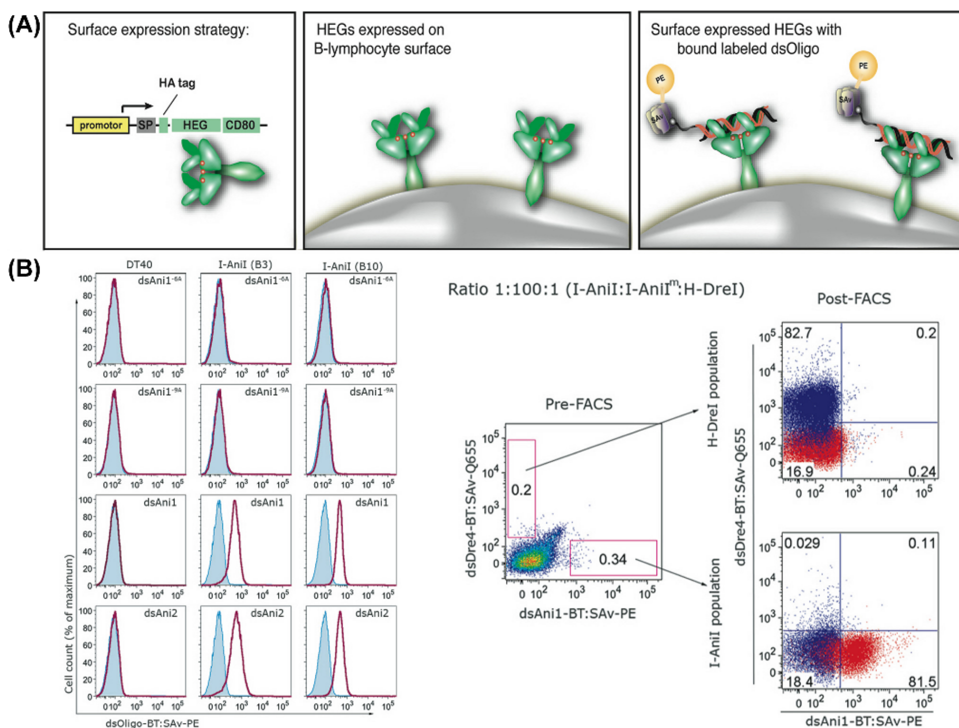


Fig. 8. DNA binding by surface displayed homing endonucleases. **(A)** An HA-tagged LAGLIDADG homing endonuclease is expressed on the surface of vertebrate cells through fusion with the transmembrane domain and tail of murine CD80. The expressed homing endonucleases I-AniI and H-DreI retain their DNA recognition, binding and cleavage activity, and binding can be quantitatively analyzed through the use of fluorescently labeled, double-stranded (ds) DNA oligonucletides and flow cytometry. "SP" = signal peptide. **(B)** *Left*: The fluorescence staining intensity using dsDNA probes is dependent on the surface expression and display of a homing endonuclease, and is capable of discriminating between the binding of a wild-type target site vs. miscognate sites harboring single base pair substitutions. DT40: parental cell line without surface HE expression. I-Ani (B3) and (B10): two individual clones expressing wild-type I-AniI homing endonuclease on the surface. dsAni1 and dsAni2: distinct dsDNA probes encoding different I-AniI target sites that are efficiently recognized, bound, and cleaved by recombinant soluble I-AniI. dsAniI-6A and dsAni1-9A: two dsDNA probes harboring I-AniI target sites that contain single base pair substitutions which eliminate cleavage activity of the recombinant soluble I-AniI. *Right*: enrichment through flow sorting of rare cells expressing a desired binding specificity from an excess (approximately 100–500 fold) of undesired HE binding activities.

produced a remarkable increase in our understanding of the biology, structure, function and mechanism of homing endonucleases. Representative structures from each of the major homing endonuclease families have been determined together with their catalytic mechanisms. At least six different methods for the modification and redesign of homing endonuclease DNA

干细胞之家www.stemcell8.cn ←点击进入

target specificity have also been developed. During this period, a small number of homing endonuclease proteins have been assayed for activity and toxicity *in vivo*, and have been used to investigate mechanistic aspects of DNA double strand break repair and recombination. The first engineered homing endonuclease variants have also been described and characterized, and the most recent of these have been shown to induce DNA cleavage and recombination at chromosomal target loci *in vivo*. This collective experience has established the feasibility of using homing endonuclease proteins for genome engineering, together with the data needed to further develop homing endonuclease proteins for basic science and engineering applications.

The development of engineered homing endonucleases as reagents for basic biology (to facilitate the introduction or analysis of double- or single-strand breaks, covalent DNA modifications or trans-acting protein factors at specific DNA target sites in living cells) has matured rapidly over the past decade, and their further use and development in this area is virtually guaranteed. In contrast, the use of homing endonucleases as therapeutic reagents for disease treatment or prevention is still in its infancy and will require many years of dedicated work. Among the most important, unanswered questions are: the specificity profile of such reagents in biologically relevant contexts (e.g., stem cells), how efficient they are in generating target site-specific DNA double strand breaks *in vivo*, the range and efficiency with which individual DNA double-strand breaks lead to desired — and undesired — molecular outcomes in populations of cells, and the long-term toxicity, mutagenicity and immunogenicity of homing endonucleases when used as genome engineering, therapeutic or disease prevention reagents. These questions are pertinent now that the feasibility of using homing endonuclease proteins for genome engineering has been demonstrated, and when, as noted by Pâques and Duchateau, "the time when therapeutic applications were pure fantasy is now gone" (Pâques and Duchateau, 2007).

References

Abbott A. (2006) Questions linger about unexplained gene-therapy trial death. *Nat Med* **12**: 597.

Agaard C, Awayez MJ, Garrett RA. (1997) Profile of the DNA recognition site of the archaeal homing endonuclease I-DmoI. *Nucl Acids Res* **25**:1523–30.

Arnould S, Chames P, Perez C, *et al*. (2006) Engineering of large numbers of highly specific homing endonucleases that induce recombination on novel DNA targets. *J Mol Biol* **355**: 443–58.

干细胞之家www.stemcell8.cn ←点击进入

Arnould S, Perez C, Cabaniols J-P, *et al*. (2007) Engineered I-CreI derivatives cleaving sequences from the human XPC gene can induce highly efficient gene correction in mammalian cells. *J Mol Biol* **371**: 49–65.

Ashworth J, Havranek JJ, Duarte CM, *et al*. (2006) Computational redesign of endonuclease DNA binding and cleavage specificity. *Nature* **441**: 656–59.

Baird PA, Anderson TW, Newcombe HB, Lowry RB. (1988) Genetic disorders in children and young adults: a population study. *Am J Hum Genet* **42**: 677–93.

Baum C. (2007) Fourth case of leukaemia in the first SCID-X1 gene therapy trial, and the diversity of gene therapy. *http://www.esgct.org/upload/4th_CaseofLeukemial.pdf*

Beerli RR, Barbas CF. (2002) Engineering polydactyl zinc-finger transcription factors. *Nat Biotechnol* **20**: 130–41.

Belfort M, Reaban ME, Coetzee T, Dalgaard JZ. (1995) Prokaryotic introns and inteins: a panoply of form and function. *J Bacter* **177**: 3897–903.

Belfort M, Roberts RJ. (1997) Homing endonucleases — keeping the house in order. *Nucl Acids Res* **25**: 3379–88.

Bertolotti R. (1996) Recombinase-mediated gene therapy: strategies based on Lesch-Nyhan mutants for gene repair/inactivation using human RAD51 nucleoprotein filaments. *Biogenic Amines* **12**: 487–98.

Bibikova M, Beumer K, Trautman JK, Carroll D. (2003) Enhancing gene targeting with designed zinc finger nucleases. *Science* **300**: 764.

Bibikova M, Carroll D, Segal DJ, *et al*. (2001) Stimulation of homologous recombination through targeted cleavage by chimeric nucleases. *Mol Cell Biol* **21**: 289–97.

Bolduc JM, Spiegel PC, Chatterjee P, *et al*. (2003) Structural and biochemical analyses of DNA and RNA binding by a bifunctional homing endonuclease and group I intron splicing cofator. *Genes Dev* **17**: 2875–88.

Bullard DC, Weaver CT. (2002) Cutting-edge technology: IV. Genomic engineering for studies of the gastrointestinal tract in mice. *Am J Physiol Gastrointest Liver Physiol* **283**: G1232–37.

Bulyk ML, Huang XH, Choo Y, Church GM. (2001) Exploring the DNA-binding specificities of zinc fingers with DNA microarrays. *Proc Natl Acad Sci USA* **98**: 7158–63.

Burt A. (2003) Site-specific selfish genes as tools for the control and genetic engineering of natural populations. *Proc R Soc Lond B Biol Sci* **270**: 921–28.

Burt A. (2005) Gates Grand Challenge Project. In: *Homing Endonuclease Genes: New Tools for Mosquito Population Engineering and Control*. *www.gcgh.org*.

Chames P, Epinat JC, Guillier S, *et al*. (2005) *In vivo* selection of engineered homing endonucleases using double-strand break induced homologous recombination. *Nucl Acids Res* **33**: e178.

Chase CD. (2006) Genetically engineered cytoplasmic male sterility. *Trends Plant Sci* **11**: 7–9.

Chevalier B, Sussman D, Otis C, *et al*. (2004) Analysis of the symmetric metal-dependent I-CreI homing endonuclease mechanism. *Biochemistry* **43**: 4015–26.

干细胞之家www.stemcell8.cn ←点击进入

Chevalier B, Turmel M, Lemieux C, *et al*. (2003) Flexible DNA target site recognition by divergent homing endonuclease isoschizomers I-CreI and I-MsoI. *J Mol Biol* **329**: 253–69.

Chevalier BS, Kortemme T, Chadsey MS, *et al*. (2002) Design, activity and structure of a highly specific artificial endonuclease. *Mol Cell* **10**: 895–905.

Chevalier BS, Monnat RJ Jr, Stoddard BL. (2001) The homing endonuclease I-CreI uses three metals, one of which is shared between the two active sites. *Nat Struct Biol* **8**: 312–16.

Chevalier BS, Stoddard BL. (2001) Homing endonucleases: structural and functional insight into the catalysts of intron/intein mobility. *Nucl Acids Res* **29**: 3757–74.

Christianson A, Howson CP, Modell B. (2006) Global Report on Birth Defects: The Hidden Toll of Dying and Disabled Children. March of Dimes, White Plains, New York.

Coates CJ, Kaminski JM, Summers JB, *et al*. (2005) Site-directed genome modification: derivatives of DNA-modifying enzymes as targeting tools. *Trends Biotechnol* **23**: 407–19.

Colleaux L, D'Auriol L, Betermier M, *et al*. (1986) Universal code equivalent of a yeast mitochondrial intron reading frame is expressed into *E. coli* as a specific double strand endonuclease. *Cell* **44**: 521–33.

Colleaux L, D'Auriol L, Galibert F, Dujon B. (1988) Recognition and cleavage site of the intron-encoded omega transposase. *Proc Natl Acad Sci USA* **85**: 6022–26.

Dalgaard JZ, Garrett RA, Belfort M. (1994) Purification and characterization of two forms of I-*Dmo*I, a thermophilic site-specific endonuclease encoded by an archaeal intron. *J Biol Chem* **269**: 28885–92.

Dalgaard JZ, Klar AJ, Moser MJ, *et al*. (1997) Statistical modeling and analysis of the LAGLIDADG family of site-specific endonucleases and identification of an intein that encodes a site-specific endonuclease of the HNH family. *Nucl Acids Res* **25**: 4626–38.

Delahodde A, Goguel V, Becam AM, *et al*. (1989) Site-specific DNA endonuclease and RNA maturase activities of two homologous intron-encoded proteins from yeast mitochondria. *Cell* **56**: 431–41.

Doetschman T, Gregg RG, Maeda N, *et al*. (1987) Targetted correction of a mutant HPRT gene in mouse embryonic stem cells. *Nature* **330**: 576–78.

Doyon JB, Pattanayak V, Meyer CB, Liu DR. (2006) Directed evolution and substrate specificity profile of homing endonuclease I-SceI. *J Am Chem Soc* **128**: 2477–84.

Duan X, Gimble FS, Quiocho FA. (1997) Crystal structure of PI-SceI, a homing endonuclease with protein splicing activity. *Cell* **89**: 555–64.

Dujon B. (1980) Sequence of the intron and flanking exons of the mitochondrial 21S rRNA gene of yeast strains having different alleles at the omega and rib-1 loci. *Cell* **20**: 185–97.

Dujon B. (1989) Group I introns as mobile genetic elements: facts and mechanistic speculations — a review. *Gene* **82**: 91–114.

干细胞之家www.stemcell8.cn ←点击进入

Dujon B, Belfort M, Butow RA, *et al*. (1989) Mobile introns: definition of terms and recommended nomenclature. *Gene* **82**: 115–18.

Durrenberger F, Rochaix J-D. (1993) Characterization of the cleavage site and the recognition sequence of the I-*Cre*I DNA endonuclease encoded by the chloroplast ribosomal intron of *Chlamydomonas reinhardtii*. *Mol Gen Genet* **236**: 409–14.

Epinat JC, Arnould S, Chames P, *et al*. (2003) A novel engineered meganuclease induces homologous recombination in yeast and mammalian cells. *Nucl Acids Res* **31**: 2952–62.

Fitzsimons-Hall M, Noren CJ, Perler FB, Schildkraut I. (2002) Creation of an artificial bifunctional intein by grafting a homing endonuclease into a mini-intein. *J Mol Biol* **323**: 173–79.

Geese WJ, Waring RB. (2001) A comprehensive characterization of a group IB intron and its encoded maturase reveals that protein-assisted splicing requires an almost intact intron RNA. *J Mol Biol* **308**: 609–22.

Ghatge M, Palaniappan N, Das Choudhuri S, Reynolds K. (2006) Genetic manipulation of the biosynthetic process leading to phoslactomycins, potent protein phosphatase 2A inhibitors. *J Ind Microbiol Biotechnol* **33**: 589–99.

Gimble FS, Moure CM, Posey KL. (2003) Assessing the plasticity of DNA target site recognition of the PI-SceI homing endonuclease using a bacterial two-hybrid selection system. *J Mol Biol* **334**: 993–1008.

Glaser S, Anastassiadis K, Stewart AF. (2005) Current issues in mouse genome engineering. *Nat Genet* **37**: 1187–93.

Gouble A, Smith J, Bruneau S, *et al*. (2006) Efficient in toto targeted recombination in mouse liver by meganuclease-induced double-strand break. *J Gene Med* **8**: 616–22.

Griesenbach U, Geddes DM, Alton EW. (2006) Gene therapy progress and prospects: cystic fibrosis. *Gene Ther* **13**: 1061–67.

Gruen M, Chang K, Serbanescu I, Liu DR. (2002) An *in vivo* selection system for homing endonuclease activity. *Nucl Acids Res* **30**: 29–34.

Hacein-Bey-Abina S, Von Kalle C, Schmidt M, *et al*. (2003) LMO2–associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* **302**: 415–19.

Heath PJ, Stephens KM, Monnat RJ, Stoddard BL. (1997) The structure of I-*Cre*I, a group I intron-encoded homing endonuclease. *Nat Struct Biol* **4**: 468–76.

Hinnen A, Hicks JB, Fink GR. (1978) Transformation of yeast. *Proc Natl Acad Sci USA* **75**: 1929–33.

Jacquier A, Dujon B. (1985) An intron-encoded protein is active in a gene conversion process that spreads an intron into a mitochondrial gene. *Cell* **41**: 383–94.

Jin Y, Binkowski G, Simon LD, Norris D. (1997) Ho endonuclease cleaves MAT DNA *in vitro* by an inefficient stoichiometric reaction mechanism. *J Biol Chem* **272**: 7352–59.

干细胞之家www.stemcell8.cn ←点击进入

Jurica MS, Monnat RJ Jr, Stoddard BL. (1998) DNA recognition and cleavage by the LAGLIDADG homing endonuclease I- CreI. *Mol Cell* **2**: 469–76.

Kolb AF, Coates CJ, Kaminski JM, *et al*. (2005) Site-directed genome modification: nucleic acid and protein modules for targeted integration and gene correction. *Trends Biotechnol* **23**: 399–406.

Koller BH, Smithies O. (1989) Inactivating the beta 2-microglobulin locus in mouse embryonic stem cells by homologous recombination. *Proc Natl Acad Sci USA* **86**: 8932–35.

Kostriken R, Strathern JN, Klar AJ, *et al*. (1983) A site-specific endonuclease essential for mating-type switching in *Saccharomyces cerevisiae*. *Cell* **35**: 167–74.

Lavery R. (2005) Recognizing DNA. *Q Rev Biophys* **38**: 339–44.

Lazowska J, Claisse M, Gargouri A, *et al*. (1989) Protein encoded by the third intron of cytochrome *b* gene in *Saccharomyces cerevisiae* is an mRNA maturase. Analysis of mitochondrial mutants, RNA transcripts proteins and evolutionary relationships. *J Mol Biol* **205**: 275–89.

Lazowska J, Jacq C, Slonimski PP. (1980) Sequence of introns and flanking exons in wild-type and box3 mutants of cytochrome *b* reveals an interlaced splicing protein coded by an intron. *Cell* **22**: 333–48.

Malik P, Arumugam PI. (2005) Gene therapy for {beta}-thalassemia. *Hematol Am Soc Hematol Educ Program*: 45–50.

Malik P, Arumugam PI, Yee JK, Puthenveetil G. (2005) Successful correction of the human Cooley's anemia beta-thalassemia major phenotype using a lentiviral vector flanked by the chicken hypersensitive site 4 chromatin insulator. *Ann N Y Acad Sci* **1054**: 238–49.

McCandless SE, Brunger JW, Cassidy SB. (2004) The burden of genetic disease on inpatient care in a children's hospital. *Am J Hum Genet* **74**: 121–27.

Moure C, Gimble F, Quiocho F. (2002) Crystal structure of the intein homing endonuclease PI-SceI bound to its recognition sequence. *Nat Struct Biol* **9**: 764–70.

Moure CM, Gimble FS, Quiocho FA. (2003) The crystal structure of the gene targeting homing endonuclease I-SceI reveals the origins of its target site specificity. *J Mol Biol* **334**: 685–96.

Orr-Weaver TL, Szostak JW, Rothstein RJ. (1981) Yeast transformation: a model system for the study of recombination. *Proc Natl Acad Sci USA* **78**: 6354–58.

Pabo CO, Nekludova L. (2000) Geometric analysis and comparison of protein-DNA interfaces: Why is there no simple code for recognition? *J Mol Biol* **301**: 597–624.

Pâques F, Duchateau P. (2007) Meganucleases and DNA double-strand break-induced recombination: perspectives for gene therapy. *Curr Gene Ther* **7**: 49–66.

Perrin A, Buckle M, Dujon B. (1993) Asymmetrical recognition and activity of the I-SceI endonuclease on its site and on intron-exon junctions. *EMBO J* **12**: 2939–47.

Porteus MH. (2006) Mammalian gene targeting with designed zinc finger nucleases. *Mol Ther* **13**: 438–46.

干细胞之家www.stemcell8.cn ←点击进入

Porteus MH, Baltimore D. (2003) Chimeric nucleases stimulate gene targeting in human cells. *Science* **300**: 763.

Posfai G, Kolisnychenko V, Bereczki Z, Blattner FR. (1999) Markerless gene replacement in *Escherichia coli* stimulated by a double-strand break in the chromosome. *Nucl Acids Res* **27**: 4409–15.

Puthenveetil G, Scholes J, Carbonell D, *et al*. (2004) Successful correction of the human beta-thalassemia major phenotype using a lentiviral vector. *Blood* **104:** 3445–53.

Rosen LE, Morrison HA, Masri S, *et al*. (2006) Homing endonuclease I-CreI derivatives with novel DNA target specificities. *Nucl Acids Res* **34**: 4791–800.

Sadelain M. (2006) Recent advances in globin gene transfer for the treatment of beta-thalassemia and sickle cell anemia. *Curr Opin Hematol* **13**: 142–48.

Samakoglu S, Lisowski L, Budak-Alpdogan T, *et al*. (2006) A genetic strategy to treat sickle cell anemia by coregulating globin transgene expression and RNA interference. *Nat Biotechnol* **24**: 89–94.

Scalley-Kim M, McConnell-Smith A, Stoddard BL. (2007) Coevolution of a homing endonuclease and its host target sequence. *J Mol Biol*, in press.

Schafer B, Wilde B, Massardo DR, *et al*. (1994) A mitochondrial group-I intron in fission yeast encodes a maturase and is mobile in crosses. *Curr Genet* **25**: 336–41.

Segal DJ, Beerli RR, Blancafort P, *et al*. (2003) Evaluation of a modular strategy for the construction of novel polydactyl zinc finger DNA-binding proteins. *Biochemistry* **42**: 2137–48.

Segal DJ, Dreier B, Beerli RR, Barbas CF. (1999) Toward controlling gene expression at will: selection and design of zinc finger domains recognizing each of the 5’-GNN-3’ DNA target sequences. *Proc Natl Acad Sci USA* **96**: 2758–63.

Seligman L, Chisholm KM, Chevalier BS, *et al*. (2002) Mutations altering the cleavage specificity of a homing endonuclease. *Nucl Acids Res* **30**: 3870–79.

Silva GH, Belfort M. (2004) Analysis of the LAGLIDADG interface of the monomeric homing endonuclease I-DmoI. *Nucl Acids Res* **32**: 3156–68.

Silva GH, Belfort M, Wende W, Pingoud A. (2006) From monomeric to homodimeric endonucleases and back: engineering novel specificity of LAGLIDADG enzymes. *J Mol Biol* **361**: 744–54.

Sitbon E, Pietrokovski S. (2003) New types of conserved sequence domains in DNA-binding regions of homing endonucleases. *Trends Biochem Sci* **28**: 473–77.

Smith J, Bibikova M, Whitby FG, *et al*. (2000) Requirements for double-strand cleavage by chimeric restriction enzymes with zinc finger DNA-recognition domains. *Nucl Acids Res* **28**: 3361–69.

Smith J, Grizot S, Arnould S, *et al*. (2006) A combinatorial approach to create artificial homing endonucleases cleaving chosen sequences. *Nucl Acids Res* **34**: e149.

Spiegel PC, Chevalier B, Sussman D, *et al*. (2006) The structure of I-CeuI homing endonuclease: evolving asymmetric DNA recognition from a symmetric protein scaffold. *Structure* **14**: 869–80.

干细胞之家www.stemcell8.cn ←点击进入

Steuer S, Pingoud V, Pingoud A, Wende W. (2004) Chimeras of the homing endonuclease PI-SceI and the homologous *Candida tropicalis* intein: a study to explore the possibility of exchanging DNA-binding modules to obtain highly speciric endonucleases with altered specificity. *Chembiochem* **5**: 206–13.

Stoddard BL. (2005) Homing endonuclease structure and function. *Q Rev Biophys* **38**: 49–95.

Sussman DJ, Chadsey M, Fauce S, *et al*. (2004) Isolation and characterization of new homing endonuclease specificities at individual target site positions. *J Mol Biol* **342**: 31–41.

Themis M, Waddington SN, Schmidt M, *et al*. (2005) Oncogenesis following delivery of a nonprimate lentiviral gene therapy vector to fetal and neonatal mice. *Mol Ther* **12**: 763–71.

Thompson AJ, Yuan X, Kudlicki W, Herrin DL. (1992) Cleavage and recognition pattern of a double-strand-specific endonuclease (I-*Cre*I) encoded by the chloroplast 23S rRNA intron of *Chlamydomonas reinhardtii*. *Gene* **119**: 247–51.

Thrasher AJ, Gaspar HB, Baum C, *et al.* (2006) Gene therapy: X-SCID transgene leukaemogenicity. *Nature* **443**: E5–E6; discussion E6–E7.

Tzfira T, White C. (2005) Towards targeted mutagenesis and gene replacement in plants. *Trends Biotechnol* **23**: 567–69.

Urnov FD, Miller JC, Lee YL, *et al*. (2005) Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature* **435**: 646–51.

Vasquez KM, Marburger K, Intody Z, Wilson JH. (2001) Manipulating the mammalian genome by homologous recombination. *Proc Natl Acad Sci USA* **98**: 8403–10.

Volna P, Jarjour J, Baxter S, *et al*. (2007) Flow cytometric analysis of DNA binding and cleavage by cell surface-displayed homing endonucleases. *Nucl Acids Res* **35**: 2748–58.

Wang L, Jackson WC, Steinbach PA, Tsien RY. (2004) Evolution of new nonantibody proteins via iterative somatic hypermutation. *Proc Natl Acad Sci USA* **101**: 16745–49.

Wintjens R, Rooman M. (1996) Structural classification of HTH DNA-binding domains and protein-DNA interaction modes. *J Mol Biol* **262**: 294–313.

Wolfe SA, Nekludova L, Pabo CO. (2000a) DNA recognition by Cys2His2 zinc finger proteins. *Ann Rev Biophys Biomol Struct* **29**:183–212.

Wolfe SA, Ramm EI, Pabo CO. (2000b) Combining structure-based design with phage display to create new Cys2His2 zinc finger dimers. *Structure* **8**: 739–50.

干细胞之家www.stemcell8.cn ←点击进入

This page intentionally left blank

干细胞之家www.stemcell8.cn ←点击进入

Chapter 7

Gene Targeting Mediated by Helper-dependent Adenoviral Vectors

Kohnosuke Mitani

Recently reported serious adverse events in the French trial of gene therapy for X-linked severe combined immunodeficiency syndrome (SCID-X1) highlighted an issue of insertional mutagenesis of cancer-related host genes caused by random-integration into host chromosomal DNA of current vector systems, such as retroviral and lentiviral vectors. For gene therapy of inherited diseases, targeted integration/gene repair via homologous recombination (HR) between exogenous and chromosomal DNA would be an ideal strategy to avoid potentially serious problems of random integration, such as cellular transformation and gene silencing. Efficient sequence-specific modification of chromosomes by HR would also advance both biological studies and therapeutic applications of a variety of stem cells. Toward these goals, viral vector-mediated approaches for gene targeting have been explored. Among them, HR mediated by helper-dependent adenoviral vectors (HDAdVs) is promising and relatively safe, and, in conjunction with emerging custom site-specific endonucleases, might be a viable and efficient option for *ex vivo* gene repair therapy as well as a powerful tool for chromosomal manipulation of various embryonic and adult stem cells.

Keywords: Gene therapy; helper-dependent adenoviral vector; homologous recombination; stem cell; gene repair; custom site-specific endonucleases.

1. Introduction

For gene therapy of inherited diseases, integrating vectors, such as retroviral, lentiviral and adeno-associated virus (AAV) vectors, have been utilized to introduce a therapeutic gene into random sites on target cell chromosomes

Gene Therapy Division, Research Center for Genomic Medicine, Saitama Medical University, Hidaka, Saitama 350-1241, Japan. mitani@saitama-med.ac.jp

干细胞之家www.stemcell8.cn ←点击进入

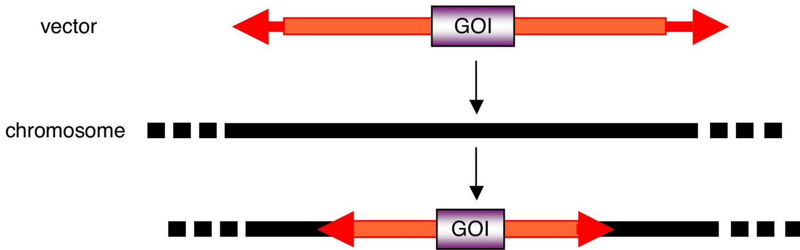
(Kay *et al.*, 2001; Verma and Weitzman, 2005). However, gene expression from randomly integrated vectors tends to be subject to integration site-specific effects, often leading to gene silencing [Fig. 1(A)]. As realized in the case of successful but controversial gene therapy for X-linked severe combined immunodeficiency syndrome (SCID-X1) in France, random integration has a potential problem of insertional mutagenesis, which might lead to cancer (Cavazzana-Calvo *et al.*, 2000; Hacein-Bey-Abina *et al.*, 2003; Baum, 2007). To overcome these problems, homologous recombination (HR) is an ideal mechanism to be exploited because it has the potential to repair mutant genes even in the case of dominant mutations [Fig. 1(B)]. It can also be applied to introduce a gene into safe and transcriptionally active chromosomal sites in order to obtain a predictable level of expression. HR between transfected exogenous DNA and chromosomal loci, termed gene targeting, has been extensively applied in mouse embryonic stem (ES) cells to produce gene knock-out mice. However, in mammalian cells, chromosomal integration of exogenous DNA is mainly random, presumably mediated by the non-homologous end-joining (NHEJ) pathway, and HR between exogenous and chromosomal DNA is extremely rare (Vasquez *et al.*, 2001). Because of their high efficiency in transducing a variety of cell types both *in vitro* and *in vivo*, viral vectors, such as AAV and adenoviral vectors (AdVs), have been used as gene targeting vectors. In this review, recent studies on the random and targeted chromosomal integration with helper-dependent (HD) AdVs, as well potential utilities for basic and therapeutic applications are discussed.

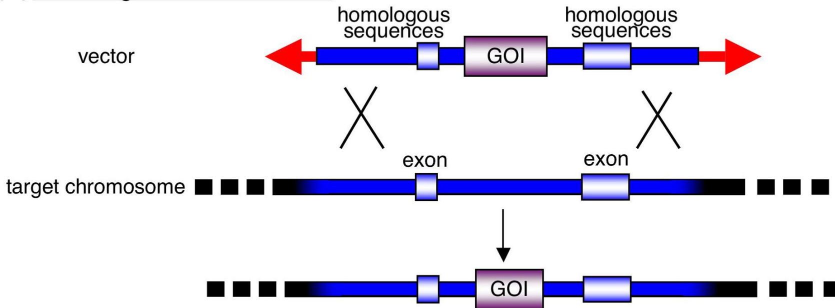
2. E1-Deleted Adenoviral Vectors

2.1. *Chromosomal Integration of E1-deleted AdVs*

In general, adenoviruses are considered as lytic viruses, and so wild-type viruses usually kill host cells upon infection. However, in the past, chromosomal integration of wild-type viruses under abortive conditions as well as that of temperature-sensitive adenovirus mutants at non-permissive temperatures has been reported (Fisher *et al.*, 1982; Hosel *et al.*, 2003). Chromosomal integration of an E1-deleted AdV has also previously been reported; the integration efficiencies were 0.4% of infected cells for rat Rat2 cells and 0.75% for simian CV-1 cells (van Doren *et al.*, 1984). In another study, both E1-deleted and HDAdVs achieved similar integration efficiencies of 10^{-2}–10^{-5} per cell in various cell lines, which were measured by using the neomycin-resistant gene as a marker (Harui *et al.*, 1999). Efficient production of transgenic mice by adenoviral gene transfer into fertilized eggs was also

干细胞之家www.stemcell8.cn ←点击进入





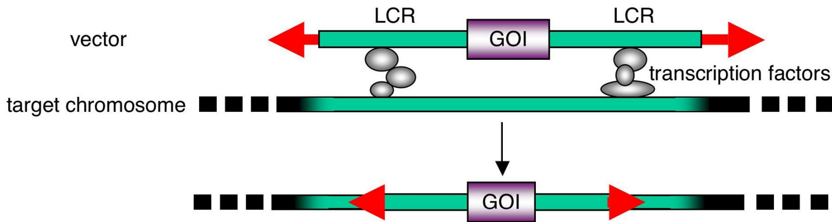


Fig. 1. Mechanisms of chromosomal integration. **(A)** Random integration. Most vector integration events are into random chromosomal sites. In this case, the majority of vector sequence (red bars and arrows) is integrated into naturally occurring chromosomal double strand breaks (DSBs) *via* the non-homologous end-joining (NHEJ) pathway. Gene expression tends to be subject to the position effects of integration sites, often leading to gene silencing. For therapeutic applications, insertional mutagenesis of nearby cancer-related genes have been a major concern. **(B)** Targeted integration via homologous recombination (HR). When a vector encodes sequences homologous to host chromosomes (blue bars), it might integrate into the homologues region via gene targeting, i.e., a DSB repair mechanism mediated by the homologous recombination pathway. In this case, vector end sequences are not co-integrated. **(C)** Targeted integration via chromatin tethering. When a vector encodes specific enhancer sequences such as locus control regions (LCR, green bar), transcription factors in a target cell are supposed to bridge the LCRs of the vector and target chromosome. As a result, when a DSB occurs in one of the DSB-proned LCR-HS regions, the vector appears to integrate via the NHEJ pathway, resulting in co-integration of vector end sequences. GOI: Gene of interest.

干细胞之家www.stemcell8.cn ←点击进入

reported (Tsukui *et al.*, 1996). Because chromosomal DNA double strand breaks (DSBs) are involved both in random integration and gene targeting, the above observations suggest that it might also be possible to utilize AdVs for gene targeting with host chromosomal DNA. Furthermore, adenoviral genome is transferred to and stays in the host nuclei as stable double-stranded linear episomal DNA (McConnell and Imperiale, 2004), and might thus be an ideal substrate for gene targeting.

2.2. *Gene Targeting with Replication-competent or E1-deleted AdVs*

Subsequently, AdVs have been used to target extrachromosomal and chromosomal loci in mammalian cells by HR, albeit with limited success. Infection of a semipermissive hemizygous CHO adenine phosphoribosyl transferase (APRT)-defective cell line containing a 3-bp deletion in the *Aprt* gene with a recombinant adenovirus containing the wild-type gene resulted in restoration of the APRT(+) phenotype at a frequency of 10^{-6}–10^{-5} per infected cell (Wang and Taylor, 1993). Six percent to 20% of the transductants appeared to result from a HR event. In another study, replication-defective E1-deleted AdVs were used for efficient delivery of donor neomycin-resistant gene for gene targeting in a mouse cell line harboring a target mutant neomycin-resistant gene on a nuclear papillomavirus shuttle vector. The structure of the neomycin-resistant recombinants was analyzed after recovery in *Escherichia coli*. All the recombinants had gone through precise correction of the gene, with HR frequencies of $1.9–8.3 \times 10^{-4}$ per cell (Fujita *et al.*, 1995). In both reports, however, either the vector (in CHO/Aprt system) or the target episomal DNA (in mouse cell line/papilloma system) replicates to high copy numbers in the corresponding target cells. Therefore, it was difficult to draw any conclusion of whether replication-incompetent AdVs could be used in HR with mammalian chromosomal DNA, which is critical for future application of the technology in gene repair therapy.

The ability of an E1, E3-defective AdV to act as a substrate for HR with chromosomal DNA was examined by including host chromosomal sequence from the mouse *Fgr* locus that also contained a neomycin-resistant gene expression cassette (Mitani *et al.*, 1995). After infection of mouse ES cells, stable integration was selected for drug resistance and the efficiency of HR was evaluated by Southern analysis. Twenty-five percent to 40% of the integration events resulted from HR, although the absolute efficiency of integration was low, with frequencies of 1.9×10^{-7}–1.2×10^{-5} per cell. These results suggested, for the first time, that it is possible to design an AdV to achieve a

干细胞之家www.stemcell8.cn ←点击进入

high gene targeting efficiency to a host chromosomal target. More recently, an E1-deleted AdV was evaluated for its ability to introduce a mutation by HR *in vivo* in the MutaMouse line, a transgenic mouse which carries 40 tandem copies of a bacteriophage λ shuttle vector comprising a wild-type bacterial *lacZ* transgene (Ino *et al.*, 2005). An 8-kb DNA corresponding to a single-point mutant *LacZ* transgene and surrounding bacteriophage λ DNA was inserted into a replication-defective AdV. This recombinant adenovirus was injected into the tail-vein of transgenic mice. Twenty-four hours later, genomic DNA was extracted from the liver and the λ shuttle was rescued by *in vitro* packaging in a GalE-defective *E. coli* mutant which supports the growth of only lacZ-defective phages when plated on a p-gal–containing agar. However, the frequency of lacZ-negative recombinant phages rescued from AdV-injected mice was the same as with control mice, and thus the frequency of AdV-mediated gene targeting in the mouse liver was less than 5×10^{-5} per copy of target gene.

These results suggest that, although replication-incompetent AdVs might be able to deliver a substrate for HR with mammalian cell chromosomal DNA, the frequencies of gene targeting per cell are low. Interestingly, however, the ratios of gene targeting to random integration were high in successful cases.

3. Helper-Dependent Adenoviral Vectors

3.1. *Development of Helper-dependent Adenoviral Vector System*

High-capacity HDAdVs were originally developed to overcome host immune responses against E1-deleted AdVs *in vivo* (Fig. 2; reviewed in: Palmer and Ng, 2005). Because all the viral genes are removed from the vector genome, the vector possesses additional advantages, such as decreased cytotoxicity and expanded cloning capacity, permitting insertion of larger segments of mammalian chromosomal DNA. These features of HDAdVs might be advantageous to obtain highly efficient site-specific integration into host chromosomes via HR. Another potential advantage of a HDAdV over an E1-deleted AdV is its ease to switch the fiber to that of other serotypes in order to change tropisms. Recently, it has been shown that, by choosing appropriate fiber serotypes, AdVs can infect almost any cell types efficiently (Havenga *et al.*, 2002). In the case of E1-deleted AdVs, vectors with different serotypes have to be propagated from each plaque isolate because the vectors themselves encode the fiber gene. On the other hand, in the case of HDAdVs, only one propagation with helper viruses with different

干细胞之家www.stemcell8.cn ←点击进入

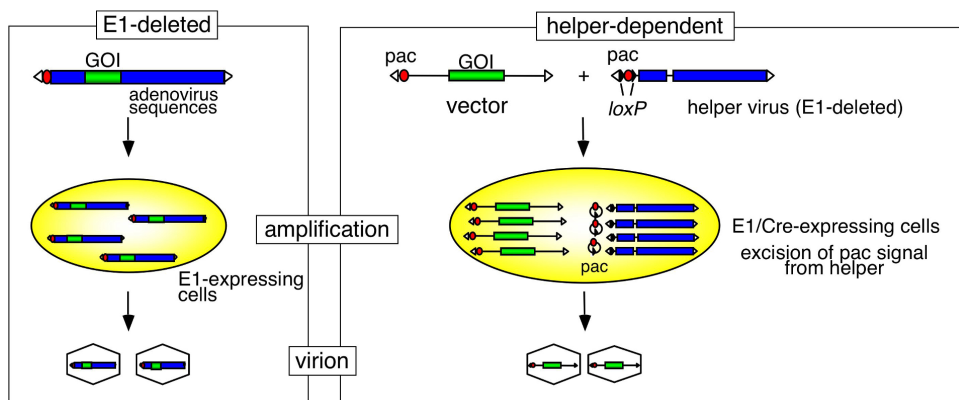


Fig. 2. Helper-dependent adenoviral vector system. Conventional E1-deleted AdVs are propagated in adenoviral E1-expressing cell lines, such as 293 cells. However, because other viral genes (blue bars) are retained on the vector genome, the cloning capacity is limited (~8.5 kb) and the vectors show cytotoxicity as well as strong immunogenicity. Helper-dependent adenoviral vectors encode only the inverted terminal repeats (white triangles) and the packaging signal (pac; red circles) as viral sequences. The vectors are propagated in the presence of E1-deleted helper virus, which supplies all the viral proteins required for propagation and packaging of the vector. The packaging signal sequence of the helper virus is sandwiched by *loxP* sites (black triangles). When the vector and helper viruses are propagated in cell lines which express both adenoviral E1 genes and P1 phage Cre genes, the helper packaging signal is excised and only the vector genome is selectively packaged into virions. Because all the viral coding sequences are deleted from the vector, the cloning capacity is large (~36 kb) and the vector shows reduced cytotoxicity and immunogenicity. GOI, Gene of interest.

fiber serotypes is necessary to switch serotypes, because the helper viruses, not the vectors, encode the fiber gene. A variety of helper AdVs with different fibers have recently become available (Parks *et al.*, 1999; Biermann *et al.*, 2001; Balamotis *et al.*, 2004; Bramson *et al.*, 2004; Shayakhmetov *et al.*, 2004).

At present, there have been three reports on HDAdV-mediated gene targeting in mammalian cells (see below). The results are summarized in Table 1.

3.2. *Attempts for Gene Targeting in Human Glioblastoma Cells*

Hillgenberg *et al.* characterized the integration pattern of a HDAdV with a neomycin-resistance gene inserted into a non-coding 27.4-kb genomic sequence derived from the human X chromosome after infection of a sex chromosome-aneuploid (X0) human glioblastoma cell line (Hillgenberg *et al.*, 2001). The integration frequency at an MOI of 1

干细胞之家www.stemcell8.cn ←点击进入

Table 1 Summary of HDAdv-mediated Gene Targeting/Tethering Experiments.

Mechanism	Target Cells	Random Integration*	Targeted Integration	Targeted to Random Ratio
HR	Human glioblastoma cell line[†]	1.7×10^{-4} (neo)	ND ($<8.5 \times 10^{-7}$)	<0.005
	Mouse ES cells[‡]	3.1×10^{-3} (neo)	2.2×10^{-3}	0.71
		5.4×10^{-2} (PCR)		0.04
Tethering	Human erythroleukemic cell line[§]	1.6×10^{-1} (GFP)	2.2×10^{-2} (HR ND)	0.14

The highest integration frequencies in each study are shown. Random and targeted integration frequencies per infected cell are indicated.

*Parenthesis indicate the method by which the frequencies of random integration were measured. It is of note that random integration frequencies measured by marker gene expression, either by neomycin-resistance or by green fluorescent protein (GFP) expression, might be underestimated because some integrated vectors are subject to gene silencing.

[†]Hillgenberg *et al.*, 2001.

[‡]Ohbayashi *et al.*, 2005.

[§]Wang *et al.*, 2005.

ND: not detected.

infectious particle/cell was 1.7×10^{-4} per infected cell and it occurred primarily by insertion of a monomer with no or little loss of sequences at the vector ends. However, out of 200 neomycin-resistant colonies analyzed, none was caused by HR (gene targeting efficiency per cell, thus, is calculated to be $<8.5 \times 10^{-7}$). Therefore, it was concluded that the potential of HDAdVs as tools to obtain efficient HR in mammalian cells is limited. Gene targeting in this human glioblastoma cell line study might have been inhibited by sequence mismatches resulting from naturally occurring polymorphisms between the vector-encoding sequences and the target chromosomal sequences. Indeed, mismatches between non-isogenic DNA have been shown to drastically inhibit gene targeting (Deng and Capecchi, 1992; Te Riele *et al.*, 1992). It is also possible that the success of HDAdV-mediated gene targeting is dependent on target cell types.

3.3. *Gene Targeting with HDAdVs in Mouse ES Cells*

The ability of HDAdVs to correct an insertional mutation at the endogenous *Hprt* (hypoxanthine phosphoribosyl transferase) locus was examined

干细胞之家www.stemcell8.cn ←点击进入

in male mouse ES cells (Ohbayashi *et al.*, 2005). The vectors contained a wild-type exon 3 sequence of the mouse *Hprt* gene with variable lengths of isogenic (ES cell line-derived) flanking intronic sequences ranging from 1.7 kb to 18.6 kb, either in E1-deleted or HDAdV backbone. HR between the vector and the mutated *Hprt* locus would restore the HPRT activity and make the cells HAT-resistant. In addition, because the β-geo gene was encoded in the HDAdVs, the frequency of random integration was examined by measuring the number of G418-resistant colonies. While no HR event was observed when E1-deleted AdV encoding a 1.7-kb homologous sequence to the *Hprt* locus or HDAdV with a 6.7-kb homology was used, the frequency obtained with a HDAdV with an 18.6-kb homology reached 0.22% per transduced cell at a surprisingly low MOI of 10 genomes per cell. Therefore, gene targeting with HDAdVs in mouse ES cells was highly efficient, and the high cloning capacity of HDAdVs was critical to achieve a successful HR. The HR frequency by electroporation of the parental plasmid DNA of the HDAdV with the 18.6 homology was 23-fold less than that by vector infection, indicating the significance of AdV-mediated DNA delivery for an efficient HR. Interestingly, in the case of AAV-mediated gene targeting, it was also reported that cell entry of the targeting cassette as a virus but not as a naked viral DNA was critical (Liu *et al.*, 2004). In a scaled-down experiment, it was also demonstrated that gene correction could be achieved relatively efficiently with a smaller number of cells by using AdVs. As for random integration, regardless of the size of homologous sequences, HDAdVs achieved similar frequencies of 10^{-4}–10^{-3} per cell, which were measured as the frequency of G418-resistant colonies. In contrast to the HR frequency, which showed an MOI-dependent increase, the random integration frequency reached a plateau at an MOI of 100. As a result, the ratio of HR to random integration was increased at an MOI of 1000 to over 50%, which would be a great advantage of using this system.

The random integration frequencies, measured as the frequency to produce neomycin-resistant colonies, might be underestimated, because integrated DNA is subject to transcriptional silencing. In order to directly detect both transcriptionally active and inactive integrated vectors at the DNA level, a PCR was carried out to amplify the vector sequences in each ES colony formed after infection. As a result, HDAdV DNA was detected at the surprisingly high frequency of 5.4% of colonies (i.e., one in every 20 infected cells). These results indicate that the random integration frequency of HDAdVs may be much higher than previously reported (Harui, *et al.*, 1999; Hillgenberg, *et al.*, 2001).

干细胞之家www.stemcell8.cn ←点击进入

3.4. *Targeted Integration Mediated by Chromatin Tethering*

Wang *et al.* reported a novel strategy to obtain site-specific chromosomal integration using HDAdVs (Wang *et al.*, 2005). They constructed a HDAdV carrying the β-globin locus control region (LCR, 22-kb; Li *et al.*, 2002) to drive green fluorescent protein (GFP) expression, whereby the LCR-GFP cassette is flanked by AAV inverted terminal repeats. This vector was rescued with a helper virus, which possesses the adenovirus type 35 fiber knob that allows efficient infection of human hematopoietic cells (Shayakhmetov, *et al.*, 2004). Initial studies on transduction and vector integration were performed in MO7e cells, a growth factor-dependent $CD34^+$ human erythroleukemic cell line. Instead of drug selection, single cell sorting of the infected cells was performed, followed by analysis of GFP expression, to determine the frequencies of vector integration, which was detected in 18.6% of the cells infected at an MOI of 300 genomes/cell. Interestingly, analysis of integration sites indicated that integration of the HDAdV in MO7e cells was not random but tethered to chromosome 11, specifically to the globin LCR: 11 out of 33 integrants were localized to chromosome 11 and 4 out of this 11 sites were within the globin LCR. Thus, more than 10% of analyzed integration sites were within the chromosomal β-globin LCR (targeted integration, Table 1). All the integrants retained vector sequences flanking the LCR sequences, indicating that site-specific integration was mediated by the NHEJ pathway but not by HR. It was speculated that both the LCR-carrying AdV and the genomic β-globin LCR were brought to discrete intranuclear foci by cell-type specific transcription factors ("transcription factories": Osborne *et al.*, 2004) and that the susceptibility of the LCR-specific DNA hypersensitive regions to DNA breaks allowed the vector DNA to integrate into the β-globin LCR [Fig. 1(C)] (Wang *et al.*, 2005). This very interesting speculation was supported by the different integration pattern of another HDAdV which contained X-chromosomal heterochromatin-derived stuffer DNA, instead of the β-globin LCR. In this case, out of 24 vector integration sites analyzed, none of them were in chromosome 11 and only one (4.1%) was in chromosome X.

Significantly, similar studies were performed with human cord blood $CD34^+$ cells, and the integration frequency at an MOI of 4000 was ~5%. Four integration sites were successfully mapped for HDAdV in GFP-positive burst-forming units — erythroid (BFU-E) colonies, among which two were on chromosome 11 and the others were on other chromosomes-suggesting that chromatin tethering-mediated targeting might also be obtained in human hematopoietic progenitors. Although the number

干细胞之家www.stemcell8.cn ←点击进入

of well-characterized LCRs is limited (Li, *et al.*, 2002), this site-specific integration strategy might be a very powerful approach to introduce therapeutic gene into safe and transcriptionally active regions. Importantly, this report presents the most successful targeted integration/correction in human primary hematopoietic progenitor cells to date.

3.5. *Integration Sites of HDAdVs*

Although the above reports suggest that HDAdV-mediated gene targeting might be an attractive strategy for safe and permanent gene therapy in inherited disorders, the frequencies of random integration are always higher than those of targeted integration, which might be unavoidable in mammalian cells. In two studies where both E1-deleted and HDAdVs were compared for random integration frequencies, HDAdVs showed even higher frequencies (Harui, *et al.*, 1999; Hillgenberg, *et al.*, 2001). This might be because adenoviral E4 genes, which inhibit double-strand break repair, are deleted from HDAdVs (Stracker *et al.*, 2002; Weitzman and Ornelles, 2005). Alternatively, because E1-deleted AdVs can replicate in some cell lines, the vector infection might have resulted in cytotoxicity to the infected cells. In any case, because frequencies of chromosomal integration are higher than 1% of the infected cells in some cases (Ohbayashi, *et al.*, 2005; Wang, *et al.*, 2005; Table 1), integration site preferences of HDAdVs could become an important issue. If random integration occurred at active gene loci at such a high frequency, it is more likely to induce cellular mutagenesis, as is the case with retroviral vectors (Hacein-Bey-Abina *et al.*, 2003; Baum, 2007). Although the results differ depending on the cell lines used, representative reports showed that 75–80%, 61%, 57% and 47% of integration sites were into genes when transductions were performed with human immunodeficiency virus, murine leukemia virus, avian sarcoma-leukosis virus vectors and human T-lymphotropic virus type I, respectively (Mitchell *et al.*, 2004; Derse *et al.*, 2007). In the case of AAV vectors, the results were quite different between two experimental systems: 53% in mouse hepatocytes *in vivo* and 39% in human fibroblasts *in vitro* (Miller *et al.*, 2005; Nakai *et al.*, 2005). The analysis of integration sites in mouse ES cells showed that HDAdVs have a tendency to integrate not in genes (5 of 18; 28%) but in intergenic regions, suggesting that HDAdVs integrate into random sites in host chromosomes, in contrast to retroviral vectors (Ohbayashi, *et al.*, 2005). In the human hematopoietic cell line, MO7e, it was reported that 10 out of 24 integrations (42%) of the HDAdV, which does not encode the β-globin LCR, were in genes (Wang, *et al.*, 2005).

干细胞之家www.stemcell8.cn ←点击进入

It was suggested that randomly integrated DNA viruses, such as AAV and hepadna virus, are located at sites of spontaneous DSB formation (Bill and Summers, 2004; Miller *et al.*, 2004). Thus, the modest preference for integration of AdVs and AAV vectors at transcribed sequences (Miller, *et al.*, 2004; Nakai, *et al.*, 2005; Ohbayashi, *et al.*, 2005; Wang, *et al.*, 2005) could be explained by a greater likelihood of DSB formation during transcription or improved access to DSBs in transcribed regions of each cell type. In addition, we recently characterized integration site preferences of linear plasmid DNA electroporated in mouse ES cells and found a modest preference in genes (K. Mitani and co-workers, unpublished data). Therefore, the use of DNA viral vectors for gene targeted chromosomal integration does not appear to add extra risks over the natural fate of exogenous DNA in terms of insertional mutagenesis of cancer-related genes and of induction of chromosomal abnormalities. Although more integration sites should be characterized, HDAdVs, therefore, might be a safe option compared to other viral vectors, even as an integrating vector.

4. Toward Endonuclease-boosted HDAdV Gene Targeting

Because the efficiency of HR mediated by HDAdVs is still in the order of 10^{-3} per cell, this strategy is currently applicable only to *ex vivo* gene therapy. Furthermore, because the frequency of random integration is higher than gene targeting, an appropriate strategy for negative selection, such as the one with the herpes simplex virus thymidine kinase gene, has to be utilized. As an attractive strategy to enhance HR-mediated gene repair at desired loci in mammalian cells, it was reported recently that zinc-finger proteins engineered to recognize a unique chromosomal site can be fused to a nuclease domain and used as a pair to generate active dimers upon biding on target DNA (Kim *et al.*, 1996). A double-strand break induced by the resulting zinc-finger nucleases (ZFNs) can create specific sequence alterations by stimulating HR between the target chromosome and an extrachromosomal DNA donor (Porteus and Baltimore, 2003; Bibikova *et al.*, 2003). It was shown that ZFNs designed against a SCID-X1 mutation in the IL2Rγ gene yielded more than 18% gene-modified human cells without selection (Urnov *et al.*, 2005). Remarkably, about 7% of the cells acquired the desired genetic modification on both X chromosomes, with cell genotype accurately reflected at the messenger RNA and protein levels. Comparably high frequencies (~5%) were observed in human T cells, raising the possibility to use strategies based on zinc-finger nucleases for the treatment of diseases. Indeed, by generating a DSB in the very target sequence of chromosomal DNA, ZFNs and

干细胞之家www.stemcell8.cn ←点击进入

emerging customized homing endonucleases are driving conventional gene targeting to efficiency levels compatible with clinical trials (Urnov *et al.*, 2005; Arnould *et al.*, 2007). As shown above, by its safety profile and gene targeting proficiency, HDAdV transduction outpasses conventional gene targeting mediated by DNA transfection. Therefore, incorporating emerging custom site-specific endonucleases into HDAdV-mediated gene targeting protocols is an obvious priority. With its ~36 kb space for transgenic sequences, HDAdVs can easily accomodate both the therapeutic DNA and the ancillary transgene(s) encoding the appropriate customized homing endonuclease or the set of paired ZFNs. Such an endonuclease-boosted HDAdV gene targeting strategy will, without a doubt, make gene correction therapy much more realistic. It is consistent with the endonuclease-boosted gene targeting arm that has been proposed as the main long-term gene therapy branch of a universal stem cell gene therapy platform (Bertolotti, 2004 and 2007). The current limitation is the availability of both types of custom site-specific endonucleases and intensive investigations are aimed at breakthrough engineering improvements that would ease the genesis of clinical grade ZFNs or customized homing endonucleases (e.g., Sepp and Choo, 2005; Arnould *et al.*, 2007; Volna *et al.*, 2007).

5. Conclusion

5.1. *Advantages of HDAdV-mediated Gene Targeting*

5.1.1. *HDAdV transduction or conventional gene targeting*

In summary, although it would be necessary to extend studies to several other loci in various cell types, it was shown that HDAdV offers a number of advantages over conventional gene targeting based on DNA transfection, thereby offering attractive potentialities for gene therapy of inherited diseases. In comparison to non-viral methods, under reasonable MOI conditions, replication-deficient viral vectors are less toxic and less damaging to target cells, because they enter cells through cell surface receptors. Obviously, one of the critical advantages of utilizing HDAdVs is their higher gene targeting efficiency as compared to conventional technology based on DNA transfection. In mouse ES cells, even at an MOI of 10 vector genomes per cell, the vector achieved gene targeting at frequency of 2.2×10^{-3} per infected cell, thereby outpassing conventional gene targeting efficiency (10^{-6}–10^{-7}; Vasquez *et al.*, 2002). Importantly enough, this advantage is a true property of HDAdVs as it is lost when one shifts from transduction to transfection of naked HDAdV DNA: in this case, gene targeting efficiency drops to the

干细胞之家www.stemcell8.cn ←点击进入

low level of conventional technology (Ohbayashi, *et al.*, 2005). The same holds true for gene targeting mediated by AAV (Liu *et al.*, 2004), indicating that viral genomic DNA, as an infectious form, might somehow attracts cellular DNA repair machinery. Interestingly enough, because gene transfer efficiency by viral vectors is not affected by the target cell number, it would also be easy to scale-down the transduction protocol. Considering difficulties in obtaining and growing a sufficient number of some rare stem cells, feasibility of manipulating chromosomes of a small number of cells is a big advantage of HDAdVs. Finally, unwanted random chromosomal integration by HDAdVs tends to be located at intergenic regions, thereby minimizing random-insertional oncogenic hazards.

5.1.2. *Viral gene targeting: HDAdV or AAV*

AAV vectors have been pioneered and are widely utilized for gene targeting in mammalian cells by Russell's group (Hendrie and Russell, 2005). The single-stranded nature of the viral genome appears to be the key to highly efficient gene targeting (Hendrie and Russell, 2005). The reported targeting frequencies were even higher than those with HDAdVs with almost 1% of the entire unselected fibroblast population undergoing targeting at the human *HPRT* locus under optimal conditions. HDAdV-mediated gene targeting might be still in its infancy, compared to AAV vectors. However, because AdVs and AAV vectors have distinct characteristics, they would be utilized in different applications. For example, currently available AAV vectors are less efficient in transducing certain types of stem cells, such as ES cells and hematopoietic stem cells, than AdVs (Smith-Arica *et al.*, 2003; Zhong *et al.*, 2004). In contrast, by choosing appropriate fiber serotypes, AdVs can infect almost any cell types efficiently (Havenga, *et al.*, 2002). The high capacity (~36 kb) of HDAdVs over a relatively small capacity of AAV (~5 kb) would also allow the delivery of a large DNA fragment for site-specific integration. On the other hand, because of its size, construction and propagation of HDAdVs is more laborious than that of AAV. To solve the technical issues, an improved helper system was developed recently (Palmer and Ng, 2003).

5.2. *Future Directions*

5.2.1. *From ex vivo to in vivo gene targeting*

An AAV vector has been used to correct dominant mutations in mesenchymal stem cells from patients with osteogenesis imperfecta (Chamberlain

干细胞之家www.stemcell8.cn ←点击进入

et al., 2004). Because AdVs have wider tropisms than AAVs, this disease as well as other monogenic disorders, especially SCID-X1, hereditary tyrosinemia type I and Fanconi anemia might be strong candidates for therapeutic HDAdV-mediated stem cell gene targeting. These diseases are ideal candidates, as corrected cells have a selectable advantage *in vivo* and therefore, after *ex vivo* transgenesis, a small starting pool of repopulating cells can be selectively amplified *in vivo* to a level consistent with a therapeutic effect (Overturf *et al.*, 1996; Battaile *et al.*, 1999; Cavazzana-Calvo *et al.*, 2000). Although hematopoietic cells are relatively refractory to AAV vectors, HDAdVs with a fiber from human group B adenoviruses can efficiently transduce human hematopoietic progenitors without affecting their clonogenic activities. More than 40% of $CD34^+$ cells were transduced and ~5% of BFU-Es, among them 50% being mixed colonies, were marker gene-positive without selection (Wang, *et al.*, 2005). These features of HDAdVs make them attractive candidates as vectors for gene repair therapy for safe and permanent correction of inherited hematopoietic diseases.

Interestingly enough, gene targeting *in vivo* in the livers of mice has recently been reported with AAV vectors (Miller *et al.*, 2006), although the frequencies were relatively low (<0.1% of hepatocytes). In another *in vivo* study, no correction of a mutant GFP gene by an AAV vector after injection of the tibialis muscle of transgenic mice was reported (Liu, *et al.*, 2004). Nonetheless, these reports strongly indicate that AAV vectors can be used to obtain HR in a variety of *in vivo* applications. Such an *in vivo* gene targeting technology perfectly fits into the broad transducing cell-specific repertoire of HDAdVs (see above) and to the low immunogenicity of these gutless AdVs (Fig. 2).

5.2.2. *Promises of endonuclease-boosted HDAdVs*

Pioneering experiments have already shown that, like for conventional gene targeting, creating a DSB in target chromosomal DNA produces a dramatic increase in AAV gene targeting efficiency (Miller *et al.*, 2003; Porteus *et al.*, 2003). AAV-mediated gene targeting frequency increased 60- to 100-fold and, under optimal conditions, was raised to the random-integration level, i.e., ~1–3% of transduced cells. Importantly enough, unlike under standard AAV gene targeting conditions (see above), these high gene targeting frequencies were obtained with relatively low MOIs. These data clearly show that endonuclease-boosted gene targeting is straightforwardly implementable to viral protocols and is therefore an emerging driving force for our HDAdV — mediated gene targeting strategy. Such an endonuclease-boosted HDAdV

干细胞之家www.stemcell8.cn ←点击进入

Table 2 Potential Applications of HDAdV-Mediated Gene Targeting.

1. Gene Therapy
   - *Ex vivo* gene correction of inherited diseases (especially hematopoietic diseases)
   - Therapy of dominantly inherited diseases
   - Gene correction *in vivo*?
2. Stem Cell Research/Regenerative Medicine
   - Establishment of cells to assay culture conditions by knocking-in of marker genes into differentiation-specific loci
   - Knock-out of immunogenic or tumorigenic genes for transplantation
   - Establishment of stem cell lines with disease-specific mutations

gene targeting technology is all the more timely as clinical grade ZFNs and customized homing endonucleases are now available (Urnov *et al.*, 2005; Arnould *et al.*, 2007).

Considering that a variety of embryonic and adult stem cells have been isolated and characterized, the development of a general strategy to obtain efficient gene targeting in various cell types would be of paramount importance for biological studies and clinical applications, such as gene therapy and regenerative medicine (Table 2). With the rapid progress in regenerative medicine, the necessity for developing an efficient tool for chromosomal manipulation of these cells has been increased. However, applications of HR in many cell types, including human ES cells, are still hampered by a lack of a suitable gene delivery method (Zwaka and Thomson, 2003). HDAdV-mediated gene targeting provides the means to manipulate a chromosome in a variety of target cells and, in conjunction with emerging custom site-specific endonucleases, we believe that this approach will be very successful both in the clinic and in basic genomic engineering.

References

Arnould S, Perez C, Cabaniols J-P, *et al*. (2007) Engineered I-CreI derivatives cleaving sequences from the human XPC gene can induce highly efficient gene correction in mammalian cells. *J Mol Biol* **371**: 49–65.

Balamotis MA, Huang K, Mitani K. (2004) Efficient delivery and stable gene expression in a hematopoietic cell line using a chimeric serotype 35 fiber pseudotyped helper-dependent adenoviral vector. *Virology* **324**: 229–37.

Battaile KP, Bateman RL, Mortimer D, *et al*. (1999) *In vivo* selection of wild-type hematopoietic stem cells in a murine model of Fanconi anemia. *Blood* **94**: 2151–58.

Baum C. (2007) Fourth case of leukaemia in the first SCID-X1 gene therapy trial, and the diversity of gene therapy. *http://www.esgct.org/upload/4th_CaseofLeukemial.pdf*

干细胞之家www.stemcell8.cn ←点击进入

Bertolotti R. (2004) Zinc finger nuclease-boosted gene targeting and synergistic transient regenerative gene therapy for long-term stem cell gene therapy. *Biogenic Amines* **18**: 503–38.

Bertolotti R. (2007) Autologous stem cell gene therapy: toward a universal platform for personalized therapy. *Gene Ther Regul* **3**: 1–14.

Bibikova M, Beumer K, Trautman JK, Carroll D. (2003) Enhancing gene targeting with designed zinc finger nucleases. *Science* **300**: 764.

Biermann V, Volpers C, Hussmann S, *et al*. (2001) Targeting of high-capacity adenoviral vectors. *Hum Gene Ther* **12**:1757–69.

Bill CA, Summers J. (2004) Genomic DNA double-strand breaks are targets for hepadnaviral DNA integration. *Proc Natl Acad Sci USA* **101**: 11135–40.

Bramson JL, Grinshtein N, Meulenbroek RA, *et al*. (2004) Helper-dependent adenoviral vectors containing modified fiber for improved transduction of developing and mature muscle cells. *Hum Gene Ther* **15**: 179–88.

Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, *et al*. (2000) Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science* **288**: 669–72.

Chamberlain JR, Schwarze U, Wang PR, *et al*. (2004) Gene targeting in stem cells from individuals with osteogenesis imperfecta. *Science* **303**: 1198–201.

Derse D, Crise B, Li Y, *et al*. (2007) Human T-cell leukemia virus type 1 integration target sites in the human genome: comparison with those of other retroviruses. *J Virol* **81**: 6731–41.

Fisher PB, Babiss LE, Weinstein IB, Ginsberg HS. (1982) Analysis of type 5 adenovirus transformation with a cloned rat embryo cell line (CREF). *Proc Natl Acad Sci USA* **79**: 3527–31.

Fujita A, Sakagami K, Kanegae Y, *et al*. (1995) Gene targeting with a replication-defective adenovirus vector. *J Virol* **69**: 6180–90.

Hacein-Bey-Abina S, Von Kalle C, Schmidt M, *et al*. (2003) LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* **302**: 415–19.

Harui A, Suzuki S, Kochanek S, Mitani K. (1999) Frequency and stability of chromosomal integration of adenovirus vectors. *J Virol* **73**: 6141–46.

Havenga MJ, Lemckert AA, Ophorst OJ, *et al*. (2002) Exploiting the natural diversity in adenovirus tropism for therapy and prevention of disease. *J Virol* **76**: 4612–20.

Hendrie PC, Russell DW. (2005) Gene targeting with viral vectors. *Mol Ther* **12**: 9–17.

Hillgenberg M, Tonnies H, Strauss M. (2001) Chromosomal integration pattern of a helper-dependent minimal adenovirus vector with a selectable marker inserted into a 27.4-kilobase genomic stuffer. *J Virol* **75**: 9896–908.

Hosel M, Webb D, Schroer J, Doerfler W. (2003) The abortive infection of Syrian hamster cells with human adenovirus type 12. *Curr Top Microbiol Immunol* **272**: 415–40.

干细胞之家www.stemcell8.cn ←点击进入

Ino A, Naito Y, Mizuguchi H, *et al*. (2005) A trial of somatic gene targeting *in vivo* with an adenovirus vector. *Genet Vaccines Ther* **3**: 8.

Kay MA, Glorioso JC, Naldini L. (2001) Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics. *Nat Med* **7**: 33–40.

Kim YG, Cha J, Chandrasegaran S. (1996) Hybrid restriction enzymes: zinc finger fusions to Fok I cleavage domain. *Proc Natl Acad Sci USA* **93**: 1156–60.

Li Q, Peterson KR, Fang X, Stamatoyannopoulos G. (2002) Locus control regions. *Blood* **100**: 3077–86.

Liu X, Yan Z, Luo M, *et al*. (2004) Targeted correction of single-base-pair mutations with adeno-associated virus vectors under nonselective conditions. *J Virol* **78**: 4165–75.

McConnell MJ, Imperiale MJ. (2004) Biology of adenovirus and its use as a vector for gene therapy. *Hum Gene Ther* **15**: 1022–33.

Miller DG, Petek LM, Russell DW. (2003) Human gene targeting by adeno-associated virus vectors is enhanced by DNA double-strand breaks. *Mol Cell Biol* **23**: 3550–57.

Miller DG, Petek LM, Russell DW. (2004) Adeno-associated virus vectors integrate at chromosome breakage sites. *Nat Genet* **36**: 767–73.

Miller DG, Trobridge GD, Petek LM, *et al*. (2005) Large-scale analysis of adeno-associated virus vector integration sites in normal human cells. *J Virol* **79**: 11434–42.

Miller DG, Wang PR, Petek LM, *et al*. (2006) Gene targeting *in vivo* by adeno-associated virus vectors. *Nat Biotechnol* **24**: 1022–26.

Mitani K, Wakamiya M, Hasty P, *et al*. (1995) Gene targeting in mouse embryonic stem cells with an adenoviral vector. *Somat Cell Mol Genet* **21**: 221–31.

Mitchell RS, Beitzel BF, Schroder AR, *et al*. (2004) Retroviral DNA integration: ASLV, HIV, and MLV show distinct target site preferences. *PLoS Biol* **2**: E234.

Nakai H, Wu X, Fuess S, *et al*. (2005) Large-scale molecular characterization of adeno-associated virus vector integration in mouse liver. *J Virol* **79**: 3606–14.

Ohbayashi F, Balamotis MA, Kishimoto A, *et al*. (2005) Correction of chromosomal mutation and random integration in embryonic stem cells with helper-dependent adenoviral vectors. *Proc Natl Acad Sci USA* **102**: 13628–33.

Overturf K, Al-Dhalimy M, Tanguay R, *et al*. (1996) Hepatocytes corrected by gene therapy are selected *in vivo* in a murine model of hereditary tyrosinaemia type I. *Nat Genet* **12**: 266–73.

Palmer D, Ng P. (2003) Improved system for helper-dependent adenoviral vector production. *Mol Ther* **8**: 846–52.

Palmer DJ, Ng P. (2005) Helper-dependent adenoviral vectors for gene therapy. *Hum Gene Ther* **16**: 1–16.

Parks R, Evelegh C, Graham F. (1999) Use of helper-dependent adenoviral vectors of alternative serotypes permits repeat vector administration. *Gene Ther* **6**: 1565–73.

干细胞之家www.stemcell8.cn ←点击进入

Porteus MH, Cathomen T, Weitzman MD, Baltimore D. (2003) Efficient gene targeting mediated by adeno-associated virus and DNA double-strand breaks. *Mol Cell Biol* **23**: 3558–65.

Sepp A, Choo Y. (2005) Cell-free selection of zinc finger DNA-binding proteins using *in vitro* compartmentalization. *J Mol Biol* **354**: 212–19.

Shayakhmetov DM, Li ZY, Gaggar A, *et al*. (2004) Genome size and structure determine efficiency of postinternalization steps and gene transfer of capsid-modified adenovirus vectors in a cell-type-specific manner. *J Virol* **78**: 10009–22.

Smith-Arica JR, Thomson AJ, Ansell R, *et al*. (2003) Infection efficiency of human and mouse embryonic stem cells using adenoviral and adeno-associated viral vectors. *Cloning Stem Cells* **5**: 51–62.

Stracker TH, Carson CT, Weitzman MD. (2002) Adenovirus oncoproteins inactivate the Mre11-Rad50-NBS1 DNA repair complex. *Nature* **418**: 348–52.

Tsukui T, Kanegae Y, Saito I, Toyoda Y. (1996) Transgenesis by adenovirus-mediated gene transfer into mouse zona-free eggs. *Nat Biotechnol* **14**: 982–85.

Urnov FD, Miller JC, Lee YL, *et al*. (2005) Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature* **435**: 646–51.

van Doren K, Hanahan D, Gluzman Y. (1984) Infection of eucaryotic cells by helper-independent recombinant adenoviruses: early region 1 is not obligatory for integration of viral DNA. *J Virol* **50**: 606–14.

Vasquez KM, Marburger K, Intody Z, Wilson JH. (2001) Manipulating the mammalian genome by homologous recombination. *Proc Natl Acad Sci USA* **98**: 8403–10.

Verma IM, Weitzman MD. (2005) Gene therapy: twenty-first century medicine. *Ann Rev Biochem* **74**: 711–38.

Volna P, Jarjour J, Baxter S, *et al*. (2007) Flow cytometric analysis of DNA binding and cleavage by cell surface-displayed homing endonucleases. *Nucl Acids Res* **35**: 2748–58.

Wang H, Shayakhmetov DM, Leege T, *et al*. (2005) A capsid-modified helper-dependent adenovirus vector containing the beta-globin locus control region displays a nonrandom integration pattern and allows stable, erythroid-specific gene expression. *J Virol* **79**: 10999–11013.

Wang Q, Taylor MW. (1993) Correction of a deletion mutant by gene targeting with an adenovirus vector. *Mol Cell Biol* **13**: 918–27.

Weitzman MD, Ornelles DA. (2005) Inactivating intracellular antiviral responses during adenovirus infection. *Oncogene* **24**: 7686–96.

Zhong L, Li W, Yang Z, *et al*. (2004) Impaired nuclear transport and uncoating limit recombinant adeno-associated virus 2 vector-mediated transduction of primary murine hematopoietic cells. *Hum Gene Ther* **15**: 1207–18.

Zwaka TP, Thomson JA (2003) Homologous recombination in human embryonic stem cells. *Nat Biotechnol* **21**: 319–21.

干细胞之家www.stemcell8.cn ←点击进入

Chapter 8

Multipotent Progenitor Cells: A New Era In Stem Cell-Mediated Gene Therapy?

Marta Serafini[†] and Catherine M. Verfaillie[*,‡]

Over the last decade, a number of studies have provided evidence of the existence of stem cells with pluripotency properties either in fresh bone marrow or following *in vitro* culture. Numerous groups have isolated non-hematopoietic cell populations from bone marrow, umbilical cord blood, amniotic fluid or fetal tissue via *in vitro* culture, which possess some molecular and biological properties comparable to embryonic stem cells. Due to their differentiation capacity into cells with features of the three germ layers, they are a novel cellular source for tissue regeneration. Because such stem cells of greater potency can be expanded for prolonged periods *ex vivo* without evidence of senescence and can be easily genetically manipulated, they can also be considered good candidates for gene therapy approaches.

Keywords: Stem cells; multipotency; plasticity; gene therapy.

1. Introduction

In recent years, the interest of the scientific world has been focussed on the biology of stem cells, the promise of stem cell technology and their future clinical applications in regenerative medicine. Studies reported by several scientists have led to the identification of various categories of stem cells, defined by their capacity for self-renewal and their differentiation potential.

[*]Corresponding author. catherine.verfaillie@med.kuleuven.be
[†]Centro Ricerca M. Tettamanti, Clinica Pediatrica Università Milano-Bicocca, Ospedale San Gerardo, Monza, Italy
[‡]Stamcelinstituut Leuven, Katholieke Universiteit Leuven, Leuven, Belgium

干细胞之家www.stemcell8.cn ←点击进入

These unique properties have defined stem cells as ideal targets for the permanent correction of genetic defects, considering that multiple cell lineages derived from stem cells can be genetically corrected. This notion is currently being tested. For instance, hematopoietic stem cells(HSCs), the best characterized somatic stem cells have, since several decades, been used for transplantation in patients with a variety of acquired and congenital diseases. In a number of trials, genetically modified HSCs have been used with variable levels of success and sometimes with unexpected toxicity (Bordignon and Roncarolo, 2002; Hacein-Bey-Abina *et al*., 2003a and 2003b; Kohn *et al*., 2003; Dave *et al*., 2004). With the discovery of multipotent stem cells in different tissues, and even cells that may differentiate across tissue boundaries, alternative cell sources for cell and gene therapy are emerging. For instance, numerous groups have isolated nonhematopoietic cell populations from bone marrow (BM), umbilical cord blood or liver via *in vitro* culture, which can differentiate into cells with mesodermal, endodermal, and ectodermal characteristics (Jiang *et al*., 2002a and 2002b; Yoon *et al*., 2005; D'Ippolito *et al*., 2004; Anjos-Afonso and Bonnet, 2006; Kucia *et al*., 2006; Kogler *et al*., 2004; Kues *et al*., 2005; De Coppi *et al*., 2007). Because such more potent stem cells can be expanded for prolonged periods *ex vivo* without evidence of senescence and can be easily genetically manipulated, they can be considered as a cell source for cell and gene therapy.

2. Methods Used for Genetic Modification

The ideal form of gene therapy, however, would be the correction of the mutant gene, without causing changes in other genes (Bertolotti, 1996). Such a strategy would overcome the difficulties encountered with gene therapy using plasmid transfection or viral vector-based approaches, such as loss of expression of the randomly inserted gene due to lack of appropriate gene regulation, loss of gene expression, mutagenesis resulting from random integration of the correcting gene into the host genome, and viral vector-mediated immunogenicity (Nienhuis *et al*., 2006; Cornetta *et al*., 1991; Dave *et al*., 2004; Noguchi *et al*., 2003; Berns, 2004; Naldini *et al*., 2006). Correction of a mutant gene can be achieved by targeting a wild type sequence to precisely recombine with its homologous mutant gene, thereby correcting it (Bishop and Schiestl, 2000). This strategy is advantageous as random insertion of the targeting DNA into the genome is infrequent in cells that have undergone homologous recombination. However, such efforts have been limited so far due to the low frequency of homologous recombination in cells and

干细胞之家www.stemcell8.cn ←点击进入

the feasibility of using the corrected cells clinically (Yanez and Porter, 1998; Urnov *et al.*, 2005).

For this reason other means of gene insertion have been developed, wherein genes are inserted randomly into the genome. Although this can be achieved by plasmid transfection, the efficiency of stable integration of the gene in the host cell genome using either lipofection or electroporation methods is too low (in the order of 1/1000–10,000 cells) to allow efficient gene transfer (Nishikawa and Huang, 2001; Lechardeur and Lukacs, 2002). Therefore, most preclinical and clinical studies have exploited gene transfer using viral vectors, such as adeno-associated virus (AAV) as well as retroviral vectors, as these allow stable integration in the host cell genome (Kay *et al.*, 2001; Richter and Karlsson, 2001). Most *ex vivo* stem cell gene therapy approaches exploit retroviruses (Kurre and Kiem, 2000; e.g., Cavazzana-Calvo *et al.*, 2000; Aiuti *et al.*, 2002; Aiuti, 2004).

Indeed, integration of proviral DNA into the host cell genome is an essential step in the life cycle of retroviruses. The retroviral RNA genome is reverse transcribed, and the proviral DNA is then inserted into the cellular chromosomal DNA via the viral encoded integrase, and the provirus is stably transmitted to all progeny of transduced cells as an integral element of the host genome (Kay *et al.*, 2001; Pages and Bru, 2004; Coffin, 1997). This feature of retroviruses underlies the usefulness of retroviruses as vectors for gene therapy. Both murine leukemia virus (MLV)-derived and human immunodeficiency virus (HIV)-derived vectors are being evaluated (Suzuki and Craigie, 2007). Two major differences exist between the two vector systems. Although retroviruses can enter the cytoplasm of non-dividing cells, they are not able to cross the nuclear membrane. During mitosis, the breakdown of this membrane allows the entry of viral retrotranscribed DNA into the nucleus (Roe *et al.*, 1993). The integration phase of lentiviruses differs from that of MLV because the preintegration complex is actively transported through the nucleopore upon recognition of the nuclear localization signals (NLS) on matrix protein, vpr and integrase by the nuclear import machinery (Zennou *et al.*, 2000; Stevenson, 2000; Lewis and Emerman, 1994). The ability to infect non-dividing cells has encouraged the creation of lentivirus-based vectors for use in clinical gene transfer studies (Thomas *et al.*, 2003; Yamashita and Emerman, 2006; Naldini *et al.*, 1996; Woods *et al.*, 2000; Salmon *et al.*, 2000; Ailles *et al.*, 2002). Hence, many important gene therapy targets such as hematopoietic stem cells, neurons and others should be susceptible to lentiviral-mediated gene transfer, without requiring *ex vivo* strategies to induce the cycling required for MLV transduction. On the other hand,

干细胞之家www.stemcell8.cn ←点击进入

the greater complexity of lentiviruses with respect to other retroviruses and the concern of their potential recombination with pathogenic viruses such as HIV-1 render the development of vectors and packaging cell lines more complicated. Secondly the insertion locations of the two vector systems differ significantly. Insertion is not random, with, for instance, MLV-based vectors inserting preferentially in the vicinity of transcription start sequences, and HIV vectors in gene rich regions of the host DNA, both of which may lead to unexpected toxicity, as will be discussed for MLV-based vectors below.

3. Hematopoietic Stem Cells in Gene Therapy Approaches

The objective of gene therapy is to deliver a therapeutic gene into cells with, in general, the goal of incorporating it into host chromosomal DNA in order to achieve stable expression. The ideal cell target is therefore a stem cell. A stem cell is an undifferentiated cell capable of: 1) long term self-renewal, or the ability to generate at least one identical copy of the original cell; 2) differentiation at the single cell level into multiple, and in some instance, only one, specialized cell type; and 3) *in vivo* functional regeneration of tissues (Verfaillie, 2002). Over the last decade, studies by many scientists have led to the identification of various categories of stem cells, defined by their capacity for self-renewal and their differentiation potential, ranging from totipotent (ability to create any type of cells for embryonic and extraembryonic development), through pluripotent (embryonic stem cells, able to give rise to all cell types of the embryo), to multi-, oligo- and unipotent adult stem cells, defined by their ability to contribute to multiple, few or only one mature cell type (Wagers and Weissman, 2004). Recent reports have shown that adult, multipotent stem cells are present in several, if not most, adult tissues, and are not confined to tissues where rapid cell turnover requires an active stem cell and progenitor cell population such as the hematopoietic tissue (Weissman *et al.*, 2001), skin and epithelial mucosa (Toma *et al.*, 2001; Toma *et al.*, 2005; Slack, 2000), but also in the central nervous system (CNS), where cell turnover is significantly lower (McKay, 1997; Gage, 2000).

HSCs have been considered a good target for gene therapy, as there is a longstanding expertise in transplantation of HSCs. The drawbacks are that HSCs cannot be readily cultured *ex vivo*. For this reason, over the past two decades, several protocols have been developed to obtain the best biological setting to culture HSCs (Bordignon, 2006; Ferguson *et al.*, 2005). Theoretically, this procedure is relatively easy, and consists of HSCs enrichment step, wherein human cells expressing the CD34 cell surface antigen are selected, followed by culture of the selected cells in the presence of

干细胞之家www.stemcell8.cn ←点击进入

cytokines to enhance susceptibility to gene transfer and then transplantation (Larochelle and Dunbar, 2004; Passegué *et al.*, 2005). Unfortunately, *ex vivo* culture of human HSCs, even in the presence of cytokines thought to support self-renewal and preservation of HSCs quality, such as FLT3 ligand and stem cell factor (SCF), along with either thrombopoietin or IL-3 and IL-6, is still associated with loss of HSCs quality (Ferguson *et al.*, 2005). Even when HSCs are cultured on extracellular matrix components, such as fibronectin which may prevent differentiation of HSCs, engraftment remains low, due to both commitment of HSCs to more mature daughter cells and difficulties of proliferating HSCs to home to the BM niche. Therefore, homologous recombination as well as plasmid transfection of means to genetically correct/alter HSCs is precluded. More recent approaches are starting to evaluate additional factors that may allow self-renewal of HSCs without loss of engraftment ability, including activation of the Notch signalling pathway (Androutsellis-Theotokis *et al.*, 2006); use of morphogens also active during HSCs development, including bone morphogenetic protein (BMP)-4 (Hutton *et al.*, 2006; Larsson and Karlsson, 2005); sonic or Indian hedgehog; or short-term overexpression of certain key regulators of self-renewal, such as the homeobox transcription factor, Hox-B4 (Antonchuk *et al.*, 2002; Krosl *et al.*, 2003; Zhang *et al.*, 2006). However, these newer approaches are still in an experimental phase.

Therefore, preclinical and clinical studies of HSCs gene therapy have exploited retroviral gene transfer. As discussed above, MLV-based vectors require that cell division occurs for the proviral DNA to be inserted into the host chromosomal DNA. Again, the need for cell division for gene insertion leads to loss of engraftable HSCs. Therefore HIV-based vectors may be the preferred means for gene transfer in HSCs. The first clinical success of HSCs-mediated gene therapy has been obtained in the late 1990s by the group led by Alain Fisher, showing the therapeutic potential of this approach (Cavazzana-Calvo *et al.*, 2000). In this trial, 11 children affected by X-linked SCID with no HLA identical sibling received autologous bone marrow $CD34^+$ cells transduced with a classic MLV vector expressing a corrective γ-chain subunit gene. Although functional T-cell immune reconstitution occurred in 9/11 treated patients, four patients developed T-cell leukaemia (Hacein-Bey-Abina *et al.*, 2003a and 2003b; Baum, 2007). Molecular studies of the leukemic cells revealed that the retroviral vector used to deliver the γ-chain gene has integrated itself near the proto-oncogene LMO-2, a transcription factor associated with human T-cell leukemia, with resulting activation of the oncogene (Kohn *et al.*, 2003; Pike-Overzet *et al.*, 2007). The risk

干细胞之家www.stemcell8.cn ←点击进入

of insertional mutagenesis has been subsequently confirmed by studies in mice and primates (Kiem *et al.*, 2004; Hematti *et al.*, 2004; Calmels *et al.*, 2005; Kustikova *et al.*, 2005; Beard *et al.*, 2007; Bushman *et al.*, 2005).

4. Adult Stem Cells with Greater Potency

Over the last five years, a variety of studies have identified novel populations of adult stem cells, that in contrast to classical somatic stem cells such as HSCs, have in common that they can be expanded *ex vivo* without signs of senescence, and may be capable of generating cell types other than the apparent tissue of origin. This phenomenon is referred to as adult stem cell plasticity or transdifferentiation (Wagers and Weissman, 2004; Theise, 2005; Serafini and Verfaillie, 2006). Under certain tissue-specific conditions, adult stem cells can acquire the fate of cell types different from the tissue of origin, even crossing germ layers. Such apparent lineage switch can be induced by adding certain growth factors to the medium when stem cells are cultured *in vitro* or by transplanting them into a different organ of the body. This concept has led to speculations that we may one day be able to use these new found capabilities clinically. To truly prove that plasticity exists, one needs to demonstrate that a single stem cell can self-renew, can give rise to the functional cell types from the tissue where the stem cell originated plus cell types of a non-related tissue, and can repopulate both tissues robustly *in vivo*.

Many of the studies that have identified cells with apparent greater potency than classical somatic stem cells have used BM as the starting material. Different populations of stem cells can be isolated from fresh BM or after culturing BM mononuclear cells for several weeks. Fresh BM is composed of a non-adherent hematopoietic and adherent stromal cell compartment, and contains multiple stem/progenitor cell populations, including HSCs, mesenchymal stem cells or marrow stromal cells (MSCs) and endothelial precursors cells (EPC).

MSCs were initially named fibroblast colony forming cells (CFU-Fs), because they are rare adherent cells that can generate distinct colonies of cells with fibroblast morphology when cultured with serum on plastic (Friedenstein *et al.*, 1976). These CFU-Fs are able to give rise to osteoblasts, chondroblasts and adipocytes (Prockop, 1997). In the late 1990s, prospective isolation methods were developed first for MSCs from bone marrow, and subsequently MSCs have also been isolated from a variety of other tissues, including umbilical cord blood (Lee *et al.*, 2004), placental tissue (In't Anker *et al.*, 2004; Yen *et al.*, 2005), fetal tissue (Campagnoli *et al.*, 2001 and 2000),

干细胞之家www.stemcell8.cn ←点击进入

adipose tissue (Zuk *et al.*, 2002), exfoliated deciduous teeth (Miura *et al.*, 2003) and amniotic fluid (In't Anker *et al.*, 2003). MSCs can be expanded for 20–40 doublings in culture and differentiate into lineages of mesenchymal tissues, including bone, cartilage, muscle, tendon, fat and marrow stroma (Prockop, 1997; Oreffo *et al.*, 2005; Rosen and MacDougald, 2006). When introduced *in vivo*, MSCs differentiate into the same array of cell types.

Over the last ten years, a number of studies have demonstrated that it is possible to purify more potent adherent stem cell populations that generate cells with characteristics of mesodermal, endodermal and ectodermal lineage, through *in vitro* culture of skin, bone marrow, muscle, umbilical cord blood, human fetal liver and other fetal tissues. These *in vitro* isolated stem cells have been named MAPC (Jiang *et al.*, 2002a and 2002b), hBMSC (Yoon *et al.*, 2005), USSC (Kogler *et al.*, 2004), FSSC (Kues *et al.*, 2005), AFS (De Coppi *et al.*, 2007), MIAMI cells (D'Ippolito *et al.*, 2004), hFLMPC (Dan *et al.*, 2006), and MASC (Beltrami *et al.*, 2007). Many of them are reported to express the embryonic stem cells (ESC) specific transcription factor Oct4 (and Nanog); they lack HSC cell surface marker expression and express a variable complement of cell surface antigens found on MSC. Despite expression of Oct4 and for some of the cells, Nanog, transcription factors known to be causally involved in the pluripotency of ESC, these more potent adult stem cell lack other typical ESC pluripotency characteristics such as formation of embryoid bodies (EBs) and teratomas. It is still unknown if MAPC, hBMSC, USSC, FSSC, AFS, MIAMI cells, hFLMPC, and MASC are a culture artefact or if they exist *in vivo*. However, Kucia *et al.* recently demonstrated that cells, termed very-small embryonic-like cells (VSEL), expressing the transcription factor Oct4 as well as the SSEA1/SSEA4 antigen on the cell membrane, can be isolated from unmanipulated murine bone marrow (Kucia *et al.*, 2006) and from human cord blood (Kucia *et al.*, 2007), suggesting that pluripotent stem cells similar to those isolated by cultivating BM may exist as such *in vivo*. Another recent report by Anjos-Afonso *et al.* has shown that cells expressing the ESC marker SSEA1 can be isolated from mesenchymal stem cell cultures after one passage *in vitro* (Anjos-Afonso and Bonnet, 2007), which they termed pre-MSCs. Pre-MSCs express high levels of Oct-4 as well as Nanog, and can differentiate into cells of the three lineages *in vitro*.

5. What are Multipotent Adult Progenitor Cells

In 2001 and 2002, our group described a new population of stem cells, named multipotent adult progenitor cells or MAPC, isolated through culture from adult BM of humans, mouse and rat and more recently from mouse muscle

干细胞之家www.stemcell8.cn ←点击进入

and brain and from swine BM. In the most updated protocol, MAPC isolation is performed under hypoxic conditions, on fibronectin coated plates, in the presence of 2% fetal calf serum and a cocktail of reagents, such as EGF, PDGF and LIF (Breyer *et al.*, 2006). The phenotype of mouse MAPC is B220, CD3, CD15, CD31, CD34, CD44, CD45, CD105, Thy1.1, Sca-1, E-cadherin, MHC class I and II, negative, EpCAM^{low} and c-Kit, VLA-6 and CD9 positive. For rat MAPC, the phenotype is CD44, CD45, MHC class I and II negative, but CD31 positive. It remains still unknown whether MAPCs exist *in vivo* or are created in culture as isolation occurs by culturing the different tissues *in vitro*, subcloning, and identification of cells with phenotypic and functional characteristics of MAPC. Hence, it is possible that the isolation of MAPCs is the result of de-differentiation of an existing classical adult stem cell to a cell with greater potency. MAPCs resemble ESCs in their self-renewal ability without obvious senescence, in their capacity to give rise to cells of mesoderm, endoderm and ectoderm *in vitro* and in the expression of ESC-specific transcription factors (Jiang *et al.*, 2002a; Breyer *et al.*, 2006). Mouse, rat, human and swine MAPCs can differentiate *in vitro* into typical mesenchymal lineage cells, including osteoblasts, chondroblasts, adipocytes and skeletal myoblasts (Jiang *et al.*, 2002a and 2002b; Zeng *et al.*, 2006; Reyes *et al.*, 2001). In addition, MAPCs can be induced to differentiate into cells with morphological, phenotypic and functional characteristics of endothelial cells, smooth muscle cells, neuroectodermal cells and hepatocytes *in vitro* (Reyes *et al.*, 2002; Aranguren *et al.*, 2006; Jiang *et al.*, 2002a; Ross *et al.*, 2006; Schwartz *et al.*, 2002). The evidence that MAPCs can contribute to tissues *in vivo* when grafted in postnatal animals comes from studies wherein MAPCs predifferentiated into endothelial cells were grafted. Reyes *et al.* reported that MAPCs could generate cells that express endothelial markers *in vitro* and contribute to neoangiogenesis *in vivo* during tumor angiogenesis (Reyes *et al.*, 2002). Moreover, Aranguren *et al.* provided evidence of the ability of human MAPC, in contrast to human AC133, to differentiate into functional arterial and venous endothelium *in vitro* and *in vivo* (Aranguren *et al.*, 2006). In a recent study by Serafini *et al.*, the initial evidence by Jiang *et al.* (Jiang *et al.*, 2002a) that MAPCs might differentiate into hematopoietic cells when grafted in sublethally irradiated animals was further confirmed (Serafini *et al.*, 2007). Tolar *et al.* reported that engraftment of MAPC that are MHC class-I negative, is inhibited by NK activity (Tolar *et al.*, 2006). Considering these findings, MAPCs were grafted in sublethally irradiated NOD-SCID mice treated with anti-NK antibody for the first three weeks. In 75% of transplanted animals, MAPCs functionally

干细胞之家www.stemcell8.cn ←点击进入

reconstituted the hematopoietic system *in vivo*, without evidence of fusion (Serafini *et al.*, 2007). High contribution from donor-derived cells could be detected in the spleen, BM, peripheral blood and lymph nodes of recipient mice. In all these tissues, the collected cells expressed appropriate B, T, and myeloid cell surface markers. Moreover, MAPC-derived progenitors were capable of migrating to the thymus, where they underwent typical differentiation processes. MAPC alone were not radioprotective, despite the fact that the hematopoietic progeny they generated *in vivo* is radioprotective. In fact, donor-derived KLS (c-Kit$^+$ Lin$^-$ Sca-1$^+$) cells purified from BM of primary recipients and transplanted into lethally irradiated secondary C57Bl/6 mice, once again led to a long-term reconstitution of the myeloid and lymphoid lineages. This data demonstrates that MAPC populations contribute to hematopoiesis *in vivo* and may be a precursors of HSCs in ontogeny, given that they generate long-term repopulating HSCs and the full complement of hematopoietic progenitors. As indicated earlier, similar *in vivo* results have been seen for the pre-MSC described by Anjos-Afonso and Bonnet (Anjos-Afonso and Bonnet, 2007). They, too, found contribution of pre-MSC to endothelium and hematopoiesis, as well as to adipose, bone and cartilage compartments of the bone following intrafemoral injection of the cells. Hence, like MAPC, pre-MSCs appear to not only be the precursor for BM derived MSCs but also HSCs.

6. Are Adult Stem Cells with Greater Potential Good Candidates for Cellular Gene Therapy?

Why are MAPC, hBMSC, USSC, FSSC, AFS, MIAMI cells, hFLMPC, MASC or pre-MSC possibly superior candidates for cellular gene therapy compared with classical somatic stem cells? As discussed in the beginning of the chapter, limitations to current cellular gene therapy include the inability to culture somatic stem cells without loss of *in vivo* engraftment ability. This precludes plasmid-based genetic modification, and to a lesser extent, viral vector-based gene transfer. It is obvious that MAPC, hBMSC, USSC, FSSC, AFS, MIAMI cells, hFLMPC, MASC or pre-MSC can be cultured for many cell doublings (ranging from $> 40 - > 100$ for human cell populations) without loss of differentiation ability *in vitro*. This should allow plasmid-based gene transfer approaches.

In fact, MAPC can be genetically modified using nucleoporation, without loss of differentiation ability (Lakshmipathy *et al.*, 2004; Table 1). The ability to expand MAPC, hBMSC, USSC, FSSC, AFS, MIAMI cells, hFLMPC, MASC or pre-MSC may also allow for homologous recombination (HR)

干细胞之家www.stemcell8.cn ←点击进入

Table 1 Efficiency of Gene Delivery using Viral and Non-viral Vectors

Category	HSCs	More Pluripotent Adult Stem Cells
RV	moderate	high
LV	high	high
Nucleoporation	low	high
Electroporation	low	high
Lipofection	low	high
HR	low	possible?

Abbreviations: RV: retroviral vectors; LV: lentiviral vectors; HSCs: hematopoietic stem cells; HR: homologous recombination.

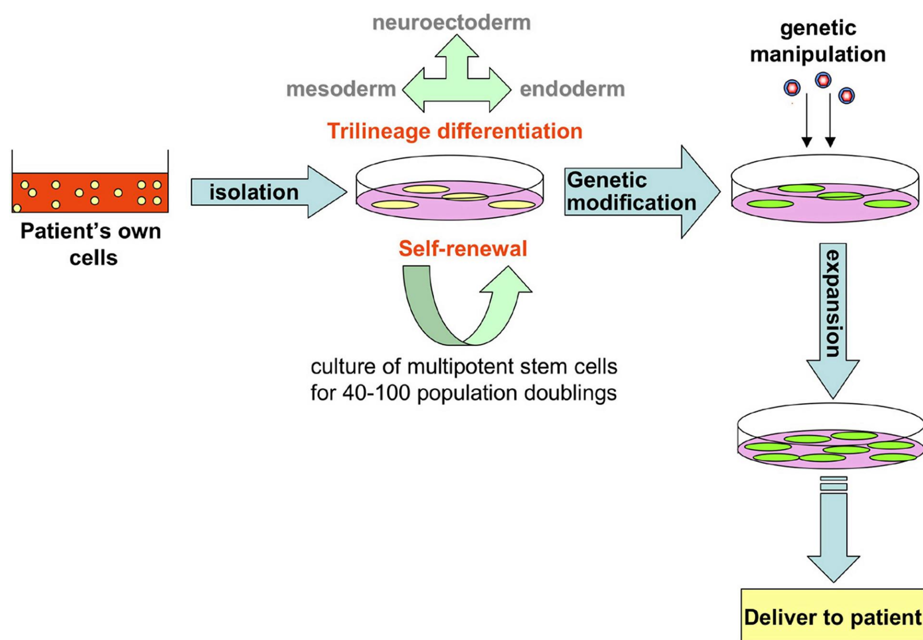


Fig. 1. Potential clinical application of genetically modified multipotent stem cells to treat a genetic abnormality. Multipotent stem cells are isolated from the patient and maintained *in vitro* for several passages. The population of cells obtained is able to differentiate into cells of the three lineages. Genetic modification of the cells can be obtained using a gene transfer vector that include a therapeutic gene. The resulting genetically modified stem cells can be propagated *in vitro* and delivered to the patient or stored for future use.

and hence *in situ* correction of a gene defect, as — even though HR can be achieved in HSCs — the very low frequency ($<1/10^5-10^6$ cells) precludes this from being a clinically useful method. As MAPC, hBMSC, USSC, FSSC, AFS, MIAMI cells, hFLMPC, MASC or pre-MSC can be expanded, low

干细胞之家www.stemcell8.cn ←点击进入

efficiency of HR should not be an impediment. Whether this will constitute a viable therapeutic approach still remains to be determined. The ability to clone and expand MAPC, hBMSC, USSC, FSSC, AFS, MIAMI cells, hFLMPC, MASC or pre-MSC may also be an advantage for viral vector-based gene therapy approaches. As discussed above, the MLV-based gene transfer is associated with insertion of the gene in the vicinity of transcription start sequences, and HIV vectors in gene rich regions of the host DNA, which can lead to insertional mutagenesis and tumorigenesis. Although the viral-vector based gene delivery would still be associated with insertions in these locations, it is not inconceivable, that following subcloning, clones of cells with the gene inserted in a harmless location could be isolated and used for transplantation.

The final conclusion that MAPC, hBMSC, USSC, FSSC, AFS, MIAMI cells, hFLMPC, MASC or pre-MSC are superior candidates for cellular gene therapy will, however, depend on the efficiency with which cells derived from human tissues will be capable of repopulating tissues *in vivo*, a question that remains unanswered.

References

Ailles L, Schmidt M, Santoni de Sio FR, *et al*. (2002) Molecular evidence of lentiviral vector-mediated gene transfer into human self-renewing, multi-potent, long-term NOD/SCID repopulating hematopoietic cells. *Mol Ther* **6**: 615–26.

Aiuti A, Slavin S, Aker M, *et al*. (2002) Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. *Science* **296**: 2410–13.

Aiuti A. (2004) Gene therapy for adenosine-deaminase-deficient severe combined immunodeficiency. *Best Pract Res Clin Haematol* **17**: 505–16.

Androutsellis-Theotokis A, Leker RR, Soldner F, *et al*. (2006) Notch signalling regulates stem cell numbers *in vitro* and *in vivo*. *Nature* **442**: 823–26.

Anjos-Afonso F, Bonnet D. (2007) Non-hematopoietic/endothelial SSEA-1pos cells defines the most primitive progenitors in the adult murine bone marrow mesenchymal compartment. *Blood* **109**:1298–306.

Antonchuk J, Sauvageau G, Humphries RK. (2002) HOXB4-induced expansion of adult hematopoietic stem cells *ex vivo*. *Cell* **109**: 39–45.

Aranguren XL, Luttun A, Clavel C, *et al*. (2007) *In vitro* and *in vivo* arterial differentiation of human multipotent adult progenitor cells. *Blood* **109**: 2634–42.

Baum C. (2007) Fourth case of leukaemia in the first SCID-X1 gene therapy trial, and the diversity of gene therapy. *http://www.esgct.org/upload/4th_CaseofLeukemial.pdf*

Beard BC, Keyser KA, Trobridge GD, Peterson LJ, *et al*. (2007) Unique integration profiles in a canine model of long-term repopulating cells transduced with gammaretrovirus, lentivirus, or foamy virus. *Hum Gene Ther* **18**: 423–34.

干细胞之家www.stemcell8.cn ←点击进入

Beltrami AP, Cesselli D, Bergamin N, *et al*. (2007) Multipotent cells can be generated *in vitro* from several adult human organs (heart, liver and bone marrow). *Blood*, in press.

Berns A. (2004) Good news for gene therapy. *N Engl J Med* **350**: 1679–80.

Bertolotti R. (1996) Recombinase-mediated gene therapy: strategies based on Lesch-Nyhan mutants for gene repair/inactivation using human RAD51 nucleoprotein filaments. *Biogenic Amines* **12**: 487–98.

Bishop AJ, Schiestl RH. (2000) Homologous recombination as a mechanism for genome rearrangements: environmental and genetic effects. *Hum Mol Genet* **9**: 2427–334.

Bordignon C. (2006) Stem-cell therapies for blood diseases. *Nature* **441**: 1100–102.

Bordignon C, Roncarolo MG. (2002) Therapeutic applications for hematopoietic stem cell gene transfer. *Nat Immunol* **3**: 318–21.

Breyer A, Estharabadi N, Oki M, *et al*. (2006) Multipotent adult progenitor cell isolation and culture procedures. *Exp Hematol* **34**: 1596–601.

Bryder D, Rossi DJ, Weissman IL. (2006) Hematopoietic stem cells: the paradigmatic tissue-specific stem cell. *Am J Pathol* 169: 338–46.

Bushman F, Lewinski M, Ciuffi A, *et al*. (2005) Genome-wide analysis of retroviral DNA integration. *Nat Rev Microbiol* 3: 848–58.

Calmels B, Ferguson C, Laukkanen MO, *et al*. (2005) Recurrent retroviral vector integration at the Mds1/Evi1 locus in nonhuman primate hematopoietic cells. *Blood* **106**: 2530–33.

Campagnoli C, Fisk N, Overton T, *et al*. (2000) Circulating hematopoietic progenitor cells in first trimester fetal blood. *Blood* **95**: 1967–72.

Campagnoli C, Roberts IA, Kumar S, *et al*. (2001) Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow. *Blood* **98**: 2396–402.

Cattoglio C, Facchini G, Sartori D, *et al*. (2007) Hot spots of retroviral integration in human CD34+ hematopoietic cells. *Blood*, in press.

Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, *et al*. (2000) Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science* **288**: 669–72.

Coffin JM. (1997) *Retroviruses*. Cold Spring Harbor Laboratory Press, NY, USA.

Cornetta K, Morgan RA, Anderson WF. (1991) Safety issues related to retroviral-mediated gene transfer in humans. *Hum Gene Ther* **2**: 5–14.

Dan YY, Riehle KJ, Lazaro C, *et al*. (2006) Isolation of multipotent progenitor cells from human fetal liver capable of differentiating into liver and mesenchymal lineages. *Proc Natl Acad Sci USA* **103**: 9912–17.

Dave UP, Jenkins NA, Copeland NG. (2004) Gene therapy insertional mutagenesis insights. *Science* **303**: 333.

De Coppi P, Bartsch G, Siddiqui MM, *et al*. (2007) Isolation of amniotic stem cell lines with potential for therapy. *Nat Biotechnol* **25**: 100–106.

干细胞之家www.stemcell8.cn ←点击进入

D'Ippolito G, Diabira S, Howard GA, *et al*. (2004) Marrow-isolated adult multilineage inducible (MIAMI) cells, a unique population of postnatal young and old human cells with extensive expansion and differentiation potential. *J Cell Sci* **117**: 2971–81.

Ferguson C, Larochelle A, Dunbar CE. (2005) Hematopoietic stem cell gene therapy: dead or alive? *Trends Biotechnol* **23**: 589–97

Fernandes KJ, McKenzie IA, Mill P, *et al*. (2004) A dermal niche for multipotent adult skin-derived precursor cells. *Nat Cell Biol* **6**: 1082–93.

Friedenstein AJ, Gorskaja JF, Kulagina NN. (1976) Fibroblast precursors in normal and irradiated mouse hematopoietic organs. *Exp Hematol* **4**: 267–74.

Gage FH. (2000) Mammalian neural stem cells. *Science* **287**: 1433–38.

Hacein-Bey-Abina S, Von Kalle C, Schmidt M, *et al*. (2003a) LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* **302**: 415–19.

Hacein-Bey-Abina S, von Kalle C, Schmidt M, *et al*. (2003b) A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *N Engl J Med* **348**: 255–56.

Hanenberg H, Xiao XL, Dilloo D, *et al*. (1996) Colocalization of retrovirus and target cells on specific fibronectin fragments increases genetic transduction of mammalian cells. *Nat Med* **2**: 876–82.

Hematti P, Hong BK, Ferguson C, *et al*. (2004) Distinct genomic integration of MLV and SIV vectors in primate hematopoietic stem and progenitor cells. *PLoS Biol* **2**: 2183–90.

Hutton JF, Rozenkov V, Khor FS, *et al*. (2006) Bone morphogenetic protein 4 contributes to the maintenance of primitive cord blood hematopoietic progenitors in an *ex vivo* stroma-noncontact co-culture system. *Stem Cells Dev* **15**: 805–13.

In 't Anker PS, Scherjon SA, Kleijburg-van der Keur C, *et al*. (2003) Amniotic fluid as a novel source of mesenchymal stem cells for therapeutic transplantation. *Blood* **102**: 1548–49.

In't Anker PS, Scherjon SA, Kleijburg-van der Keur GM, *et al*. (2004) Isolation of mesenchymal stem cells of fetal or maternal origin from human placenta. *Stem Cells* **22**: 1338–45.

Jiang Y, Jahagirdar BN, Reinhardt RL, *et al*. (2002a) Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* **418**: 41–49.

Jiang Y, Vaessen B, Lenvik T, *et al*. (2002b) Multipotent progenitor cells can be isolated from postnatal murine bone marrow, muscle, and brain. *Exp Hematol* **30**: 896–904.

Kay MA, Glorioso JC, Naldini L. (2001) Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics. *Nat Med* **7**: 33–40.

Kiem HP, Sellers S, Thomasson B, *et al*. (2004) Long-term clinical and molecular follow-up of large animals receiving retrovirally transduced stem and progenitor cells: no progression to clonal hematopoiesis or leukemia. *Mol Ther* **9**: 389–95.

干细胞之家www.stemcell8.cn ←点击进入

Kogler G, Sensken S, Airey JA, *et al*. (2004) A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential. *J Exp Med* **200**:123–35.

Kohn DB, Sadelain M, Glorioso JC. (2003) Occurrence of leukaemia following gene therapy of X-linked SCID. *Nat Rev Cancer* **3**: 477–88.

Krosl J, Austin P, Beslu N, *et al*. (2003) *In vitro* expansion of hematopoietic stem cells by recombinant TAT-HOXB4 protein. *Nat Med* **9**:1428–32.

Kucia M, Reca R, Campbell FR, *et al*. (2006) A population of very small embryonic-like (VSEL) CXCR4(+)SSEA-1(+)Oct-4+ stem cells identified in adult bone marrow. *Leukemia* **20**: 857–69.

Kucia M, Halasa M, Wysoczynski M, *et al*. (2007) Morphological and molecular characterization of novel population of CXCR4(+) SSEA-4(+) Oct-4(+) very small embryonic-like cells purified from human cord blood — preliminary report. *Leukemia* **21**: 297–303.

Kues WA, Petersen B, Mysegades W, *et al*. (2005) Isolation of murine and porcine fetal stem cells from somatic tissue. *Biol Reprod* **72**: 1020–28.

Kurre P, Kiem HP. (2000) Progress towards hematopoietic stem cell gene therapy. *Curr Opin Mol Ther* **2**: 400–11.

Kustikova O, Fehse B, Modlich U, *et al*. (2005) Clonal dominance of hematopoietic stem cells triggered by retroviral gene marking. *Science* **308**: 1171–74.

Lakshmipathy U, Pelacho B, Sudo K, *et al*. (2004) Efficient transfection of embryonic and adult stem cells. *Stem Cells* **22**: 531–43.

Larochelle A, Dunbar CE. (2004) Genetic manipulation of hematopoietic stem cells. *Semin Hematol* **41**: 257–71.

Larsson J, Karlsson S. (2005) The role of Smad signaling in hematopoiesis. *Oncogene* **24**: 5676–92.

Lechardeur D, Lukacs GL. (2002) Intracellular barriers to non-viral gene transfer. *Curr Gene Ther* **2**: 183–94.

Lee OK, Kuo TK, Chen WM, *et al*. (2004) Isolation of multipotent mesenchymal stem cells from umbilical cord blood. *Blood* **103**: 1669–75.

Lewis PF, Emerman M. (1994) Passage through mitosis is required for oncoretroviruses but not for the human immunodeficiency virus. *J Virol* **68**: 510–16.

McKay R. (1997) Stem cells in the central nervous system. *Science* **276**: 66–71.

Miura M, Gronthos S, Zhao M, *et al*. (2003) SHED: stem cells from human exfoliated deciduous teeth. *Proc Natl Acad Sci USA* **100**: 5807–12.

Naldini L, Blomer U, Gallay P, *et al*. (1996) *In vivo* gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* **272**: 263–67.

Naldini L. (2006) Inserting optimism into gene therapy. *Nat Med* **12**: 386–88.

Nienhuis AW, Dunbar CE, Sorrentino BP. (2006) Genotoxicity of retroviral integration in hematopoietic cells. *Mol Ther* **13**: 1031–49.

Nishikawa M, Huang L. (2001) Nonviral vectors in the new millennium: delivery barriers in gene transfer. *Hum Gene Ther* **12**: 861–70.

干细胞之家www.stemcell8.cn ←点击进入

Noguchi P. (2003) Risks and benefits of gene therapy. *N Engl J Med* **348**: 193–94.

Onodera M. (2004) Delivery of genes to hematopoietic stem cells. *Meth Mol Biol* **246**: 527–39.

Oreffo RO, Cooper C, Mason C, Clements M. (2005) Mesenchymal stem cells: lineage, plasticity, and skeletal therapeutic potential. *Stem Cell Rev* **1**: 169–78.

Pages JC, Bru T. (2004) Toolbox for retrovectorologists. *J Gene Med* **Suppl 1**: S67–S82.

Passegué E, Wagers AJ, Giuriato S, *et al*. (2005) Global analysis of proliferation and cell cycle gene expression in the regulation of hematopoietic stem and progenitor cell fates. *J Exp Med* **202**: 1599–611.

Pike-Overzet K, de Ridder D, Weerkamp F, *et al*. (2007) Ectopic retroviral expression of LMO2, but not IL2Rgamma, blocks human T-cell development from CD34+ cells: implications for leukemogenesis in gene therapy. *Leukemia* **21**: 754–63.

Prockop DJ. (1997) Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science* **276**: 71–74.

Reyes M, Lund T, Lenvik T, *et al*. (2001) Purification and *ex vivo* expansion of postnatal human marrow mesodermal progenitor cells. *Blood* **98**: 2615–25.

Reyes M, Dudek A, Jahagirdar B, *et al*. (2002) Origin of endothelial progenitors in human postnatal bone marrow. *J Clin Invest* **109**: 337–46.

Richter J, Karlsson S. (2001) Clinical gene therapy in hematology: past and future. *Int J Hematol* **73**: 162–69.

Roe T, Reynolds TC, Yu G, Brown PO. (1993) Integration of murine leukemia virus DNA depends on mitosis. *EMBO J* **12**: 2099–108.

Rosen ED, MacDougald OA. (2006) Adipocyte differentiation from the inside out. *Nat Rev Mol Cell Biol* **7**: 885–96.

Ross JJ, Hong Z, Willenbring B, *et al*. (2006) Cytokine-induced differentiation of multipotent adult progenitor cells into functional smooth muscle cells. *J Clin Invest* **116**: 3139–49.

Salmon P, Kindler V, Ducrey O, *et al*. (2000) High-level transgene expression in human hematopoietic progenitors and differentiated blood lineages after transduction with improved lentiviral vectors. *Blood* **96**: 3392–98.

Schwartz RE, Reyes M, Koodie L, *et al*. (2002) Multipotent adult progenitor cells from bone marrow differentiate into functional hepatocyte-like cells. *J Clin Invest* **109**: 1291–302.

Serafini M, Verfaillie CM. (2006) Pluripotency in adult stem cells: state of the art. *Semin Reprod Med* **24**: 379–88.

Serafini M, Dylla SJ, Oki M, *et al*. (2007) Hematopoietic reconstitution by multipotent adult progenitor cells: precursors to long-term hematopoietic stem cells. *J Exp Med* **204**: 129–39.

Slack JM. (2000) Stem cells in epithelial tissues. *Science* **287**: 1431–33.

Stevenson M. (2000) HIV nuclear import: What's the flap? *Nat Med* **6**: 626–28.

Suzuki Y, Craigie R. (2007) The road to chromatin — nuclear entry of retroviruses. *Nat Rev Microbiol* **5**: 187–96.

干细胞之家www.stemcell8.cn ←点击进入

Theise ND. (2005) On experimental design and discourse in plasticity research. *Stem Cell Rev* **1**: 9–13.

Thomas CE, Ehrhardt A, Kay MA. (2003) Progress and problems with the use of viral vectors for gene therapy. *Nat Rev Genet* **4**: 346–58.

Tolar J, O'Shaughnessy MJ, Panoskaltsis-Mortari A, *et al*. (2006) Host factors that impact the biodistribution and persistence of multipotent adult progenitor cells. *Blood* **107**: 4182–88.

Toma JG, Akhavan M, Fernandes KJ, *et al*. (2001) Isolation of multipotent adult stem cells from the dermis of mammalian skin. *Nat Cell Biol* **3**: 778–84.

Toma JG, McKenzie IA, Bagli D, Miller FD. (2005) Isolation and characterization of multipotent skin-derived precursors from human skin. *Stem Cells* **23**: 727–37.

Urnov FD, Miller JC, Lee YL, *et al*. (2005) Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature* **435**: 646–51.

VandenDriessche T, Collen D, Chuah MK. (2003) Biosafety of onco-retroviral vectors. *Curr Gene Ther* **3**: 501–15.

Verfaillie CM. (2002) Adult stem cells: assessing the case for pluripotency. *Trends Cell Biol* **12**: 502–508.

Wagers AJ, Weissman IL. (2004) Plasticity of adult stem cells. *Cell* **116**: 639–48.

Weissman IL, Anderson DJ, Gage F. (2001) Stem and progenitor cells: origins, phenotypes, lineage commitments, and transdifferentiations. *Ann Rev Cell Dev Biol* **17**: 387–403.

Woods NB, Fahlman C, Mikkola H, *et al*. (2000) Lentiviral gene transfer into primary and secondary NOD/SCID repopulating cells. *Blood* **96**: 3725–33.

Yanez R J, Porter AC. (1998) Therapeutic gene targeting. *Gene Ther* **5**: 149–59.

Yamashita M, Emerman M. (2006) Retroviral infection of non-dividing cells: old and new perspectives. *Virology* **344**: 88–93.

Yen BL, Huang HI, Chien CC, *et al*. (2005) Isolation of multipotent cells from human term placenta. *Stem Cells* **23**: 3–9.

Yokota T, Oritani K, Mitsui H, *et al*. (1998) Growth-supporting activities of fibronectin on hematopoietic stem/progenitor cells *in vitro* and *in vivo*: structural requirement for fibronectin activities of CS1 and cell-binding domains. *Blood* **91**: 3263–72.

Yoon YS, Wecker A, Heyd L, *et al*. (2005) Clonally expanded novel multipotent stem cells from human bone marrow regenerate myocardium after myocardial infarction. *J Clin Invest* **115**: 326–38.

Zeng L, Rahrmann E, Hu Q, *et al*. (2006) Multipotent adult progenitor cells from swine bone marrow. *Stem Cells* **24**: 2355–66.

Zennou V, Petit C, Guetard D, *et al*. (2000) HIV-1 genome nuclear import is mediated by a central DNA flap. *Cell* **101**: 173–85.

Zhang XB, Beard BC, Beebe K, *et al*. (2006) Differential effects of HOXB4 on nonhuman primate short- and long-term repopulating cells. *PLoS Med* **3**: 687–98.

Zuk PA, Zhu M, Ashjian P, *et al*. (2002) Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* **13**: 4279–95.

干细胞之家www.stemcell8.cn ←点击进入

Chapter 9

Leukemia Stem Cells, A Pioneering Paradigmatic Model

Daniel J. Pearce and Dominique Bonnet*

A fundamental problem in cancer research is the identification of the cell type capable of initiating and sustaining the growth of the neoplastic clone *in vivo*. The key to solving this problem lies on the observation made over 40 years ago that tumors are heterogeneous and thus might be maintained only by a rare subset of cells called "cancer stem cells" (CSCs). However, the proof of this principle was only possible after the development of modern research tools for investigating the behavior of defined cell populations *in vivo*. The blood-related cancer leukemia was the first disease where human CSCs, or leukemic stem cells (LSCs), were isolated. The development of quantitative xenotransplantation assays using immune-deficient mouse recipients to detect primitive human HSCs with *in vivo* repopulating ability and the adaptation of this model to leukemia has been instrumental. Leukemia can now be viewed as aberrant hematopoietic processes initiated by rare LSCs that have maintained or reacquired the capacity for indefinite proliferation through accumulated mutations and/or epigenetic changes. Yet, despite their critical importance, much remains to be learned about the developmental origin of LSC and the mechanisms responsible for their emergence in the course of the disease. This chapter will review our current knowledge on normal and leukemic stem cell development and finally demonstrate how these discoveries provide a paradigm for identification of cancer stem cell (CSC) from solid tumors.

Keywords: Hematopoietic stem cell (HSC); xenotransplantation model; leukemic stem cell (LSC); cancer stem cell (CSC); self-renewal.

*Corresponding author. dominique.bonnet@cancer.org.uk
Hematopoietic Stem Cell Laboratory, Cancer Research UK, London Research Institute, London, WC2A 3PX, UK

干细胞之家www.stemcell8.cn ←点击进入

1. Introduction

The hallmark properties of the hematopoietic stem cells (HSC) are their ability to balance self-renewal versus differentiation cell fate decisions to provide sufficient primitive cells to sustain hematopoiesis, while generating more mature cells with specialized capacities. Through the process of asymmetric cell division, a single division can result in the formation of both an identical stem cell and a more highly mature cell (Ho, 2005). In order to assure a persistent pool of regenerating cells without outgrowth of immature cell types, a tight regulation of HSC division is required. Unchecked growth of immature cells is thought to represent a paradigm for malignant outgrowth, at least for acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) (Passegue *et al.*, 2003; Reya *et al.*, 2001). Thus, determining the composition and relationship of the cell types that constitute the human stem cell compartment may help both to identify the cellular and molecular factors that govern normal and leukemic stem cell (LSC) development, and to advance clinical applications of transplantation, gene therapy, stem cell expansion and tumor cell purging. This review will introduce the notion of LSCs, the potential origin of these cells with an emphasis on myeloid leukemia, the mechanisms that regulate normal hematopoietic stem cell, how dysregulation of these key processes are involved in leukemogenesis and finally, to examine the impacts these discoveries may have clinically and in understanding the organization of cancer of other tissues.

2. Normal Hematopoietic Stem Cell

Most mature blood cells have a short life (granulocytes — a few hours in circulation and a few days in tissues; erythrocytes — a few weeks). To maintain a sufficient number of mature cells, new cells must be produced to replace them. The turnover is immense; a 70 kg man may produce almost 1 trillion cells per day, including approximately 200 billion erythrocytes and 70 billion neutrophils. At any one time during hematopoiesis, cells are at various stages of development, from recognizable mature cell types to more primitive undifferentiated precursor cells. One common cell is thought to give rise to all these precursors and is known as the pluripotent hematopoietic stem cell (HSC). Hematopoiesis is thought to occur via progressive lineage restriction as a successive hierarchy of differentiation from a single pluripotent stem cell to multipotent progenitors to bipotent and unipotent progenitors and ultimately to many different committed mature cell types (see Fig. 1). It may be inferred that since hematopoiesis continues mostly unchanged throughout

干细胞之家www.stemcell8.cn ←点击进入

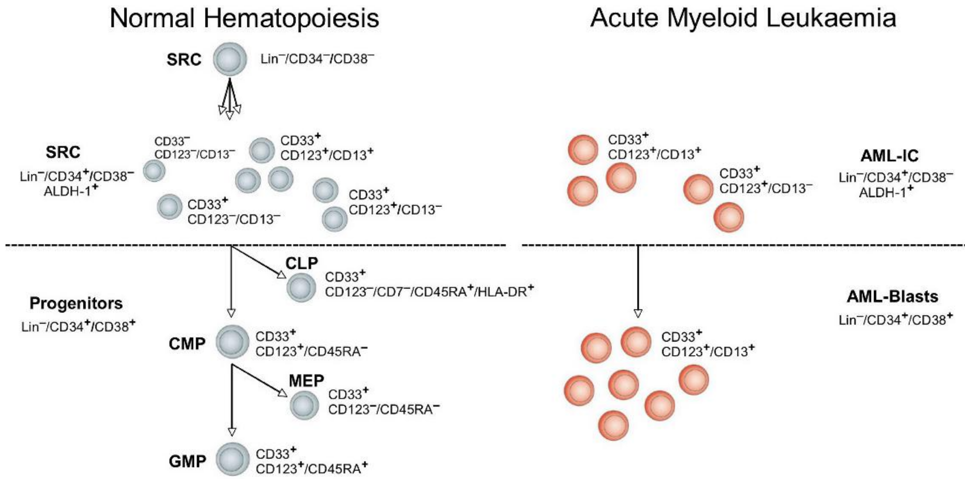


Fig. 1. Diagrammatic representation of myeloid markers expressed in hematopoiesis and acute myeloid leukemia. This figure compares the phenotype of normal, non-malignant hematopoietic cells and AML cells. Phenotypes above the dotted line are capable of 12-week engraftment in NOD/SCID mice. Normal non-malignant SRC are a heterogenous population. Most SRCs are $CD34^+$, but a rare population of $Lin^-/CD34^-/CD38^-$ cells exist that precede $CD34^+$ cells in the hierarchy of hematopoiesis. $Lin^-/CD34^+/CD38^-$ SRCs are a heterogenous population, which includes subsets that are positive or negative for CD33, CD123 and CD13. The number and size of cells in the diagram represent the relative frequencies of SRC subsets. The most prevalent subsets of $Lin^-/CD34^+/CD38^-$ SRCs are $CD33^+/CD123^+/CD13^+$ and $CD33^+/CD123^+/CD13^-$. The phenotype of AML-IC most closely resembles these two subsets of normal SRC. In contrast, AML-ICs do not resemble normal progenitors as these co-expressed CD38. This data is consistent with a leukemic transformation event that causes most AMLs occurring in a subset of normal HSCs. SRC — SCID repopulating cells, CLP — common lymphoid progenitor, CMP — common myeloid progenitor, MEP — megakaryocyte/erythroid progenitor, GMP — granulocyte/macrophage progenitor. AML-IC — acute myeloid leukemia initiating cell. See main text for further information. (Based on Fig. 1 of: Pearce and Bonnet, *Gene Ther Regul*, **3**: 65–90 (2007), with permission from World Scientific Publishing.)

life, the cells ultimately responsible for hematopoiesis are constantly being renewed.

This was first described by Till and McCulloch in 1961, who demonstrated that a single cell can repopulate the spleen of myeloablated mice with cells of all hematopoietic lineages (Till and McCulloch, 1961). These colonies were deemed to have been derived from a single cell by Becker *et al.* in 1963, who demonstrated that when radiation damaged stem cells (resulting in chromosomal abnormalities) were transplanted into lethally irradiated mice, each spleen colony contained cells with only one chromosomal abnormality (Becker *et al.*, 1963). Till and McCulloch also proposed

干细胞之家www.stemcell8.cn ←点击进入

the aforementioned concept that is central to our definition of stem cells: self-renewal. They and colleagues observed that rare cells within the spleen colonies were capable of forming further spleen colonies and hence were still spleen colony-forming cells (CFU-S) (Siminovitch *et al.*, 1963).

2.1. *Characterization of the Hematopoietic Stem Cell*

Over the years, various assays have been developed to examine hematopoietic stem and progenitor cells. As these assays were improved and progressively became more selective for stem cells, our definition of stem cells and understanding of hematopoiesis has developed. Here, we describe these assays, starting with those that assess the proliferation and differentiation of mature progenitors before moving on to describe more recent HSC-specific assays.

The colony formation cell (CFC) assay involves the use of a semi-solid, methylcellulose-based medium supplemented with cytokines that promote differentiation and proliferation of hematopoietic progenitor cells. Long-term cultures of hematopoietic cells with stromal feeder layers are an attempt to recreate hematopoiesis *in vitro*. Within this system, committed cells and progenitors exhaust their proliferative potential within a few weeks and are no longer part of the culture. More primitive cells attach to the feeder layer and some actually pass through to underneath the stromal layer. The cells that grow underneath the layer have a simple morphology and areas of growth of primitive cells under the feeder layers in a long-term culture are known as cobblestone areas. The cells that produce these cobblestone areas are termed cobblestone area forming cells (CAFC) and are thought to be more primitive than progenitor cells (Breems *et al.*, 1994; Ploemacher *et al.*, 1989). Later in the long-term culture, some CAFC go on to produce non-adherent cells that produce progenitor colonies in the CFC assay. Cells that produce CFC at 5 weeks of long-term culture are termed long-term culture initiating cells (LTC-IC) (Sutherland *et al.*, 1989 and 1990). An extended LTC-IC has also been described that produces CFCs after 10 weeks of culture (Hao *et al.*, 1996).

The long-term culture system assesses the proliferation and myeloid differentiation potential of hematopoietic cells. Unfortunately, lymphoid cells do not grow under the conditions of feeder layer long-term cultures or in the standard CFC assay so the multilineage (lymphoid and myeloid) potential of cells is not assessed. Furthermore, only limited self-renewal is required to produce colonies after 5 or even 10 weeks in long-term culture.

干细胞之家www.stemcell8.cn ←点击进入

Culture systems have since been developed that support the growth of both myeloid and lymphoid cells and hence can examine the multilineage potential of primitive hematopoietic cells. These systems involve the transfer of hematopoietic cells from stromal cells that support the growth of lymphoid cells to a stromal cell line that support myeloid growth (Hao *et al.*, 1998; Miller *et al.*, 1999). Limited self-renewal of multilineage stem/progenitor cells may be demonstrated by performing secondary experiments on to determine whether rare cells are capable of re-initiating such multilineage cultures (Punzel *et al.*, 1999; Theunissen and Verfaillie, 2005).

Although self-renewal of multilineage cells may be demonstrated *in vitro*, cytokines are required to stimulate primitive cells to divide and cells are quickly exhausted compared to their apparent endogenous potential. Since we do not know the concentrations or composition of all the cytokines signals involved, this lack of long-term potency may be a due to the artificial nature of *in vitro* culture systems.

The ideal way to assess hematopoietic stem cells would be to observe their growth in the endogeneous microenvironment where they would receive the appropriate signals. With colonies of inbred mice it is possible to myeloablate recipients and transplant hematopoietic cells from genetically similar mice and observe the growth of primitive hematopoietic cells in their endogenous environment for a significant proportion (10 months) of an organisms lifespan (29 months average) (Osawa *et al.*, 1996; Spangrude *et al.*, 1988). It is not possible to perform these experiments in humans, but xenotransplantation models may provide the closest paradigm for the human hematopoietic environment.

The greatest challenge to the development of xenotransplantation models is immune rejection of the transplanted cells. This may be overcome by transplanting cells before the immune system has developed or by modulation of the hosts' immune response. The injection of primitive cells into foetal sheep that have a naive immune system has allowed the growth of hematopoietic cells to be observed in an *in vivo* environment for over one year (Zanjani *et al.*, 1999 and 1997). However, this model is technically difficult, expensive to manage and hence is limited to small sample numbers.

Immuno-compromised mouse models offer the most suitable environment available for the study of human hematopoietic cells. The SCID mutation in mice causes a lack of functional T and B lymphocytes and with cytokine stimulation, human cells may develop in the marrows of these mice (Lapidot *et al.*, 1992). When the SCID mutation is combined with the NOD mutation, mice also have an impaired natural killer (NK) and

干细胞之家www.stemcell8.cn ←点击进入

antigen-presenting cell function and the growth of human cells in these mice is possible without cytokine stimulation (Shultz *et al.*, 1995; Cashman *et al.*, 1997; Larochelle *et al.*, 1996). Given ideal conditions, the NOD/SCID model can support multilineage (B-cell and myeloid) for over 3 months and can support secondary engraftment (Hogan *et al.*, 1997). In this way, the NOD/SCID assay can assess the self-renewal, proliferation and differentiation of human hematopoietic stem cells.

The ideal demonstration of human stem cell potential would be the ability of a single cell to produce all the hematopoietic lineages for a significant period of time. Due to apparent inefficiencies in transplantation of human single cells, xenotransplantation studies are still elusive. Indeed, although transplantation in a mouse-mouse setting is very efficient (Matsuzaki *et al.*, 2004), transplantation is not efficient in the human-mouse situation. This may be partially due to an inefficiency in homing given that injection of cells directly into the bone increases the frequency of repopulating cells when compared to intravenous administration (Yahata *et al.*, 2003). In spite of this, it has been demonstrated via viral tracking experiments that a single cell is capable of multi-lineage differentiation in the NOD/SCID model (Guenechea *et al.*, 2001).

Other mouse models have been developed to support the growth of human hematopoietic cells. The NOD/SCID-β_2 microglobulin null ($\beta 2m^{-/-}$) mouse has a further defect in NK cell activity and is more tolerant of human grafts than the NOD/SCID model (Kollet *et al.*, 2000; Glimm *et al.*, 2001; Christianson *et al.*, 1997). However, in these mice limited T-lymphoid reconstitution is achieved. Furthermore all these immunodeficient mice are problematic as lymphomas limit their lifespan preventing long-term reconstitution assessment. These hurdles were recently overcome in three new strains: NOD/Shi-Scid Il2Rγ^{null} (Hiramatsu *et al.*, 2003; Yahata *et al.*, 2002), NOD/SCID IL2Rγ^{null} (Ishikawa *et al.*, 2005; Shultz *et al.*, 2005) and BALB/c-Rag2^{null} IL2Rγ^{null} (Traggiai *et al.*, 2004), which all lack the Il-2 family common cytokine receptor gamma chain gene. The absence of functional receptors for IL2, IL-7 and other cytokines may prevent the expansion of NK cells and early lymphoma cells in NOD/SCID IL2Rg^{null} mice, resulting in better engraftment of transplanted human cells and longer lifespan of the mice. It was reported recently that human HSCs and progenitor cells engraft successfully in these mice and produce all human myeloid and lymphoid lineages. T and B cells migrate into lymphoid organs and mount HLA-dependent allogeneic responses, and generate antibodies against T cell-dependent antigens such as ovalbumin and tetanus toxin (Ishikawa *et al.*, 2005; Traggiai *et al.*, 2004).

干细胞之家www.stemcell8.cn ←点击进入

2.2. *Identification/Isolation of Hematopoietic Stem Cells*

To fully understand HSC biology and the hierarchy of hematopoiesis one must be able to separate the extremely rare HSC from the rest of hematopoiesis and ideally be able to identify/isolate their progenies. HSC isolation is also important for the development of novel strategies such as gene therapy and stem cell expansion.

Many approaches have been employed to isolate hematopoietic stem cells via flow cytometry, the most popular of which are based on the expression of a combination of antigenic markers. Lineage committed cells express markers that may be used to negatively identify a population of primitive, non-committed cells. The resulting lineage-negative (lin$^-$) population may be further purified for hematopoietic stem cells by the addition of other surrogate markers.

For almost 20 years, the CD34 antigen has been the marker of choice for the identification and isolation of the human hematopoietic stem cell. The CD34$^+$ cell population is a heterogeneous subset consisting of most of the stem/progenitor cells present in hematopoietic tissue. Self-renewing stem cells capable of long-term reconstitution of conditioned hosts may be distinguished from the more mature progenitors via their lack of CD38 co-expression (Bhatia *et al.*, 1997). Such CD34$^+$ repopulating cells also co-express CD133 and CD318 (Buhring *et al.*, 2004; Yin *et al.*, 1997).

In addition to this well-characterized CD34$^+$ subset, hematopoietic stem cells that do not possess any detectable CD34 expression exist. Direct sorting of cells negative for CD34 as well as a cocktail of lineage antigens into NOD/SCID mice has yielded long-term hematopoietic reconstitution. NOD/SCID repopulation derived from CD34$^-$ cells occurred at a different time-point to CD34$^+$ cell repopulation and involved the generation of CD34$^+$ cells. These results demonstrate that lin$^-$/CD34$^-$ cells contain a subset of hematopoietic stem cells that are separate and distinct to those previously identified and actually precede CD34$^+$ cells in the hierarchy of hematopoiesis (Bhatia *et al.*, 1998). This observation has been reproduced in the foetal sheep model with very similar results (Zanjani *et al.*, 1999).

Using a monoclonal antibody directed against human CD93 (known previously as C1qRp and homologue to the mouse stem cell marker AA4.1), we reported that human Lin$^-$CD34$^-$CD38$^-$CD93$^+$ are highly enriched in CD34$^-$ SCID repopulating cells (SRC) (Danet *et al.*, 2002). Indeed, the transplantation of as few as 5000 Lin$^-$CD34$^-$CD38$^-$CD93$^+$ cord blood cells is capable of giving rise to both myeloid and lymphoid cells in NOD/SCID mice, whereas as many as 840,000 Lin$^-$CD34$^-$CD38$^-$CD93$^-$ are unable to

干细胞之家www.stemcell8.cn ←点击进入

engraft. The presence of CD93 on CD34$^-$-SRC might provide a new means of defining the most primitive human repopulating cells. Furthermore, we demonstrated that CD93 is a positive marker of all human repopulating stem cells because it is expressed on both CD34$^-$ and CD34$^+$ stem cells from umbilical cord blood and adult bone marrow.

As mentioned above, HSCs are generally regarded as being devoid of lineage specific antigen (Andrews *et al.*, 1990, 1989 and 1986). As HSCs commit to specific blood cell lineages, lineage markers are expressed. However, it has been noted that genes associated with specific lineages are expressed in cells with a stem cell phenotype (Hu *et al.*, 1997). This led to the development of the "lineage priming" hypothesis that postulates that HSCs promiscuously express lineage specific genes prior to commitment (Orkin, 2003). Using a conditional knockout mouse, one group recently demonstrated that the myeloid gene lysozyme is transcribed in HSCs (Ye *et al.*, 2003). This appeared to confirm the "lineage priming" hypothesis.

Recently we show that CD33, CD13 and/or CD123, well-established myeloid markers, are expressed on most human long-term repopulating cells from cord blood and bone marrow (Taussig *et al.*, 2005). This study changes our views of HSCs and the process of differentiation.

SRCs within the Lin$^-$CD34$^+$CD38$^-$ population are thus heterogeneous. This heterogeneity is consistent with the heterogeneity observed within SRCs using viral tracking studies (Guenechea *et al.*, 2001). Some repopulating cells do not contribute to hematopoiesis until later time points while others appear to be relatively short lived. The phenotypes of the cells with different repopulation characteristics have not yet been defined. The CD13$^-$ CD123$^-$fraction of Lin$^-$ CD34$^+$ CD38$^-$ cord blood cells may be the HSCs that contribute to hematopoiesis only at later time points. These might be more primitive and equivalent to the long-term repopulating cells characterized in mice, though it should be noted that other fractions also engrafted at 12 weeks.

The presence of myeloid markers on HSCs is consistent with the lineage-priming hypothesis mentioned previously (Enver and Greaves, 1998; Orkin, 2003). This hypothesis states that HSCs transcribe lineage specific genes prior to commitment, in readiness for differentiation (HSCs are "primed"). Evidence for the lineage-priming hypothesis comes from a number of sources (Ford *et al.*, 1992; Jimenez *et al.*, 1992; Miyamoto *et al.*, 2002; Ye *et al.*, 2003).

Therefore, the default position of HSCs may be myeloid. HSCs may be primed to differentiate down one of the myeloid lineages. This is consistent

干细胞之家www.stemcell8.cn ←点击进入

with the observation that the lymphoid system evolved later than the myeloid system. Lymphocytes are present in all jawed vertebrates, whereas lower organisms such as the hagfish (jawless vertebrate) do not have a lymphoid system (Klein and Nikolaidis, 2005), but do possess granulocytes and red cells (Newton *et al.*, 1994).

Based on the heterogeneity and complexity of the HSC compartment, it appears that no universal markers might be identifiable. Nevertheless, using a micro-array approach, two groups identified independently two new murine HSC markers. One of those genes, encoding the murine endothelial protein C receptor (EPCR), is expressed at high levels within the bone marrow in hematopoietic stem cells. Bone marrow cells isolated on the basis of EPCR expression alone are highly enriched for hematopoietic reconstitution activity, showing levels of *in vivo* engraftment comparable to that of stem cells purified using the most effective conventional methods. Moreover, evaluation of cell populations first enriched for stem cell activity by conventional methods and subsequently fractionated on the basis of EPCR expression indicates that stem cell activity is always associated with EPCR-expressing cells (Balazs *et al.*, 2005). Morrison's group on the other hand demonstrates that cell surface receptors of the SLAM family, including CD150, CD244, and CD48, were differentially expressed among functionally distinct progenitors. HSCs were highly purified as $CD150^{+}CD244^{-}CD48^{-}$ cells while most restricted progenitors were $CD48^{+}CD244^{+}CD150^{-}$. The primitiveness of hematopoietic progenitors could thus be predicted based on the combination of SLAM family members they expressed (Kiel *et al.*, 2005). They also showed in another study that SLAM family markers are conserved among hematopoietic stem cells during ageing or G-CSF mobilization (Yilmaz *et al.*, 2006).

Matsubara *et al.* also established recently that endomucin, a CD34-like sialomucin, as a novel cell-surface marker for LTR-HSCs throughout development and provide a powerful tool in understanding HSC ontogeny (Matsubara *et al.*, 2005). They show that endomucin^{+} cells, were confined to the murine bone marrow c-Kit^{+} Sca-1^{+} Lin^{-} (KSL) HSCs and progenitor cells, and, importantly, long-term repopulating HSCs were exclusively present in the Endomucin^{+}CD34^{-}KSL population.

Further work will be needed to determine the overlap between these new stem cell markers in mice but also whether any of these markers are also expressed in human HSC.

In addition to the use of these surrogate markers, alternative methods to identify hematopoietic stem cells have been developed. These attempt to

干细胞之家www.stemcell8.cn ←点击进入

highlight a physiological property of hematopoietic stem cells that is different to the rest of hematopoiesis. This approach may be more accurate as the expression of markers may change, whereas a functional assay by its very nature identifies HSCs (Sato *et al.*, 1999).

Aldehyde Dehydrogenase (ALDH) is a cytosolic enzyme that is responsible for the oxidation of intracellular aldehydes. Over 17 human ALDH genes have been identified and the ALDH superfamily is highly conserved across a variety of species (Yoshida *et al.*, 1998; Sophos and Vasiliou, 2003). This enzyme is thought to have an important role in oxidation of alcohol and vitamin A, and in cyclophosphamide chemoresistance (Wang *et al.*, 2001; Duester, 2000).

Elevated levels of ALDH have been demonstrated in murine and human progenitor cells when compared to other hematopoietic cells (Kastan *et al.*, 1990). More recently, a method has been developed for the assessment of ALDH activity in viable cells. This non-cytotoxic method utilizes a cell-permeable fluorescent substrate to identify cells with high ALDH activity (Jones *et al.*, 1995). Substrate converted by ALDH is a charged molecule and is unable to leave the cell as freely as the unconverted substrate. In this way, converted ALDH substrate accumulates in cells with a high ALDH activity. This approach has allowed the analysis of viable murine and human $ALDH^+$ progenitors by flow cytometry. Human cord blood hematopoietic cells with high ALDH activity are highly enriched for primitive $CD34^+$ cells and depleted for lineage positive cells (CD3, CD14, CD20 and CD56), indicating that they do indeed represent a primitive hematopoietic cell population (Storms *et al.*, 1999). Although the $Lin^-/ALDH^+$ population almost completely overlaps with the $Lin^-/CD34^+$ cell population (Pearce *et al.*, 2005), there is an extremely small population of $Lin^-/ALDH^+/CD34^-$ cells that is capable of NOD/SCID repopulation (unpublished observations).

In addition to ALDH activity, the selective expression of detoxification pumps has been used to identify HSC. The fluorescent dye Rhodamine 123 is a substrate for the MDR pump, which is expressed on hematopoietic stem and progenitor cells and a flow cytometric method based efflux of the dye has been developed (Uchida *et al.*, 1996). A possibly more HSC selective assay has been devised that involves the identification of the side population (SP), which is formed by preferential efflux of the DNA binding dye Hoechst 33342 (Goodell *et al.*, 1996). ABCG2 transporters selectively expressed on the surface of hematopoietic stem cells are thought to be responsible for this preferential efflux (Zhou *et al.*, 2001; Kim *et al.*, 2002). SP cells have been identified in adult BM from several species including human (Goodell *et al.*, 1997). The hematopoietic stem cell status of murine SP cells has been

干细胞之家www.stemcell8.cn ←点击进入

established (Goodell *et al.*, 1996; Pearce *et al.*, 2004), but to date, description of the functional activities of human SP cells in normal individuals has been limited to an *in vitro* study of cord blood and more recently to an *in vivo* study of human foetal liver (Uchida *et al.*, 2001).

3. Leukemia Stem Cells

3.1. *Concept of the Leukemia-Initiating Cell*

Whereas it was previously believed that most or all cancer cells possess the property to self-renew and replenish new cancer cells, it has recently become clear that cancers, as most normal tissues, are organized in a hierarchical fashion, and that only a small fraction of tumor cells have the ability to reconstitute a new tumor (Reya *et al.*, 2001). The existence of such cancer stem cells (CSCs) with self-renewal potential was first documented in leukemias (Bonnet and Dick, 1997; Lapidot *et al.*, 1994), but has later been extended to solid tumors, including breast and brain raising the possibility that such cells are the apex of all neoplastic systems (Singh *et al.*, 2004, Al-Hajj *et al.*, 2003). As these rare CSCs are both required and sufficient to reconstitute a new tumor, they have immediate and important clinical implications. A better identification and characterization of CSCs should provide a better understanding of tumor developmental biology and the genetic events involved in the transformation process. Recent studies have shown that CSCs, as normal somatic stem cells, express high levels of multidrug resistance (MDR) pumps that efficiently efflux cytotoxic drugs (Zhou *et al.*, 2001; Wulf *et al.*, 2001), making them particularly difficult to target with cytotoxic therapies. Similarly, they appear to be less immunogenic than more differentiated cancer cells in the tumor (Costello *et al.*, 2000), and therefore also more difficult to eradicate with immunotherapy. Thus, it has become evident, that CSCs might prove not only to be the most important but also the most difficult cancer cells to eliminate with conventional therapies, and that a specific monitoring and targeting of these elusive CSCs could become an important tool towards identification and characterization of improved cellular and molecular targets for development of improved cancer therapies. This has opened a new translational research field bridging stem cell and cancer biology (Reya *et al.*, 2001), which is likely to have considerable impact on the development of clinical oncology.

Based on these recent studies, the paradigm of cancer as a hierarchical disease, sustained by a rare population of CSCs has emerged. Implicit in this model of cancer development is the notion that CSCs are biologically distinct from other cells in the tumor and are able to initiate and sustain

干细胞之家www.stemcell8.cn ←点击进入

tumor growth *in vivo* whereas the bulk cells are not. Despite of the clear importance of CSCs in the genesis and perpetuation of cancers, little is currently known about the biological and molecular properties that make CSCs distinct from normal stem cells, the developmental/cellular origin of CSCs, the mechanisms responsible for their emergence in the course of the disease, and identification of candidate molecular targets for therapeutic intervention.

3.2. *Leukemic Initiating Cells or Leukemic Stem Cells*

The adaptation of the available quantitative assays for normal human stem cells capable of repopulating hematopoiesis *in vivo* allowed the identification of leukemic initiating cells. Transplantation of primary AML cells into NOD/SCID mice led to the finding that only rare cells, termed AML-initiating cells (AML-IC), are capable of initiating and sustaining growth of the leukemic clone *in vivo*, and serial transplantation experiments showed that AML-IC possess high self-renewal capacity, and thus can be considered to be the leukemic stem cells. By LSC we refer to a cell that has self-renewal and differentiation potential and is able to reinitiate the leukemia when transplanted into NOD/SCID mice. This definition does not preclude the nature of the cells that is being transformed (i.e., normal HSC, progenitors or mature cells).

Importantly, AML-IC can be prospectively identified and purified as $CD34^{+}/CD38^{-}$cells in AML patient samples, regardless of the phenotype of the bulk blast population, and represented the only AML cells capable of self-renewal (Bonnet and Dick, 1997). However, subsequently, considerable heterogeneity has been revealed. Using lentiviral gene marking to track the behavior of individual LSCs, following serial transplantation, has revealed heterogeneity in their ability to self-renew, similar to what is seen in the normal HSC compartment (Hope *et al.*, 2004).

3.3. *Identification/Isolation of the Leukemia-Initiating Cell*

The adaptation of xenotransplantation assays to examine the propagation of AML *in vivo* has allowed the phenotypic identification of the AML-IC. As mentioned above the first demonstration of an *in vivo* repopulating AML-IC involved assessment of the phenotype. In this study it was demonstrated that the only cells capable of engrafting NOD/SCID mice possessed the $CD34^{+}/CD38^{-}$ immunophenotype. This phenotype has been extended via the use of immunodeficient mice to include an absence of

干细胞之家www.stemcell8.cn ←点击进入

CD71, HLA-DR and CD117, but include expression of CD123 (Blair *et al.*, 1997 and 1998; Blair and Sutherland, 2000; Jordan *et al.*, 2000). In a recent study we have extended this phenotype further to include expression of CD33 and CD13 on AML-IC from the vast majority of patients (Taussig *et al.*, 2005). Hence, the extended immunophenotype of the leukemic stem cell as defined by *in vivo* propagation is $CD34^{+}/CD38^{-}/CD71^{-}/HLA\text{-}DR^{-}/CD117^{-}/CD33^{+}/CD13^{+}/CD123^{+}$ (see Fig. 1 for a summary). However, the exclusivity of some markers is debatable. For instance, CD123 is indeed expressed on AML-IC, but is also expressed on the vast majority of AML blasts (unpublished observations) from most patients and hence, could be excluded from the above phenotype of AML-IC.

Apart from immunophenotype, other methods have been utilized to identify the AML-IC. For instance, we have recently published data on the ALDH-1 activity of AML-IC from various AML patients. It seems that in approximately one third of patients, at least a subset of AML-IC possess a high ALDH activity that is detectable using the fluorescent ALDH substrate mentioned previously (Pearce *et al.*, 2005).

4. Key Mechanisms that Regulate HSC Development

An understanding of the factors that regulate normal hematopoietic cell development is essential for a more complete comprehension of leukemogenesis and leukemia. As discussed in the previous section, AML-ICs are very similar to HSCs in terms of phenotype and self-renewal/proliferation, and as will be presented here, many of the mechanisms/factors that are associated with the growth of HSCs are important for the development of AML-ICs and the propagation of leukemia.

4.1. *The Hematopoietic Stem Cell Niche*

The hematopoietic microenvironment consists of bone cells, stromal cells, macrophages, fat cells and extracellular matrix. Stem cells and their immediate progeny interact with the hematopoietic microenvironment. Stem/progenitor cells adhere to stromal cells via adhesion molecules. Cytokines and chemokines are also retained in the microenvironment; they bind to extracellular matrix and some are presented on the surface of stromal cells (Slanicka Krieger *et al.*, 1998). This co-adhesion of progenitors and cytokines to the microenvironment results in the co-localization of progenitors at a specific stage of differentiation with the appropriate array of cytokines, in niches (Verfaillie, 1998).

干细胞之家www.stemcell8.cn ←点击进入

Both cellular as well as extracellular matrix components of the stem cell microenvironment or niche are critical in stem cell regulation. By labelling stem cells and re-infusing them, it has been shown that HSCs reside in close proximity to the endosteal bone surface (Haylock and Nilsson, 2005; Potocnik *et al.*, 2000). Using a combinatorial Gata-2 and Sca-1 expression system, Suzuki *et al.*, confirmed these results using *in vivo* imaging (Suzuki *et al.*, 2006).

The microenvironment has been described to influence survival, proliferation, and differentiation (Haylock and Nilsson, 2005; Rafii *et al.*, 1997). Recently, two genetic studies identify that specialized spindle-shaped N-cadherin$^+$ osteoblasts are a key component of the BM stem cell niche (Calvi *et al.*, 2003; Zhang *et al.*, 2003). Subsequently, advanced imaging studies have demonstrated that the HSCs reside in close proximity to the bone lining osteoblasts (Arai *et al.*, 2004) as well as blood vessels, which may constitute an alternative niche (also called vascular niche) (Kiel *et al.*, 2005). HSCs are thought to anchore to osteoblasts via N-cadherin interaction. However, the molecules that regulate stem cell niche interactions and how these may influence the balance between self-renewal and differentiation are just being to be revealed. Indeed, Arai *et al.* show that the Angiopoietin-1/Tie-2 signalling pathway maintains repopulating quiescent HSC in the niche, presumably via the activation of cell adhesion molecules such as N-cadherin (Arai *et al.*, 2004). Furthermore, the contrasting behavior of HSCs under or overexpressing c-Myc *in vitro* and *in vivo* reported recently, together with the identification of c-Myc-regulated specific cell adhesion molecules suggests that c-Myc controls the interactions between HSCs and their stem cell niche environment (Wilson *et al.*, 2004). This thus raises the possibility that c-Myc may be a negative regulator downstream of the Tie-2 pathway repressing N-cadherin and activating the cell cycle. Two recent studies demonstrate that another osteoblast secreted molecule, osteopontin (Opn), also play a key role in the attraction, retention and regulation of HSC (Nilsson *et al.*, 2005; Stier *et al.*, 2005).

Although osteoblasts have been shown to have a central role in HSC regulation, other stromal and micro-environnmental cell types and extracellular matrix proteins also contribute to this process. Hyaluronic acid as well as the membrane bound form of stem cell factor are also key components of this niche (Driessen *et al.*, 2003; Nilsson *et al.*, 2003). More recently, it has been shown that calcium sensing receptor (CaR) has a function in retaining HSCs in close physical proximity to the endosteal surface and the regulatory niche components associated with it (Adams *et al.*, 2006).

干细胞之家www.stemcell8.cn ←点击进入

Adams *et al.* (2006) demonstrated that antenatal mice deficient in CaR had primitive hematopoietic cells in the circulation and spleen, whereas few were found in bone marrow. $CaR^{-/-}$ HSCs from foetal liver were normal in number, in proliferative and differentiative function, and in migration and homing to the bone marrow. Yet they were highly defective in localizing anatomically to the endosteal niche, behavior that correlated with defective adhesion to the extracellular matrix protein, collagen I.

Knockout of the Rho GTPase Cdc42 results in a lack of HSC retention in the bone marrow and a greater number of HSCs in cycle. This effect is intrinsic to transplantable HSCs and severely affects the transplantation and homing ability of the cells. Thus it seems that Cdc42 is a critical regulator of the adhesion interaction between HSCs and their niche (Yang *et al.*, 2007).

Interestingly, it seems that HSCs are organized in the bone marrow in relation to localized blood flow and oxygenation levels. Utilizing techniques usually employed to assess hypoxia in solid tumors, it has been demonstrated that HSCs localize to areas of least oxygenation in the murine bone marrow. There seems to be a gradient within HSCs, where the most primitive subsets of HSCs, have the greatest hypoxia (Parmar *et al.*, 2007).

Overall, it appears that the regulation of hematopoiesis is the result of multiple processes involving cell-cell and cell-extracellular matrix interactions, the action of specific growth factors and others cytokines as well as intrinsic modulators of hematopoietic development.

4.2. *Role of Negative Regulators of HSC Expansion*

The ability to self-renew is central to the function of normal hematopoietic cells and to the propagation of leukemia. It is thought that although the whole HSC pool is slowly dividing, individual cells are often in quiescence (Bradford *et al.*, 1997). Apart from apoptosis and senescence (which we will not discussed here), a stem cell has three fates: quiescence, self-renewal or differentiation. Since both self-renewal and differentiation require a cell to enter the cell cycle, the control mechanisms of a stem cell that maintain quiescence for the appropriate period and situation is critical for the correct regulation of hematopoietic stem cells and AML-IC.

4.2.1. *Cell cycle regulators*

Assessing the relative expression of various cell cycle regulatory molecules in single HSC, Cheng *et al.* (2001) determined that primitive quiescent HSC had markedly elevated levels of p21 compared with more differentiated

干细胞之家www.stemcell8.cn ←点击进入

cells. Recently using BM from animals deficient in cyclin-dependent — kinase-inhibitors (CKIs), different groups have demonstrated that CKIs exerted a negative control on HSCs.

It has been reported that p21-deficient mice do not exhibit any gross changes in hematopoietic cell output (Deng *et al.*, 1995; Taniguchi *et al.*, 1999). However, analysis of more purified populations of stem cells has revealed some significant changes in the absence of p21. Interestingly, the proportion of murine SP cells that is quiescent (low pyronin-Y staining) is lower in mice that lack the p21 gene when compared to wild-type mice. This increase in proliferating stem cells is consistent with the observed increased sensitivity to the cytotoxic drug 5-FU in $p21^{-/-}$ mice when compared to wild-type mice. In addition, upon serial transplantation, cells from mice that are deficient for the p21 gene are exhausted before cells from wild-type mice. Serial transplant is thought to be an assay for hematopoietic stem cell self-renewal and hence, p21 may have a role in the regulation of proliferation in stem cells: self-renewal. Since knocking out p21 causes a lack of quiescence and exhaustion in stem cells, it may be postulated that p21 usually functions as a negative regulator of proliferation in stem cells.

P18 is also a CKI but is a member of the INK4 gene family (INK4c). P18 and other members of the INK4 family are also involved in the regulation of the entry into the cell cycle. INK4 family members (e.g., p15, p16, p18 and p19) prevent the CDK4/6/cyclinD complex from phosphorylating the RB gene product, and thus blocks cell cycle progression. Hence, p18 is a negative regulator of cell cycle progression that indirectly acts on the same Rb repressor complex as p21, but via a different CDK complex.

P18 is expressed in high amounts in human $CD34^{+}$ cells but not in mature cell types. Mice deficient for p18 exhibit organomegaly with hypocellularity as well as an increased sensitivity to carcinogens (Franklin *et al.*, 1998; Bai *et al.*, 2003). When transplanted, cells from p18-deficient mice exhibit an increase in self-renewal as demonstrated by an increased ability to repopulate irradiated hosts in competition with wild-type cells. $P18^{-/-}$ mice exhibit a 14-fold increase in long-term repopulation and have maintained their multilineage potential in multiple serial transfer recipients for over 3 years with no apparent exhaustion (Yuan *et al.*, 2004; Yu *et al.*, 2005). Hence, it seems that p18 is a negative regulator of self-renewal in HSCs that usually functions to regulate the amount of self-renewal in HSCs.

Intriguingly, it seems that p18 is able to override the effects of p21 knockout on serial transplantation ability. When $p18^{-/-}p21^{-/-}$ mice are competed *in vivo* with $p21^{-/-}$ mice, the double knockout cells out-compete the p21 knockout cells, almost to the level of $p18^{-/-}$ cells. The authors hypothesize

干细胞之家www.stemcell8.cn ←点击进入

that serial transplantation exhausts stem cells in a similar fashion to aging, and that p18 and p21 have opposing roles in stem cell exhaustion. They postulate that p21 normally serves to prevent exhaustion by maintaining cells in quiescence and that p18 deficiency removes control on this exhaustion by an unknown mechanism (Yu *et al.*, 2005).

P16 (INK4A), P19 (INK4D) and p15 (INK4B) are members of the same family and are thought to function in a similar fashion to p18. P16 is highly expressed in $CD34^+$ cells and not in more mature differentiated cells (Furukawa *et al.*, 2000). Although p16 knockout mice do not exhibit any obvious deficiency in hematopoiesis or repopulation (Nakayama and Nakayama, 1998; Serrano *et al.*, 1996), it has been recently demonstrated that p16 has a subtle role in cell cycle control of hematopoietic stem cells. Cells from single knockouts of p16 or p19 do not exhibit any significant hematopoietic deficiency. However, when combined, the dual p16/p19 knockout causes a significant loss in hematopoietic control. Specifically, cells from the dual p16/p19 knockout demonstrated an increased ability to serially repopulate irradiated hosts. Furthermore, mice with the dual p16/p19 knockout had an increased incidence of spontaneous tumors (Stepanova and Sorrentino, 2005).

The MYC-antagonist MAD1 and p27 (Kip1) have been also shown to have a role in regulating the quiescence of HSC (Foley *et al.*, 1998; McArthur *et al.*, 2002). More recently, both molecules seem to regulate HSC self-renewal and differentiation decision in a context-dependent manner (Cheng *et al.*, 2000; Walkley *et al.*, 2005). Under homeostatic conditions, HSC in $MAD1^{-/-}p27^{-/-}$ mice increase in frequency due to an increase of these cells to enter into cell cycle. Under stress conditions, the enhanced capacity of $MAD1^{-/-}p27^{-/-}$ HSCs to enter cell cycle allows for more rapid generation of differentiated cells (Walkley *et al.*, 2005). C-Myb, through p300, has also recently been revealed as a key player in the maintenance of HSC. Using a genome wide N-ethyl-N-nitrosourea (ENU) mutagenesis and phenotype screening, Sandberg *et al.* (2005) identified a mouse line that harbours a point mutation in the trans-activating domain of c-Myb leading to a disruption of its interaction with the transcriptional co-activator p300. HSCs from these mice contain 5 to 10 fold increase in long-term repopulating cells due to an increase in the number of actively cycling stem cells.

4.2.2. *Polycomb genes*

When a hematopoietic stem cell has entered the cell cycle, two fates are possible: self-renewal and differentiation with associated proliferation. Self-renewal is under the control of various regulators. Dysregulated

干细胞之家www.stemcell8.cn ←点击进入

self-renewal would involve an expansion of primitive cells and is probably very important in leukemogenesis.

The polycomb (PcG) group of proteins negatively regulate the expression of key genes in development and differentiation. Two discrete PcG complexes have been identified and are named polycomb repressive complex one (PRC1) and PRC2. PRC2 contains histone deacetylases and histone methyltransferases and hence is involved in the repression of gene expression. The role of PRC1 is less defined, but is thought to be involved in the maintenance of gene repression and can directly interact with PCR2 (Cao *et al.*, 2002; Czermin *et al.*, 2002; van der Vlag and Otte, 1999).

Bmi-1 is a polycomb gene that is a member of the PCR1 complex. Mice deficient for Bmi-1 produce embryonic hematopoiesis, but have a profound defect in adult hematopoiesis. Long-term repopulation is severely impaired when compared to wild-type mice. Indeed, adult hematopoiesis cannot be maintained post-natally and a progressive pan-cytopaenia eventually occurs (Park *et al.*, 2003). Enforced expression of Bmi-1 in adult hematopoietic cells results in a marked increase in long-term repopulation mediated by an increased symmetrical cell division by hematopoietic stem cells (Iwama *et al.*, 2004). Although it is not known how the PCR1 complex acts *in vivo*, Bmi-1 target genes have been identified as p16 and p19 (Molofsky *et al.*, 2005). Bmi-1 is thought to usually repress the expression of p16 and p19 and to negatively regulate progression into the cell cycle. Hence, Bmi-1 may be thought of as a regulator of hematopoietic stem cell self-renewal in adult hematopoiesis.

Enhancer of zeste homolog 2 (Ezh2), is another member of the polycomb group that has been shown to be an important regulator of stem cell exhaustion (Kamminga *et al.*, 2006). De Haan's group showed that whereas normal HSCs were rapidly exhausted after serial transplantations, overexpression of Ezh-2 in mice completely conserved long-term repopulating potential. This data suggests that stabilization of the chromatin structure preserves HSC potential after replicative stress. Similar impaired repopulation has been reported in Rae28 (another Polycomb gene)-deficient mice following transplantation of foetal liver (Ohta *et al.*, 2002).

4.2.3. *Other master regulators of HSC self-renewal*

Recent work on Gfi-1 (growth factor independent-1) performed by Zeng *et al.* and Hock *et al.* revealed that Gfi-1 deficiency leads to an exhaustion of the HSCs in adult mouse bone marrow, presumably through unrestricted cycling of these cells (Hock *et al.*, 2004; Zeng *et al.*, 2004).

干细胞之家www.stemcell8.cn ←点击进入

Nephroblastoma overexpressed 3 (NOV3, CCN3) is a signalling protein that is associated with the undifferentiated stem cell state. RNAi knockdown studies have revealed a central role for NOV3 in the regulation of HSC self-renewal. Under-expression of NOV3 severely impairs the ability of HSCs to repopulate primary hosts, whereas over-expression enhances the potential of HSCs. Since NOV3 under-expression causes a severe reduction in the expression of both Bmi-1 and Gfi-1, it may be stated that NOV3 functions as a master regulator of HSC self-renewal (Gupta *et al.*, 2007).

A recent paper has also implicated the protein E4F1 as a key regulator of Bmi-1. It seems that expression of Bmi-1 and E4F1 have opposing effects on cellular growth. A reduction in the expression of E4F1 serves to rescue the proliferative defects caused by Bmi-1 knockout, without changing the levels of downstream effectors such as INK4a/arf and p53. Thus, E4F1 directly interacts with Bmi-1 to regulate cellular proliferation (Chagraoui *et al.*, 2006).

Zfx is a zinc finger protein that is expressed in HSCs and has recently been reported to have a fundamental function in their self-renewal. A conditional knockout of Zfx resulted in increase in apoptosis and an up-regulation of stress response genes, which led to a progressive loss of HSCs in adult hematopoiesis. Hence, it seems that proper Zfx function is required for the maintenance of adult hematopoiesis, possibly for a correct cellular response to stress (Galan-Caridad *et al.*, 2007).

Forkhead box O (FoxO) transcription factors are also involved in the cellular response to stress but seem to have a role in the control of HSC self-renewal. Simultaneous conditional knockout of FoxO1, FoxO3 and FoxO4 affects the sensitivity of HSCs to reactive oxygen species (ROS), increases their apoptosis rate and stimulates HSCs out of quiescence and into the cell cycle. This results in a reduction in the numbers of HSCs and a severe impairment of their repopulation ability, ultimately leading to stem cell exhaustion. Much of this phenotype can be corrected by administration of an anti-oxidant that reduces cellular concentration of ROS. Hence, it seems that the normal function of FoxO1, FoxO3 and FoxO4 is to maintain HSCs in an appropriate, mostly quiescent, but self-renewing state in response to cellular levels of ROS (Tothova *et al.*, 2007).

4.3. *Positive Regulators of HSCs*

4.3.1. *SCL/TAL*

SCL or TAL-1 is a class II basic helix-loop-helix (bHLH) transcription factor. SCL mRNA is expressed at low levels in primitive human $CD34^+/CD38^-$

干细胞之家www.stemcell8.cn ←点击进入

stem cells, is up-regulated in CMP and MEP progenitor cell populations, but is down-regulated in the GMP cell population (Zhang *et al.*, 2005). SCL knockout mice fail to develop either primitive or definitive hematopoiesis, indicating an important role in very early hematopoiesis, possibly in its initiation (Shivdasani *et al.*, 1995). A central role for SCL in adult definitive hematopoiesis has been confirmed by the failure of $SCL^{-/-}$ embryonic stem cells injected into normal blastocysts to form any hematopoietic cells (Robb *et al.*, 1996). Conditional knock-in experiments utilizing the Cre-LoxP system have revealed that although required for the initiation of hematopoiesis, SCL is not required for the self-renewal of long-term repopulating cells (LTRCs) as defined by serial transplantation. Furthermore, SCL is required for erythroid/megakaryocyte differentiation, but not for the development of myeloid (macrophage/granulocyte) or lymphoid cells (Mikkola *et al.*, 2003). However, enforced expression of SCL in human $CD34^{+}$ cells does significantly affect their *in vivo* repopulation ability (NOD/SCID). This indicates that SCL expression levels may regulate self-renewal in human stem cells (Reynaud *et al.*, 2005). In summary, although SCL has an important role in the initiation of hematopoiesis, its role in the self-renewal of HSCs is dispensable but important.

4.3.2. *Homeobox genes: Hox B3, B4 and A9*

Mammalian Hox gene family members encode DNA binding transcription factors, which all contain a conserved homeobox region homologous to *Drosophila* proteins and play a crucial role in mammalian embryonic axis formation. Human Hox genes are present on different chromosomes in four major clusters, A, B, C, and D, each of which consists of 9-12 tandem genes (Scott, 1992). Many of these genes are involved in hematopoiesis including, HoxA5, HoxA9, HoxA10, HoxB3, HoxB4 and HoxB6.

Mice deficient for HoxB3 and HoxB4 have a reduced overall hematopoietic cell output but normal differentiation of progenitors occurs (Bjornsson *et al.*, 2003). HoxB3 and HoxB4 are expressed in primitive hematopoietic cells, but not in mature cell types. Consistently, HoxB4 over-expression causes an increase in the regeneration of transplantable HSCs. Interestingly, this increase in repopulation potential did not include an increase in mature cells and progenitors, but was restricted to primitive HSCs. Hence, it is thought that HoxB4 functions as a positive regulator of HSC self-renewal (Sauvageau *et al.*, 1995). It has been determined that the DNA binding domain of HoxB4 is required for this function (Beslu *et al.*, 2004). More recently, in an effort to avoid possible deleterious effects of genetically manipulating HSCs,

干细胞之家www.stemcell8.cn ←点击进入

stromal cells have been engineered to secrete HoxB4. Co-cultures of human CD34$^+$CD38$^-$ cells on stromal cells secreting HoxB4 underwent a 20-fold increase in long-term initiating cells (LTC-IC) and 2.5 fold increase in SRC (Amsellem *et al.*, 2003). Similar results were obtained when mouse BM cells were grown on stromal cells engineered to express HoxB4 fused to the HIV transactivating protein TAT (Krosl *et al.*, 2003).

Over-expression of HoxB3 causes an impairment in lymphoid cell production, but increases myelopoiesis and eventual myeloproliferation (Sauvageau *et al.*, 1995). HoxA9 is expressed in CD34$^+$ hematopoietic cells and in maturing lymphocytes. Mice deficient for HoxA9 have a reduced multilineage hematopoietic cell output and smaller hematopoietic organs such as the spleen and thymus when compared to wild-type mice (Lawrence *et al.*, 1997). Furthermore, although initially thought to be unaffected, the long-term repopulation ability of HSCs from mice deficient for HoxA9 is severely impaired (Lawrence *et al.*, 2005).

4.3.3. *Mesodermal inducing factors in the regulation of HSC fate*

The Notch pathway is an environmental signalling system that may play an important role in HSC niche (Suzuki and Chiba, 2005). Notch can affect cells' fate by regulating transcription directly via association with nuclear factors and can thus affect the growth and differentiation of HSCs. Incubation of human HSCs with the soluble Notch ligands, Jagged1, have been shown recently to drive *in vitro* HSC self-renewal. When injected into NOD/SCID mice, Jagged1-treated HSCs were capable of reconstitute lymphoid and myeloid differentiation (Karanu *et al.*, 2000). Furthermore, the constitutive expression of Notch 1C using retroviral vector is sufficient to generate clonal population of immortalized HSCs (Varnum-Finney *et al.*, 2000). Recently, Notch signalling has been shown to play a dominant function in differentiation inhibition. Using transgenic Notch reporter mice, Duncan *et al.* (2005) found that Notch signalling is active in HSCs *in vivo* and downregulates as HSCs differentiate. Furthermore, intact Notch signalling is required for Wnt-mediated maintenance of undifferentiated HSCs but not for survival or entry into cell cycle *in vitro* (Duncan *et al.*, 2005).

Other factors that play a role in the organization of early mesoderm and embryonic specification of non-hematopoietic tissues are the Hedgehog (Hh) family of proteins which comprises three proteins [Sonic Hedgehog (Shh), Indian Hedgehog (Ihh) and Desert Hedgehog (Dhh)] and two primary receptors [Smoothened (Smo) and Patched (Ptc)]. Hh ligand binding results in Smo activation, with subsequent signalling events that modulate

干细胞之家www.stemcell8.cn ←点击进入

the expression of genes such as Wnt2A, BMP-4 inhibitor, Noggin (Polakis, 2000; Ruiz i Altaba *et al.*, 2002). Expression of Shh, Ptc and Smo has been detected in primitive human HSCs (Bhardwaj *et al.*, 2001). Antibodies to Hh blocked cytokine-induced proliferation of HSCs, while addition of soluble Shh resulted in an increase in stem cell numbers as measured by the NOD/SCID repopulating cell (SRC) assays. The mitogenic effect of Smo was abrogated by Noggin, demonstrating that Shh acts via BMP-4 in primitive HSC proliferation (Bhardwaj *et al.*, 2001). High levels of BMP4 has also been shown to enhance stem cell survival in an *ex vivo* culture (Bhatia *et al.*, 1999).

Another system involves the frizzled receptors and their Wnt ligands. Transduction of primitive mouse HSCs with an activated form of β-catenin results in improved survival and expansion of HSCs *in vitro* which is accompanied by an increase of Notch1 and HoxB4 levels, implying these molecules as downstream effectors (Reya *et al.*, 2003). More recently, the direct application of purified Wnt3A has been utilized to successfully expand HSCs (Willert *et al.*, 2003). Glycogen synthase kinase 3 (GSK-3), a constitutively active serine threonine kinase, has been thought to be involved in the regulation of several pathways, including Wnt, Hedgehog and Notch pathways. Recently, it has been shown that upon GSK-3 kinase inhibitor treatment, primitive HSCs and progenitors increase without altering secondary repopulation, suggesting that the HSC pool is maintained while overall hematopoietic reconstitution is increased (Trowbridge *et al.*, 2006).

The molecular mechanisms by which all these factors interact to regulate stem and progenitor cell self-renewal remain to be elucidated.

4.4. *Differentiation Control*

Once a cell has left quiescence and entered the cell cycle, three fates are possible: self-renewal, differentiation and apoptosis. Factors that affect the cell fates of differentiation will be presented next.

AML-1/Runx-1 is a transcription factor that is situated on chromosome 21. AML-1 dimerizes with core binding factor beta (CBFβ) subunit to form a transcriptional protein complex that regulates the expression of lineage-specific genes such as IL-3, GM-CSF, M-CSF, elastase and myeloperoxidase (Lutterbach *et al.*, 2000). Mice deficient in AML-1 do develop primitive hematopoiesis, but fail to produce any detectable definitive hematopoiesis (Okuda *et al.*, 1996; Wang *et al.*, 1996a). Conditional knockout experiments have revealed that AML-1 is not required for the self-renewal

干细胞之家www.stemcell8.cn ←点击进入

of HSCs, as evidenced by long-term repopulation of irradiated hosts. However, in these experiments, megakaryocyte and lymphocyte development is dependent on the presence of AML-1, whereas other myeloid cells do not require AML-1 for their development (Ichikawa *et al.*, 2004).

As mentioned previously, core binding factor beta (CBFβ) is a one of the co-factors that is required for the function of AML-1/Runx-1. Not surprisingly, CBFβ deficiency results in a very similar phenotype in mutated mice to AML-1 deficiency (Wang *et al.*, 1996b).

The members of the GATA family of genes (GATA-1 to 6) all contain a homologous region that binds to a DNA sequence in the target gene called a GATA motif (Trainor *et al.*, 1996). GATA-1 is expressed in erythroid cells/megakaryocytes and mast cells as well as low levels in hematopoietic stem cells (Pevny *et al.*, 1991). Disruption of the GATA-1 gene results in severe impairment of differentiation to the erythroid/megakaryocyte lineage. Conditional knockouts have confirmed the essential role of GATA-1 in the control of thrombopoiesis/erythropoiesis (Shivdasani *et al.*, 1997; Takahashi *et al.*, 1997).

GATA-2 is expressed in high amounts in hematopoietic stem cells and early stages of erythroid/megakaryocyte and mast cell differentiation (Dorfman *et al.*, 1992; Leonard *et al.*, 1993; Masuda *et al.*, 2004; Orlic *et al.*, 1995). Knockout of GATA-2 results in a total hematopoietic deficit and embryonic lethality at day 10 (Tsai *et al.*, 1994). Conditional knockout of GATA-2 has suggested that GATA-2 regulates the growth of primitive hematopoietic cells (Heyworth *et al.*, 1999). Recently, it has been demonstrated that GATA-2 inhibits the degradations of p21 and p27 and cause their accumulation in cells. In this way, GATA-2 may be a regulator of the growth of hematopoietic stem cells (Ezoe *et al.*, 2002). GATA-3 is expressed exclusively in T-cells and GATA 4 to 6 are not expressed in the hematopoietic system (Patient and McGhee, 2002).

PU.1 is a basic Helix Loop Helix transcription factor that is a member of the Ets family of proteins. PU.1 is highly expressed in mature B-cells and myeloid cells and at a lower level in HSCs and CLP and CMP cell populations (Akashi *et al.*, 2000; Klemsz *et al.*, 1990). Mice deficient for the PU.1 gene have a multilineage deficit in hematopoietic output that is most profound in neutrophils/monocytes and results in embryonic lethality (McKercher *et al.*, 1996; Scott *et al.*, 1994). Conditional knockouts of the PU.1 gene (Cre-loxp) and subsequent sorting/assessment of various stages of hematopoiesis have revealed some interesting results. Although originally thought to be a myeloid-specific factor, PU.1 also has an important role in HSCs. HSCs

干细胞之家www.stemcell8.cn ←点击进入

deficient in PU.1 competed poorly with wild-type HSCs in a long-term repopulation model. Formation and function of the primitive CMP and CLP cell populations were severely reduced in cells deficient for PU.1. Hence, although only expressed at lower levels in HSCs and CLPs, PU.1 has an indispensable role in their function (Iwasaki *et al*., 2005).

Antisense nucleotides to HoxA5 reduce myeloid output and increase erythroid cell production (Fuller *et al*., 1999). Conversely but consistently, enforced expression of HoxA5 in human hematopoietic cells causes a reduction in erythroid differentiation and an increase in myeloid cells (Crooks *et al*., 1999). Hence, HoxA5 may play a role in early progenitors in the switch between erythroid and myeloid differentiation. HoxA10 overexpression causes some profound changes in hematopoiesis. Lymphoid and macrophage progenitors were reduced but many progenitors with megakaryocyte potential were present (Thorsteinsdottir *et al*., 1997). Mice deficient for the HoxB6 gene feature an increase in erythroid progenitors, but not in total erythoid cell numbers (Kappen, 2000). Consistent with a function in erythroid cell differentiation, HoxB6 expression decreases during erythroid differentiation of K562 cells (Shen *et al*., 1992). Furthermore, HoxB6 represses the expression of both alpha and beta globin genes in K562 cells (Shen *et al*., 2004).

HoxC4 mRNA is present in HSCs and during B and T-cell differentiation. HoxC6 is also found in maturing B and T-cells. HoxC5 is present in erythroleukemia cell lines and some cases of non-Hodgkin's lymphoma but is not detectable in normal $CD34^+$ cells (Bijl *et al*., 1996).

Hox gene products function *in vivo* as members of a multiprotein complex that includes other homeobox proteins. Two major families have been identified as members of this complex and are encoded by the myeloid ectopic insertion site (Meis 1-3) and pre-B-cell homeobox (Pbx) genes (Mann and Affolter, 1998). Although Hox proteins may bind to DNA directly, enhanced DNA binding is thought to occur when Hox gene products function as part of this complex (Lu and Kamps, 1997). Although there are 39 members of the Hox gene family in humans, there are only 3 Meis and 4 Pbx gene products that interact with Hox proteins (Grier *et al*., 2005; Mann and Affolter, 1998). Hence, Meis and Pbx proteins may be thought of as co-factors of Hox proteins and may serve to affect the activity/specificity of Hox proteins (Mann and Affolter, 1998). Although Pbx mutants do exhibit mild abnormalities, they do not seem to have affected hematopoiesis, indicating that Pbx may not be important for the control of Hox genes (DiMartino *et al*., 2001). Mice deficient for Meis-1 do not produce

干细胞之家www.stemcell8.cn ←点击进入

any definitive hematopoiesis and die mid-gestation (Azcoitia *et al.*, 2005). Indeed, Meis-1 is usually expressed in the aorta-gonad-mesonephros (AGM) mesenchyme, in hemogenic endothelium and in the first hematopoietic clusters to appear in the AGM (Azcoitia *et al.*, 2005).

CAAAT/enhancer-binding protein alpha (C/EBPα) is a transcription factor that has a central role in myeloid differentiation. C/EBPα is expressed in primitive myeloid cells and levels decrease as cells mature (Scott, 1992). Mice deficient for the CEBPA gene do not develop neutrophils and exhibit reduced G-CSF signalling (Zhang *et al.*, 1997). C/EBPα is produced in two forms, a complete form that inhibits cell proliferation and differentiation (p42) and a truncated isoform that lacks the N-terminus, which does not (p30) (Lin *et al.*, 1993).

Transcription factors can repress or allow expression of target genes. As mentioned above, they often require co-factors or interact with other transcription factors. Some transcription factors regulate the expression of other transcription factors. Indeed, PU.1, GATA-1 and SCL all have *cis* regulatory elements, suggesting transcriptional regulation (Rosenbauer *et al.*, 2005 and 2006). In this way, additional levels of control are exerted on gene expression regulation. Furthermore, it is now becoming apparent that there is considerable, more direct interplay between transcription factors.

As mentioned above, PU.1 is required for myeloid differentiation and GATA-1 is indispensable for erythroid/megakaryocyte differentiation. Enforced GATA-1 expression suppresses the myeloid phenotype induced by PU.1 without interfering with PU.1 gene expression. GATA-1 protein directly interacts with PU.1 protein in a dose dependent manner via a discrete domain (Nerlov *et al.*, 2000; Zhang *et al.*, 2000). Additional examples of cross-antagonisms between lineage specific transcription factors, or their cofactors are beginning to emerge and reveal novel mechanisms (see review: Cantor and Orkin, 2001). Thus, it appears to be a dynamic balance of forces that ultimately determine the phenotype of a cell.

4.5. *Epigenetic Factors*

Further to the regulation of transcription factors expression and function by other transcription factors, there are other levels of gene-expression regulation. Epigenetic alterations to histones and DNA-methylation states have an important role in the regulation of gene expression in hematopoiesis. Such chromatinian modifications are mitotically inherited, but are not passed onto offspring. DNA methylation is thought to prevent gene expression via the establishment of a silent chromatin state by collaborating with

干细胞之家www.stemcell8.cn ←点击进入

molecules that modify nucleosomes (Wolffe and Hayes, 1999). Methylation occurs on the cytosine residues of the dinucleotide sequence CpG and is performed by DNA methyltransferases (Dnmt). Histone modifications occur post-translation and involve histone methyltransferases and acetyltransferases.

4.5.1. *Methylation and histone modifications*

The mixed lineage leukemia gene-1 (MLL1) is a histone methyltransferase that has an important role in the regulation of gene expression in hematopoietic stem and progenitor cells, possibly in an antagonistic role to the polycomb gene products. The MLL1 gene is a member of the family that shows homology to the trithorax group of *Drosophila* genes. These genes are thought to be responsible for the positive maintenance of gene expression through histone modifications. Indeed, the MLL1 gene product has a SET domain (named in *Drosophila* as Su(var)3-9, enhancer of zeste and trithorax) that is thought to be responsible for histone methyltransferase activity (Milne *et al.*, 2002; Nakamura *et al.*, 2002). It is thought that MLL1 adds methyl groups to the 4th lysine on histone H3. Hyper (tri) methylation allows the transcription of certain genes (Santos-Rosa *et al.*, 2002). MLL1 is part of a very large complex that interacts with too many proteins to list here, but we will list some of the interactions that may be important in hematopoiesis and leukemogenisis. MLL1 interacts with DNA via three AT hooks on the N-terminus (Zeleznik-Le *et al.*, 1994). The repressor domain contains elements that are responsible for Dnmt activity, recruitment of the Bmi-1-containing complex and mediating transcriptional repression through recruitment of histone deacetylases (Xia *et al.*, 2003; Zeleznik-Le *et al.*, 1994). For proper function, MLL1 is cleaved into two polypeptides that subsequently re-associate non-covalently and this is thought to confer stability to the N-terminus portion and the correct localization to the C-terminus fragment (Hsieh *et al.*, 2003).

The MLL1 gene is thought to be responsible for the regulation of Hox gene expression (Popovic and Zeleznik-Le, 2005). Indeed, the direct interaction of HoxA9 and HoxC8 with the SET domain of MLL1 has been reported (Milne *et al.*, 2002; Nakayama and Nakayama, 1998). Mice deficient in the MLL1 gene fail to develop beyond day 11 of gestation and have various defects in limb development (Yu *et al.*, 1998). Heterozygous mutants develop poorly and are anaemic, indicating a role for both copies of the MLL1 gene (Yu *et al.*, 1995).

干细胞之家www.stemcell8.cn ←点击进入

4.5.2. *Micro-RNAs and hematopoietic regulation*

Micro-RNAs (miRNAs) are endogeneously expressed, non-coding RNAs, approximatively 22 nucleotides in length that interact with native coding mRNAs to inhibit translation. miRNAs regulatory roles in diverse developmental and physiological events, and disease pathogenesis have become evident in the last few years (Bartel, 2004). However, it remains essential to define their roles in normal haematopoeisis and leukemogenesis. *In vitro* and *in vivo* ectopic expression of miRNAs (miR-142, 181 and 233) in murine hematopoietic stem cells dramatically altered the proportion of differentiated lineages (Chen *et al.*, 2004). These data suggest that miRNA are lineage-specific and important components in murine lineage commitment. Indeed, it seems that genes responsible for lineage commitment are expressed by human hematopoietic stem and progenitor cells, but are repressed by miRNAs. Specifically, miRNA-155 blocks the expression of genes involved with myeloid and erythroid differentiation of human $CD34^+$ cells (Georgantas *et al.*, 2007).

Studies from the last three years have demonstrated that there is altered expression of miRNA genes in several human blood cancer, including chronic lymphoblastic leukemia, pediatric Burkitt lymphoma, large cell lymphoma (review in: Gregory and Shiekhattar, 2005; McManus, 2003). One recent study reveals that miRNAs may be important for enabling stem cells to overcome the G1/S checkpoint of the cell cycle (Hatfield *et al.*, 2005). Hatfield *et al.* show that the miRNA pathway regulates stem cell division in the fruit fly *Drosophila melanogaster*. It is clear now that the over-expression or down-regulation of miRNA genes is important in the development of cancer. Changes in miRNA gene expression between normal and leukemic cells could be exploited, not only for diagnostic and prognostic purposes but also to investigate the specific roles of various miRNAs in leukemogenesis. Given that miRNAs regulate gene expression and enter readily into cells, they might represent new therapeutic targets to treat leukemia. Nevertheless, before this step is taken, important questions remain to be answered. For example, which mechanisms regulate miRNA gene expression in normal hematopoietic cell development and which go awry during leukemogenesis? What are the miRNAs that are differentially expressed between normal and leukemic stem cells? It is becoming clear that some miRNAs act as tumor suppressors and thus an improved understanding of this class of RNAs and their potential function is required (Hammond, 2007).

干细胞之家www.stemcell8.cn ←点击进入

5. Dysregulation of Key Processes in AML

Not surprisingly, when disrupted, processes so central to the function of hematopoietic stem cells are often lethal or can cause leukemia. Indeed, many of the genes and their products presented in the previous section were originally identified due to their association with or dysregulation in AML. In this section, we will discuss some examples of such dysregulaton.

Knockout of p18 and p21 does not cause leukemia directly in mice (Deng *et al.*, 1995; Yu *et al.*, 2005). Direct mutations of p21, p16, p15 are rare in AML and p27 deletions are present in a minority of AML cases (5%) (Drexler, 1998; Hayette *et al.*, 1997). However, P18 is expressed in higher levels in acute myeloid leukemia cells when compared to non-malignant differentiated hematopoietic cells indicating that this gene may be dysregulated in AML. In fact, it is often the upstream regulators of these fundamental mechanisms that are dysregulated in AML.

Situated on chromosome 11 band q23, the MLL1 gene is an extremely common target of chromosomal translocations in AML. Indeed, over 50 fusion partners have been identified for MLL1translocations. The involvement of MLL1 in a translocation generally indicates a poor prognosis regardless of the fusion partner (Ayton and Cleary, 2001). Over 80% of all MLL1-associated leukemias are caused by fusion to one of six genes: AF10, AF9, ENL, ELL, AF6 and AF4. The remaining 20% of MLL1-associated leukemia involve various other fusion proteins.

It has recently been established that either MLL-ENL or MLL-AF9 fusion proteins alone are sufficient to initiate human leukemia. Retroviral-mediated transduction of MLL-ENL into human, lineage-negative cord blood cells that are transplanted into immunodeficient mice, results in a pro B-cell acute lymphoblastic leukemia. Transduction of MLL-AF9 generates both a pro B-cell acute lymphoblastic leukemia and myelomoncytic and monoblastic acute myeloid leukemias, consistent with the disease that occurs in the majority of patients with MLL-AF9 leukemias. Interestingly, these experimental models were capable of responding to environmental cues and switching lineages from lymphoid to myeloid, also consistent with the behavior of human disease (Barabe *et al.*, 2007).

Fusion partners may provide a domain that is usually present in the MLL1 gene, but is lost in the truncated version. Alternatively, MLL1 may be directly fused to a protein that usually interacts with the MLL1 gene product. For instance, MLL1 usually interacts with the p300 complex and in the MLL-CBP fusion this association in maintained (Ernst *et al.*, 2001). Another possible mechanism for the leukemogenic properties of MLL1 fusion proteins

干细胞之家www.stemcell8.cn ←点击进入

is dimerizaton. The LacZ gene product is a β-galactosidase, an enzyme that only functions as a tetramer. When LacZ is knocked into the MLL1 gene locus at the main break point (MBR), AML is initiated. These knock-in mice express an MLL-β-Galactosidase that has enzyme activity, indicating that the MLL-β-Galactosidase protein must exist as a tetramer (Dobson *et al.*, 2000). Truncation of the MLL1 gene at the same MBR did not produce leukemia, suggesting that it is the dimerization of MLL1 that is important for leukemogenesis (Corral *et al.*, 1996). Indeed, many of the pathogenic MLL1 fusion proteins may facilitate its dimerization. For instance, AF10 and AF17 both have leucine zipper motifs (Chaplin *et al.*, 1995; Prasad *et al.*, 1994) and in the case of AF10, this motif has actually been demonstrated to be required for MLL-mediated leukemogenesis (DiMartino *et al.*, 2002).

As mentioned previously, the MLL1 gene is thought to be responsible for the regulation of Hox gene expression (Popovic and Zeleznik-Le, 2005). Indeed, it is thought that the dysregulation of Hox gene expression is the reason for the leukemogenic properties of MLL1 fusion proteins. Expression levels of HoxA7, HoxA9, HoxA10 and HoxA11 are increased when cell lines are transduced with the MLL1 fusion genes MLL-ENL, MLL-AF6, MLL-CBP, MLL-ELL and MLL-AF10. Interestingly, expression of Meis-1, a known cofactor of Hox protein function, is also increased when murine cell lines are transduced with various MLL1 fusion proteins (Ayton and Cleary, 2003). Furthermore, through the use of mice deficient for Hox genes, it has been demonstrated that HoxA7 and HoxA9 are important for the leukemic transformation of hematopoietic progenitors by the MLL-GAS7 protein (So *et al.*, 2004).

Menin, a product of the MEN1 gene, usually interacts with MLL1 *in vivo*. Menin may be thought of as a tumor suppressor gene as mutations in this gene have been linked to various solid tumors (Chandrasekharappa *et al.*, 1997). Knockout of Menin results in embryonic lethality in mice, but heterogeneous mutants develop a variety of endocrine tumors associated with a loss of heterozygosity (Bertolino *et al.*, 2003a and 2003b).

It has recently been demonstrated that menin is involved with the pathogenic role of MLL1 fusion proteins. It has been revealed that menin interacts with the MLL1 fusion protein MLL-AF6 and bind to the Hox genes HoxA7, HoxA9 and HoxA10 in human cell lines (Yokoyama *et al.*, 2005). It appears that menin is required for the transformation and maintenance of leukemia generated by the MLL-ENL, MLL-GAS7 and MLL-AF10 fusion proteins. Conditional knockout of menin in transformed cells resulted in a loss of leukemic clonogenic potential in cells transformed by MLL1

干细胞之家www.stemcell8.cn ←点击进入

oncoproteins. The loss of menin after MLL-based transformation occurred, resulted in the removal of the differentiation block and the onset of terminal differentiation (Yokoyama *et al.*, 2005).

The caudel-type homeobox (Cdx2) gene forms a transcription factor that acts directly on Hox gene regulatory elements to control certain aspects of embryonic development. Cdx2 is expressed in leukemic cells from the vast majority (90%) of AML cases, but not in non-malignant HSCs. Overexpression of Cdx2 in non-malignant murine HSCs causes a transplantable myeloid leukemia. Whereas knockdown of Cdx2 in certain human leukemia cell lines resulted in profound growth retardation. These data indicate that CDx2 is dysregulated and important in the pathogenesis of AML (Scholl *et al.*, 2007).

The t[8,21] mutation is one of the most common abnormalities in AML, accounting for almost 10% of cases (Grimwade *et al.*, 1998). The t[8,21] mutation results in the AML-1-ETO fusion protein. ETO is the human homologue of the drosophila NERVY protein (Era *et al.*, 1995). As mentioned previously, the AML-1 fusion gene product usually serves to positively regulate the expression of various lineage-specific genes. The AML-1-ETO fusion protein may also serve as a transcriptional activator, but interestingly, has been demonstrated to actually repress the function of wild-type AML-1 protein via an interaction with C/EBPα (Pabst *et al.*, 2001). Consequently, most patients have only one allele that is affected (Lowenberg *et al.*, 1999). Further, downstream affects have been reported for AML-1-ETO. The AML-1-ETO fusion protein directly inactivates the myeloid transcription factor PU.1. This interaction occurs due to the displacement of c-jun from the PU.1 protein by AML-1-ETO (Vangala *et al.*, 2003).

Interestingly, the AML-1-ETO fusion protein is thought to downregulate C/EBPα and this may contribute to leukemogenesis. Patients with the AML-1-ETO fusion protein have a lower expression of C/EBPα than patients from other groups. Expression of AML-1-ETO in U937 cells causes a down-regulation of CEBPα while enforced expression of CEBPα in AML-1-ETO^{+} Kasumi cells causes their neutrophil differentiation (Pabst *et al.*, 2001).

Mutations in the C/EBPα gene are reportedly found in almost 9% of AML patients, mostly within patients without another detectable abnormality (Nerlov, 2004). Mutations cluster primarily to two regions. N-terminal mutations cause production of the p30 isoform. Mutations within the C-terminal region interfere with protein dimerization and DNA binding (Leroy *et al.*, 2005). The p30 form of C/EBPα interferes with the normal control of cell proliferation during myeloid differentiation by inhibiting DNA binding

干细胞之家www.stemcell8.cn ←点击进入

of wild-type C/EBPα (Pabst *et al.*, 2001). Interestingly, analysis of familial AML has revealed that C/EBPα mutations may be a primary event in some leukemias (Smith *et al.*, 2004).

Inversion of chromosome 16 is the second most common abnormality in AML, accounting for 8% of cases. The inversion results in a fusion of most of CBFβ and a smooth muscle myosin heavy chain (MYH-11) (Liu *et al.*, 1993). As mentioned previously, CBFβ is a cofactor of AML-1 that is required for proper DNA binding and transcriptional regulation by the CBF heterodimer. Myosin usually exists as a dimer and the CBFβ-MYH-11 fusion results in dimerization of most of CBFβ. The CBFβ-MYH-11 fusion protein can still interact with AML-1 and this dimerization is thought to be important for leukemogensis. Possible mechanisms include: sequestering AML-1 to the cytoplasm, prevention of degradation of AML-1 and direct repression of gene expression (Huang *et al.*, 2001 and 2003; Lukasik *et al.*, 2002; Shigesada *et al.*, 2004). Interestingly, it has also been reported that HoxA9 levels are low in inv(16) cases, when compared to other subtypes of leukemia (Debernardi *et al.*, 2003).

6. The cell of Origin in Leukemia

An important question for a more complete understanding of leukemogenesis is the cellular origin of the leukemic stem cell. Although candidate CSCs have been identified in many hematological as well as solid tumors, their normal cellular origins remains in most cases elusive (Fialkow *et al.*, 1978; Nilsson *et al.*, 2000). The importance of establishing this is not only to better understand how the functional properties (such as self renewal potential) of the cellular targets of primary and subsequent transforming genetic events (which might occur at the same or distinct developmental and commitment stages) can impact on the biology of the disease, but also on therapeutic strategies. As will be discussed later, there are various pieces of logical and observational evidence to support a stem cell origin for leukemia, but surely the leukemogenic abnormality plays an important role.

The fundamental point is whether the leukemogenic event dictates the cell phenotype or whether the cell type in which this mutation occurs may influence the phenotype of the abnormality. One can envisage different situations where either or both the cell type and mutation may dictate the phenotype of the malignancy. The expression of a particular mutation could be controlled by a cell specific transcription factor. It is also possible that an abnormality only has the opportunity to occur in a certain cell type. Furthermore, it may be that a mutation is molecularly dependent on a cell-specific

干细胞之家www.stemcell8.cn ←点击进入

co-factor. Alternatively, if to cause leukemia, a mutation did not require a cell-specific co-factor or required a co-factor that was expressed in all cell types, then the cell type this abnormality occurred in would be less influential on the cell phenotype of the malignancy. One can imagine how this last "dependency" situation is particularly applicable to mutations that occur in HSC, but are only leukemogenic in certain cell types.

SPI-B is usually expressed in lymphocytes, but can interchange with PU.1 in myeloid development. So the role of PU.1/SPI-B in cells is interchangeable, but specificity of their expression is dependent on cell type (Rosenbauer *et al.*, 2005). The same may be true for fusion proteins — some may affect the same cell processes in multiple cell types and some may only affect certain cell types due to the lack of expression of co-factors in other cell types.

This question has been addressed in a more direct fashion through the use of retroviral-mediated gene transfer and cell fractionation. Cozzio *et al.* have tested the leukemogenic potential of the MLL-ENL fusion protein in purified HSC, CMP, GMP and MEP cell populations (Cozzio *et al.*, 2003). Transduction of HSC, CMP and GMP cells by MLL-ENL reportedly produces a similar leukemia as defined by phenotype and leukemogenic latency, whereas transduction of MEP cells did not confer any increase in proliferative or leukemogenic potential (see Fig. 2). Hence, in the case of MLL-ENL fusion protein, it seems that transformation of stem cells and myeloid progenitors is possible, but transformation of erythroid progenitors is not. Presumably, this is due to the selective expression of a factor that interacts with the MLL-ENL fusion protein in myeloid but not erythroid progenitors. In a similar study by So *et al.* (2003), almost identical results were generated with the MLL-GAS7 fusion protein. HSC, CMP and GMP cell subsets were susceptible to MLL-GAS7 — mediated transformation, whereas MEP cell populations could not be transformed (Fig. 2). Interestingly, bi-phenotypic leukemia was generated with the MLL-GAS7 fusion protein when HSCs were transformed, whereas transduction of CMP and GMP populations resulted in myeloid leukemia only (So *et al.*, 2003).

These results confirm that the cell type is important for the leukemogenic effect of certain fusion proteins. However, retroviral-mediated transduction of fusion proteins into various stages of hematopoiesis is a very artificial model, may not represent the leukemogenic event *in vivo* and hence the leukemia obtained may not behave in a similar fashion. For instance, the phenotype of leukemias generated from HSC, CMP and GMP cell populations is very uniform with no apparent hierarchy (Cozzio *et al.*, 2003).

干细胞之家www.stemcell8.cn ←点击进入

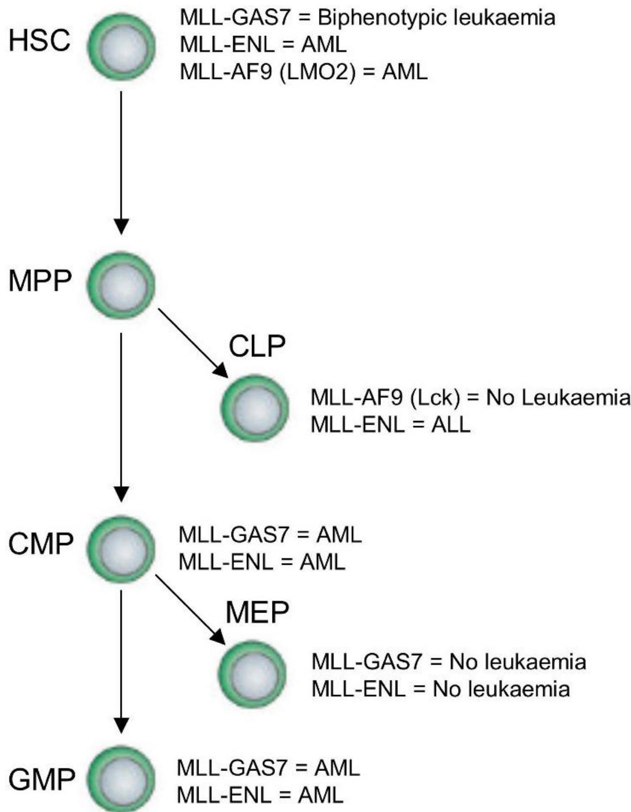


Fig. 2. Relationship between cell type and leukemic potential of various fusion proteins. This figure summarizes the results of various experiments that have examined the ability of certain fusion proteins to transform particular stages of normal, murine hematopoietic cell development. Although some fusion proteins can indeed transform most stages of hematopoietic cell development, certain fusion proteins are only capable of transforming particular cell types or produce different leukemias in different cell types. For instance, MLL-ENL can induce an AML in HSCs, CMPs and GMPs, but not MEPs. Similarly, although MLL-AF9 can cause AML in HSCs, this fusion protein cannot transform CLPs. MLL-GAS7 can cause only AML in CMP, GMP and MEP populations, but causes bi-phenotypic leukemia when HSCs are transduced. These experiments demonstrate that the cell type is very important for leukemic transformation. (Based on Fig. 2 of: Pearce and Bonnet, *Gene Ther Regul*, **3**: 65–90 (2007), with permission from World Scientific Publishing.)

In contrast, there is much greater heterogeneity and hierarchy within human AML samples (Bonnet and Dick, 1997). Related to this observation, the frequency of AML-IC within the leukemias generated by MLL-ENL fusions is extremely high (Cozzio *et al.*, 2003). Indeed, the transfer of 13 to 46 transduced cells into irradiated mice resulted in high-level engraftment of all five recipients. This suggests a frequency of AML-IC that is completely different

干细胞之家www.stemcell8.cn ←点击进入

to the $1/10^6$ to $1/10^7$ reported for human AML cell populations (Pearce *et al.*, 2006; Rombouts *et al.*, 2000).

A model that is possibly more analogous to the human AML situation is the Cre-loxP-mediated translocations described by the group of Terry Rabbitts (Rabbitts *et al.*, 2001). In this translocator mouse model, *de novo* translocations are generated *in vivo* in adult mice by Cre-mediated excision of LoxP sites. The expression of Cre can be regulated by various lineage-specific promoters to direct the excision of LoxP and hence translocation to a specific cell lineage. These Cre-loxP-mediated translocation experiments confirm the ability of the MLL-ENL fusion protein to transform LMO2-expressing HSCs and reveal that the MLL-AF9 fusion protein can also transform HSCs when Cre is under the control of the LMO2 promoter. MLL-ENL translocations may also cause leukemia when the T-cell specific promoter, Lck, controls Cre expression. Interestingly, the MLL-AF9 fusion protein, which usually causes myeloid leukemia, cannot produce any malignancy when Cre is under the control of the Lck promoter (Drynan *et al.*, 2005). These data seem to be consistent with the known incidence of either lymphoid or myeloid malignancies with each of these fusion proteins: MLL-ENL is associated with both ALL and AML, whereas MLL-AF9 is associated with myeloid leukemias only. This data provides an example that leukemogenesis depends of the cell type and the mutation as some fusion proteins may cause leukemia regardless of cell type (MLL-ENL), whereas some fusion proteins are dependent on cell type (MLL-AF9).

Apart from molecular considerations, there are other pieces of logical evidence that may give a clue as to the cellular origin of AML. As mentioned previously, the concept that AML is organized as a hierarchy, which is maintained by AML-IC, is distinct to the question of the cellular origin of AML. Direct analysis of the leukemogenic potential of certain fusion proteins has determined that it is possible to generate AML from either HSCs or myeloid progenitors (Cozzio *et al.*, 2003; So *et al.*, 2003) (see Fig. 2). Whether this is possible *in vivo* and how AML is generated in humans, still remains to be determined. The immunophenotype and morphology of the bulk of AML cells is very similar to various stages of hematopoietic progenitor development. However, the AML-IC reportedly has a phenotype that is very similar to a subset of non-malignant HSCs (Fig. 1). The phenotype that is common between AML-IC and hematopoietic stem cells is: $CD34^{+}CD38^{-}HLA\text{-}DR^{-}CD71^{-}CD123^{+}$ $CD33^{+}$ $CD13^{+}$ (Bonnet and Dick, 1997; Blair *et al.*, 1998; Jordan *et al.*, 2000; Taussig *et al.*, 2005; Bhatia *et al.*, 1997). There are some antigens (Thy-1 and c-Kit) that are different between HSCs and AML-IC,

干细胞之家www.stemcell8.cn ←点击进入

but not all HSCs express these antigens (Bhatia *et al.*, 1998; Blair *et al.*, 1997; Blair and Sutherland, 2000).

This is indirect evidence that relies in the premise that the phenotype of the AML-IC does not change significantly during leukemogenesis. Some reported observations support this hypothesis. For instance, the immunophenotype of primitive hematopoietic cells does not change significantly when transformed with the AML-ETO fusion protein (Delaney and Bernstein, 2004). However, other studies have described profound alterations in immunophenotype upon MLL-ENL and MLL-GAS7 transformations of HSCs (Cozzio *et al.*, 2003; So *et al.*, 2003). One consideration however, is that since only bulk immuno-phenotyping has been reported, it may be that the AML-IC in these MLL1-generated leukemias has a different immunophenotype to the majority of the leukemia.

A major argument against a progenitor origin for AML is opportunity. The hematopoietic cell turnover is immense; most myeloid cells terminally differentiate and die by apoptosis within a few days. Whereas stem cells are the longest lived cells in the hematopoietic system and hence, will be exposed to the most damage over time. This is consistent with the multi-hit hypothesis of leukemogenesis. Although certain fusion proteins may directly cause leukemia in mice, it is thought that translocations/inversions and insertions/deletions are the primary events that confers some advantage to mutated cells and provide an opportunity for secondary events and eventual progression to frank malignancy. Indeed, it has been reported that congenital defects are present in neonates and that these do not always progress to frank leukemia, indicating that a pre-leukemic state may exist (Greaves *et al.*, 2003).

Furthermore, there are more similarities between AML-IC and HSC than between AML-IC and progenitors. Hallmark features of both cell types are the ability to self-renew and proliferate extensively. The cellular properties of HSCs are very close to the behavior of AML-IC and therefore less changes are required to transform an HSC into an AML-IC.

Another aspect of the multi-hit hypothesis is that the original hit may occur in one cell type, but the final leukemogenic event may occur in a more differentiated progeny of the original cell. In this situation, the first hit could predispose in which cell the second hit may be leukemogenic. For instance, if the first hit was a differentiation block that avoided apoptosis, then the second hit may well occur in the cell type where differentiation was blocked. Alternatively, if the first hit affected self-renewal, then the second hit may occur in HSCs and an HSC-type leukemia could be generated.

干细胞之家www.stemcell8.cn ←点击进入

It seems that the pathways that regulate normal commitment/differentiation and self-renewal are not completely abolished in AML-IC. Rather, the effects of transforming mutations could be layered onto the normal developmental framework of HSC, resulting in the leukemic clone having an aberrant developmental hierarchy that retains aspects of its normal counterpart. This concept is supported by the correlation between genes required for normal hematopoietic development and those perturbed in leukemia (Tenen, 2003), and by the recent demonstration that Bmi-1 plays a key role in self-renewal determination in both normal and leukemic murine stem cells (Lessard and Sauvageau, 2003; Park *et al.*, 2003).

7. The Heterogeneity of Leukemia-Initiating Cells

Part of the confusion regarding the origin of the AML-IC may be due to the extreme heterogeneity of AML. Given the various possible routes to AML from a normal hematopoietic cell, it is not surprising that there is great heterogeneity in AML. Indeed, AML may be thought of as a large collection of different diseases that merely share a similar morphology. Indeed, the most effective risk stratification approach so far has been to examine the genetic abnormalities associated with a particular case of AML and compare to previous experience of AML cases with the same abnormality (Grimwade *et al.*, 2001 and1998). Although cytogenetic analysis allows the definition of the hierarchical groups with favourable, intermediate and poor prognosis, the intermediate risk group contains patients with variable outcomes. Assessing the prognosis of this large group of patients is currently difficult.

We have recently reported that the ability of a particular AML to engraft in the NOD/SCID model is related to the prognosis of individual AML cases (Pearce *et al.*, 2006). Specifically, examination of the follow-up of younger patients with intermediate-risk AML revealed a significant difference in overall survival between NOD/SCID-engrafting and non-engrafting cases. We could not detect a difference between engrafting and non-engrafting cases in various engraftment variables including: homing ability, AML-IC frequency, immune rejection by the host or alternative tissue sources. Hence, the ability to engraft NOD/SCID recipients seems to be an inherent property of the cells that is directly related to prognosis.

It is extremely interesting to note that although the NOD/SCID model assesses AML independent of the response to chemotherapy, engraftment still correlates with the response to this treatment (prognosis group). A possible explanation is that NOD/SCID engraftment reflects the stem cell nature

干细胞之家www.stemcell8.cn ←点击进入

of each individual AML case. AML cases that engraft in the NOD/SCID assay at six weeks may represent diseases driven by potent leukemia-initiating cells with stem cell-like self-renewal and proliferation abilities whereas non-engrafting AML cases may involve less potent leukemia-initiating cells with more restricted progenitor-type self-renewal and proliferation abilities.

We are not suggesting that there are simply two groups of AML cases that are derived from two different cell types. It is far more likely that there is a great range of cell potential between different AML samples and a large spectrum of cells that each case originated in. The NOD/SCID assay may provide a tool to examine the potential of AML cells by providing a threshold. Below this threshold, AML cases that cannot self-renew or proliferate enough to provide detectable engraftment at six weeks have a favourable prognosis, whereas AML cases that can engraft at six weeks have a worse prognosis.

Alternatively, it may be that certain AML cases require a cytokine or cell-cell interaction that cannot be provided by the NOD/SCID microenvironment. Most cytokines cross react, but some do not and a lack of a certain cytokine or cellular interaction may be important for the growth of particular AML cases.

8. Cytokines and AML

Various cytokine receptors that are important in hematopoiesis are dysregulated in leukemia and a few key receptors will be presented here. The FLT3 gene encodes a class III receptor kinase that belongs to the immunoglobulin superfamilly (Rosnet *et al.*, 1991). It is usually expressed on multipotential, myelomonocytic, and primitive B cell progenitors (Rosnet *et al.*, 1991). Point mutations in codons 835 and 836 of the FLT3 gene have been described. (Moreno *et al.*, 2003; Spiekermann *et al.*, 2002; Yamamoto *et al.*, 2001). An internal tandem duplication (ITD) is found in almost 30% of patients with AML and is associated with a poor prognosis (Nakao *et al.*, 1996). Mutations in the nucleocytoplasmic shuttle protein nucleophosmin (NPM1) are found in approximately 48% of patients with AML. NPM1 mutations are statistically associated with a FLT3-ITD mutation and only predict a favourable prognosis in the absence of a FLT3-ITD (Dohner *et al.*, 2005).

c-Kit is also a member of the type III receptor kinase family that is the receptor for stem cell factor (SCF) and has a central role in the regulation of hematopoietic cells. Mutations in exon 8 of the gene and a point mutation at codon 816 (D816) have been described in patients that involve CBF

干细胞之家www.stemcell8.cn ←点击进入

mutations (inv(16) and t[8,21]) and are associated with a poor prognosis (Care *et al.*, 2003; Schnittger *et al.*, 2006). Mutations in the extracellular exon 8 portion of c-Kit cause an increased response to SCF and this is thought to be important in the development of leukemia (Kohl 2005). Mutations in the intracellular portion of the c-Kit gene product (D816) are associated with factor-independent growth of myeloid cells and may contribute to leukemogenesis (Ma *et al.*, 2002).

It has been suggested that many of these alterations in cytokine receptors may be secondary events in leukemogenesis. Consistently, most cytokine mutations are associated with particular leukemogenic abnormalities. For instance, c-Kit and FLT3 mutations are associated with CBF mutations suggesting that they have a role in the leukemogenesis (Schnittger *et al.*, 2006). The precise mechanisms that cause leukemia from an initial AML-1-ETO fusion are still unknown. Indeed, it seems that AML-1-ETO is not sufficient to cause leukemia in a variety of models and it has been suggested that further genetic abnormalities are required for leukemogenesis (Fenske *et al.*, 2004). Interestingly, it has been reported that leukemia can be generated when AML-1-ETO is combined with a FLT3 mutation, but not when cell possess an altered AML-1-ETO gene only (Schessl *et al.*, 2005).

9. Competition Between Normal and Leukemia Cells

Distinct to the influence of cytokines receptors on the growth of AML cells is the influence of AML cells on normal hematopoiesis. This could have an affect on both the pathogenesis and one of the major symptoms of the disease: bone marrow failure. It is clear that even at presentation of a severe AML, there are normal, non-malignant HSCs present in the patient's marrow. However, one of the major symptoms of AML is bone marrow failure.

Indeed, the presence of primitive hematopoietic cells has been demonstrated from bone marrow samples of AML patients at presentation (Haddad *et al.*, 2004). Furthermore, upon chemotherapy, the vast majority of patients recover their non-malignant cell counts to more normal levels. This suggests that HSCs are present in the marrows of AML patients, but these are somehow dormant.

A complex network of cytokines, adhesion molecules and other cellular interactions controls normal hematopoiesis (see previous stem cell niche section). AML cells resemble myeloid blasts and may well release cytokines appropriate to this stage of hematopoietic cell development. The presence of a large "progenitor" population in the form of AML may provide

干细胞之家www.stemcell8.cn ←点击进入

a large negative feedback signal to developing HSCs. This signal could affect developing HSCs and progenitors directly or through changes to the hematopoietic microenvironment. In addition to the presence of a dormant HSC population in AML patients, a negative feedback pressure may also explain the absolute lymphocytosis observed in many AML patients. This could be the result of a "lineage switch" to the lymphoid pathway induced by the presence of too many myeloid progenitors (in the form of AML).

If this negative feedback does occur then this may be very important for the pathogenesis of the disease. As mentioned previously, leukemia is thought to be generated by multiple genetic abnormalities in a multi-step process from a slightly abnormal HSC to frank leukemia. It has been reported that although many people develop a population of cells with a certain genetic abnormality, not all go on to develop leukemia (Kim-Rouille *et al.*, 1999; Wiemels *et al.*, 1999 and 2002). This suggests that a pre-leukemic state exists and that in some individuals, this pre-leukemia cell population is prevented from developing to leukemia.

If this is the case, then the number of AML cells present in the marrow and their effect on normal hematopoiesis may be very important during the pathogenesis of AML. Once a cell has acquired sufficient abnormalities to start growing abnormally, it may be that another criteria for full leukemia is cell numbers. Initially, abnormal HSC clone cell numbers may be controlled by the immune system and by competition with normal hematopoiesis. Once an abnormal cell clone reaches a certain cell number, a negative feedback signal may be generated by the abnormal cells and received by normal HSCs. In this way, there may be a threshold of cell numbers, below which the AML is still controlled by the immune system and above which the immune system is overwhelmed, partly by sheer cell numbers and partly by a lack of immune cell production by the hematopoietic system. There is evidence for such interactions in the literature. For instance, elastase is produced by AML cells and its level is significantly elevated in the blood of AML patients (Tavor *et al.*, 2005; El Ouriaghli *et al.*, 2003). Furthermore, theoretical mathematical models of clonal succession have suggested that there is indeed a threshold of malignant cell numbers, above which clonal dominance of the malignant clone is inevitable (Catlin *et al.*, 2005).

10. Conclusive Remarks

The insights into the molecular pathogenesis of AML have led recently to the development of more specific targeted agents and have ushered in an exciting new era of anti-leukemia therapy. Such agents include the

干细胞之家www.stemcell8.cn ←点击进入

immunoconjugate gemtuzumab ozogamicin (GO, anti-CD33), multidrug resistance inhibitors, farnesyl transferase inhibitors, histone deacetylase, proteosome inhibitors, Fms-like tyrosine kinase 3 (*FLT3*) inhibitors, and apoptosis inhibitors (see more details in review by Tallman *et al.*, 2005). Nevertheless, none of these treatments have been tested on AML-ICs and/or HSCs. As demonstrated before, CD33 is expressed on both normal and malignant HSCs and this may explain the efficacy but toxicity observed in patients treated with GO.

Identification of AML-ICs has important implications for future research as well as for the development of novel therapies. In order to learn more about the nature of the events involved in leukemia, research should focus more on AML-ICs and not on the blast population that makes up the majority of the leukemic clone. Existing therapies have been developed largely against the bulk blasts population. Thus, the lack of durable response in most cases, suggests that the treatment used can usually ablate the leukemic blasts but may not effectively target the AML-IC population. Indeed, the failure of the current therapeutic regimens is likely related to the resistance and persistence of AML-ICs. Attention has turned to the self-renewal potential of AML-ICs as a therapeutic target. Several genes and pathways have been identified that are important for this critical property of self-renewal, including the WNT/β-catenin pathway, Notch, Bmi-1, and Hox family members (as mentioned here). Although it is not yet certain how these pathways might be effectively targeted therapeutically, or whether it will be possible to target these pathways without excessive toxicities of the normal hematopoietic system, AML-ICs are ultimately the most important target cells to ablate. Furthermore, combinations of several agents, targeting more than one gene mutation or antigenic determinant, may hold greater promise. The future also rests on gene expression profiling of highly purified AML-ICs, which might allow the identification of genes that reflect the biology of these cells that are actually driving the leukemia. Thus, this technology will likely provide important insights into the molecular pathogenesis of AML-IC and identify critical genes that may be targeted.

As mentioned above, recent studies in solid tumors indicate that the concept of cancer as a hierarchy that is initiated and maintained by a rare population of stem cells may have broader implications beyond the field of hematopoiesis. The increasing evidence that rare cancer stem cells drive the formation of a number of different tumors types raises the question of whether all cancers originate from and are maintained by cancer stem cells.

干细胞之家www.stemcell8.cn ←点击进入

Acknowledgements

We apologise to authors whose work we may have omitted in an effort to focus on issues that we feel are important. We thank Christopher Ridler for his assistance in the preparation of this manuscript.

References

Adams GB, Chabner KT, Alley IR, *et al*. (2006) Stem cell engraftment at the endosteal niche is specified by the calcium-sensing receptor. *Nature* **439**: 599–603.

Akashi K, Traver D, Miyamoto T, Weissman IL. (2000) A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature* **404**: 193–97.

Al-Hajj M, Wicha MS, Benito-Hernandez A, *et al*. (2003) Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci USA* **100**: 3983–88.

Amsellem S, Pflumio F, Bardinet D, *et al*. (2003) *Ex vivo* expansion of human hematopoietic stem cells by direct delivery of the HOXB4 homeoprotein. *Nat Med* **9**: 1423–27.

Andrews RG, Singer JW, Bernstein ID. (1989) Precursors of colony-forming cells in humans can be distinguished from colony-forming cells by expression of the CD33 and CD34 antigens and light scatter properties. *J Exp Med* **169**: 1721–31.

Andrews RG, Singer JW, Bernstein ID. (1990) Human hematopoietic precursors in long-term culture: single CD34+ cells that lack detectable T cell, B cell, and myeloid cell antigens produce multiple colony-forming cells when cultured with marrow stromal cells. *J Exp Med* **172**: 355–58.

Andrews RG, Takahashi M, Segal GM, *et al*. (1986) The L4F3 antigen is expressed by unipotent and multipotent colony-forming cells but not by their precursors. *Blood* **68**: 1030–35.

Arai F, Hirao A, Ohmura M, *et al*. (2004) Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. *Cell* **118**: 149–61.

Ayton PM, Cleary ML. (2001) Molecular mechanisms of leukemogenesis mediated by MLL fusion proteins. *Oncogene* **20**: 5695–707.

Ayton PM, Cleary ML. (2003) Transformation of myeloid progenitors by MLL oncoproteins is dependent on Hoxa7 and Hoxa9. *Genes Dev* **17**: 2298–307.

Azcoitia V, Aracil M, Martinez AC, Torres M. (2005) The homeodomain protein Meis1 is essential for definitive hematopoiesis and vascular patterning in the mouse embryo. *Dev Biol* **280**: 307–20.

Bai F, Pei XH, Godfrey VL, Xiong Y. (2003) Haploinsufficiency of p18(INK4c) sensitizes mice to carcinogen-induced tumorigenesis. *Mol Cell Biol* **23**: 1269–77.

Balazs AB, Fabian AJ, Esmon CT, Mulligan RC. (2005) Endothelial protein C receptor (CD201) explicitly identifies hematopoietic stem cells in murine bone marrow. *Blood* **107**: 2317–21.

Barabe F, Kennedy JA, Hope KJ, Dick JE. (2007) Modeling the initiation and progression of human acute leukemia in mice. *Science* **316**: 600–604.

干细胞之家www.stemcell8.cn ←点击进入

Bartel DP. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**: 281–97.

Becker AJ, Mc CE, Till JE. (1963) Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. *Nature* **197**: 452–54.

Bertolino P, Radovanovic I, Casse H, *et al*. (2003a) Genetic ablation of the tumor suppressor menin causes lethality at mid-gestation with defects in multiple organs. *Mech Dev* **120**: 549–60.

Bertolino P, Tong WM, Galendo D, *et al*. (2003b) Heterozygous Men1 mutant mice develop a range of endocrine tumors mimicking multiple endocrine neoplasia type 1. *Mol Endocrinol* **17**: 1880–92.

Beslu N, Krosl J, Laurin M, *et al*. (2004) Molecular interactions involved in HOXB4-induced activation of HSC self-renewal. *Blood* **104**: 2307–14.

Bhardwaj G, Murdoch B, Wu D, *et al*. (2001) Sonic hedgehog induces the proliferation of primitive human hematopoietic cells via BMP regulation. *Nat Immunol* **2**: 172–80.

Bhatia M, Bonnet D, Murdoch B, *et al*. (1998) A newly discovered class of human hematopoietic cells with SCID-repopulating activity. *Nat Med* **4**: 1038–45.

Bhatia M, Bonnet D, Wu D, *et al*. (1999) Bone morphogenetic proteins regulate the developmental program of human hematopoietic stem cells. *J Exp Med* **189**: 1139–48.

Bhatia M, Wang JC, Kapp U, *et al*. (1997) Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. *Proc Natl Acad Sci USA* **94**: 5320–25.

Bijl J, Van Oostveen JW, Kreike M, *et al*. (1996) Expression of HOXC4, HOXC5, and HOXC6 in human lymphoid cell lines, leukemias, and benign and malignant lymphoid tissue. *Blood* **87**: 1737–45.

Bjornsson JM, Larsson N, Brun AC, *et al*. (2003) Reduced proliferative capacity of hematopoietic stem cells deficient in Hoxb3 and Hoxb4. *Mol Cell Biol* **23**: 3872–83.

Blair A, Hogge DE, Ailles LE, *et al*. (1997) Lack of expression of Thy-1 (CD90) on acute myeloid leukemia cells with long-term proliferative ability *in vitro* and *in vivo*. *Blood* **89**: 3104–12.

Blair A, Hogge DE, Sutherland HJ. (1998) Most acute myeloid leukemia progenitor cells with long-term proliferative ability *in vitro* and *in vivo* have the phenotype CD34(+)/CD71(−)/HLA-DR. *Blood* **92**: 4325–35.

Blair A, Sutherland HJ. (2000) Primitive acute myeloid leukemia cells with long-term proliferative ability *in vitro* and *in vivo* lack surface expression of c-kit (CD117). *Exp Hematol* **28**: 660–71.

Bonnet D, Dick JE. (1997) Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med* **3**: 730–37.

Bradford GB, Williams B, Rossi R, Bertoncello I. (1997) Quiescence, cycling, and turnover in the primitive hematopoietic stem cell compartment. *Exp Hematol* **25**: 445–53.

干细胞之家www.stemcell8.cn ←点击进入

Breems DA, Blokland EA, Neben S, Ploemacher RE. (1994) Frequency analysis of human primitive hematopoietic stem cell subsets using a cobblestone area forming cell assay. *Leukemia* **8**: 1095–104.

Buhring HJ, Kuci S, Conze T, *et al*. (2004) CDCP1 identifies a broad spectrum of normal and malignant stem/progenitor cell subsets of hematopoietic and non-hematopoietic origin. *Stem Cells* **22**: 334–43.

Calvi LM, Adams GB, Weibrecht KW, *et al*. (2003) Osteoblastic cells regulate the hematopoietic stem cell niche. *Nature* **425**: 841–46.

Cantor AB, Orkin SH. (2001) Hematopoietic development: a balancing act. *Curr Opin Genet Dev* **11**: 513–19.

Cao R, Wang L, Wang H, *et al*. (2002) Role of histone H3 lysine 27 methylation in Polycomb-group silencing. *Science* **298**: 1039–43.

Care RS, Valk PJ, Goodeve AC, *et al*. (2003) Incidence and prognosis of c-KIT and FLT3 mutations in core binding factor (CBF) acute myeloid leukemias. *Br J Haematol* **121**: 775–77.

Cashman JD, Lapidot T, Wang JC, *et al*. (1997) Kinetic evidence of the regeneration of multilineage hematopoiesis from primitive cells in normal human bone marrow transplanted into immunodeficient mice. *Blood* **89**: 4307–16.

Catlin SN, Guttorp P, Abkowitz JL. (2005) The kinetics of clonal dominance in myeloproliferative disorders. *Blood* **106**: 2688–92.

Chagraoui J, Niessen SL, Lessard J, *et al*. (2006) E4F1: a novel candidate factor for mediating BMI1 function in primitive hematopoietic cells. *Genes Dev* **20**: 2110–20.

Chandrasekharappa SC, Guru SC, Manickam P, *et al*. (1997) Positional cloning of the gene for multiple endocrine neoplasia-type 1. *Science* **276**: 404–407.

Chaplin T, Bernard O, Beverloo HB, *et al*. (1995) The t(10;11) translocation in acute myeloid leukemia (M5) consistently fuses the leucine zipper motif of AF10 onto the HRX gene. *Blood* **86**: 2073–76.

Chen CZ, Li L, Lodish HF, Bartel DP. (2004) MicroRNAs modulate hematopoietic lineage differentiation. *Science* **303**: 83–86.

Cheng J, Luo J, Zhang X, *et al*. (2000) Inhibition of cell proliferation in HCC-9204 hepatoma cells by a c-myc specific ribozyme. *Cancer Gene Ther* **7**: 407–12.

Cheng T, Shen H, Rodrigues N, *et al*. (2001) Transforming growth factor beta 1 mediates cell-cycle arrest of primitive hematopoietic cells independent of p21(Cip1/Waf1) or p27(Kip1). *Blood* **98**: 3643–49.

Christianson SW, Greiner DL, Hesselton RA, *et al*. (1997) Enhanced human CD4+ T cell engraftment in beta2-microglobulin-deficient NOD-scid mice. *J Immunol* **158**: 3578–86.

Corral J, Lavenir I, Impey H, *et al*. (1996) An Mll-AF9 fusion gene made by homologous recombination causes acute leukemia in chimeric mice: a method to create fusion oncogenes. *Cell* **85**: 853–61.

Costello RT, Mallet F, Gaugler B, *et al*. (2000) Human acute myeloid leukemia CD34+/CD38-progenitor cells have decreased sensitivity to chemotherapy and

干细胞之家www.stemcell8.cn ←点击进入

Fas-induced apoptosis, reduced immunogenicity, and impaired dendritic cell transformation capacities. *Cancer Res* **60**: 4403–11.

Cozzio A, Passegue E, Ayton PM, *et al*. (2003) Similar MLL-associated leukemias arising from self-renewing stem cells and short-lived myeloid progenitors. *Genes Dev* **17**: 3029–35.

Crooks GM, Fuller J, Petersen D, *et al*. (1999) Constitutive HOXA5 expression inhibits erythropoiesis and increases myelopoiesis from human hematopoietic progenitors. *Blood* **94**: 519–28.

Czermin B, Melfi R, Mccabe D, *et al*. (2002) Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. *Cell* **111**: 185–96.

Danet GH, Luongo JL, Butler G, *et al*. (2002) C1qRp defines a new human stem cell population with hematopoietic and hepatic potential. *Proc Natl Acad Sci USA* **99**: 10441–45.

Debernardi S, Lillington DM, Chaplin T, *et al*. (2003) Genome-wide analysis of acute myeloid leukemia with normal karyotype reveals a unique pattern of homeobox gene expression distinct from those with translocation-mediated fusion events. *Genes Chromosomes Cancer* **37**: 149–58.

Delaney C, Bernstein ID. (2004) Establishment of a pluripotent preleukaemic stem cell line by expression of the AML1-ETO fusion protein in Notch1-immortalized HSCN1cl10 cells. *Br J Haematol* **125**: 353–57.

Deng C, Zhang P, Harper JW, *et al*. (1995) Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. *Cell* **82**: 675–84.

Dimartino JF, Ayton PM, Chen EH, *et al*. (2002) The AF10 leucine zipper is required for leukemic transformation of myeloid progenitors by MLL-AF10. *Blood* **99**: 3780–85.

Dimartino JF, Selleri L, Traver D, *et al*. (2001) The Hox cofactor and proto-oncogene Pbx1 is required for maintenance of definitive hematopoiesis in the fetal liver. *Blood* **98**: 618–26.

Dobson CL, Warren AJ, Pannell R, *et al*. (2000) Tumorigenesis in mice with a fusion of the leukemia oncogene Mll and the bacterial lacZ gene. *EMBO J* **19**: 843–51.

Dohner K, Schlenk RF, Habdank M, *et al*. (2005) Mutant nucleophosmin (NPM1) predicts favorable prognosis in younger adults with acute myeloid leukemia and normal cytogenetics — interaction with other gene mutations. *Blood* **106**: 3740–46.

Dorfman DM, Wilson DB, Bruns GA, Orkin SH. (1992) Human transcription factor GATA-2. Evidence for regulation of preproendothelin-1 gene expression in endothelial cells. *J Biol Chem* **267**: 1279–85.

Drexler HG. (1998) Review of alterations of the cyclin-dependent kinase inhibitor INK4 family genes p15, p16, p18 and p19 in human leukemia-lymphoma cells. *Leukemia* **12**: 845–59.

干细胞之家www.stemcell8.cn ←点击进入

Driessen RL, Johnston HM, Nilsson SK. (2003) Membrane-bound stem cell factor is a key regulator in the initial lodgment of stem cells within the endosteal marrow region. *Exp Hematol* **31**: 1284–91.

Drynan LF, Pannell R, Forster A, *et al*. (2005) Mll fusions generated by Cre-loxP-mediated *de novo* translocations can induce lineage reassignment in tumorigenesis. *EMBO J* **24**: 3136–46.

Duester G. (2000) Families of retinoid dehydrogenases regulating vitamin A function: production of visual pigment and retinoic acid. *Eur J Biochem* **267**: 4315–24.

Duncan AW, Rattis FM, Dimascio LN, *et al*. (2005) Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance. *Nat Immunol* **6**: 314–22.

El Ouriaghli F, Fujiwara H, Melenhorst JJ, *et al*. (2003) Neutrophil elastase enzymatically antagonizes the *in vitro* action of G-CSF: implications for the regulation of granulopoiesis. *Blood* **101**: 1752–58.

Enver T, Greaves M. (1998) Loops, lineage, and leukemia. *Cell* **94**: 9–12.

Era T, Asou N, Kunisada T, *et al*. (1995) Identification of two transcripts of AML1/ETO-fused gene in t(8;21) leukemic cells and expression of wild-type ETO gene in hematopoietic cells. *Genes Chromosomes Cancer* **13**: 25–33.

Ernst P, Wang J, Huang M, *et al*. (2001) MLL and CREB bind cooperatively to the nuclear coactivator CREB-binding protein. *Mol Cell Biol* **21**: 2249–58.

Ezoe S, Matsumura I, Nakata S, *et al*. (2002) GATA-2/estrogen receptor chimera regulates cytokine-dependent growth of hematopoietic cells through accumulation of p21(WAF1) and p27(Kip1) proteins. *Blood* **100**: 3512–20.

Fenske TS, Pengue G, Mathews V, *et al*. (2004) Stem cell expression of the AML1/ETO fusion protein induces a myeloproliferative disorder in mice. *Proc Natl Acad Sci USA* **101**: 15184–89.

Fialkow PJ, Denman AM, Jacobson RJ, Lowenthal MN. (1978) Chronic myelocytic leukemia. Origin of some lymphocytes from leukemic stem cells. *J Clin Invest* **62**: 815–23.

Foley KP, McArthur GA, Queva C, *et al*. (1998) Targeted disruption of the MYC antagonist MAD1 inhibits cell cycle exit during granulocyte differentiation. *EMBO J* **17**: 774–85.

Ford AM, Bennett CA, Healy LE, *et al*. (1992) Immunoglobulin heavy-chain and CD3 delta-chain gene enhancers are DNase I-hypersensitive in hemopoietic progenitor cells. *Proc Natl Acad Sci USA* **89**: 3424–28.

Franklin DS, Godfrey VL, Lee H, *et al*. (1998) CDK inhibitors p18(INK4c) and p27(Kip1) mediate two separate pathways to collaboratively suppress pituitary tumorigenesis. *Genes Dev* **12**: 2899–911.

Fuller JF, Mcadara J, Yaron Y, *et al*. (1999) Characterization of HOX gene expression during myelopoiesis: role of HOX A5 in lineage commitment and maturation. *Blood* **93**: 3391–400.

Furukawa Y, Kikuchi J, Nakamura M, *et al*. (2000) Lineage-specific regulation of cell cycle control gene expression during haematopoietic cell differentiation. *Br J Haematol* **110**: 663–73.

干细胞之家www.stemcell8.cn ←点击进入

Galan-Caridad JM, Harel S, Arenzana TL, *et al*. (2007) Zfx controls the self-renewal of embryonic and hematopoietic stem cells. *Cell* **129**: 345–57.

Georgantas RW 3rd, Hildreth R, Morisot S, *et al*. (2007) CD34+ hematopoietic stem-progenitor cell microRNA expression and function: a circuit diagram of differentiation control. *Proc Natl Acad Sci USA* **104**: 2750–55.

Glimm H, Eisterer W, Lee K, *et al*. (2001) Previously undetected human hematopoietic cell populations with short-term repopulating activity selectively engraft NOD/SCID-beta2 microglobulin-null mice. *J Clin Invest* **107**: 199–206.

Goodell MA, Brose K, Paradis G, *et al*. (1996) Isolation and functional properties of murine hematopoietic stem cells that are replicating *in vivo*. *J Exp Med* **183**: 1797–806.

Goodell MA, Rosenzweig M, Kim H, *et al*. (1997) Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. *Nat Med* **3**: 1337–45.

Greaves MF, Maia AT, Wiemels JL, Ford AM. (2003) Leukemia in twins: lessons in natural history. *Blood* **102**: 2321–33.

Gregory RI, Shiekhattar R. (2005) MicroRNA biogenesis and cancer. *Cancer Res* **65**: 3509–12.

Grier DG, Thompson A, Kwasniewska A, *et al*. (2005) The pathophysiology of HOX genes and their role in cancer. *J Pathol* **205**: 154–71.

Grimwade D, Walker H, Harrison G, *et al*. (2001) The predictive value of hierarchical cytogenetic classification in older adults with acute myeloid leukemia (AML): analysis of 1065 patients entered into the United Kingdom Medical Research Council AML11 trial. *Blood* **98**: 1312–20.

Grimwade D, Walker H, Oliver F, *et al*. (1998) The importance of diagnostic cytogenetics on outcome in AML: analysis of 1,612 patients entered into the MRC AML 10 trial. The Medical Research Council Adult and Children's Leukemia Working Parties. *Blood* **92**: 2322–33.

Guenechea G, Gan OI, Dorrell C, Dick JE. (2001) Distinct classes of human stem cells that differ in proliferative and self-renewal potential. *Nat Immunol* **2**: 75–82.

Gupta R, Hong D, Iborra F, *et al*. (2007) NOV (CCN3) functions as a regulator of human hematopoietic stem or progenitor cells. *Science* **316**: 590–93.

Haddad R, Guardiola P, Izac B, *et al*. (2004) Molecular characterization of early human T/NK and B-lymphoid progenitor cells in umbilical cord blood. *Blood* **104**: 3918–26.

Hammond SM. (2007) MicroRNAs as tumor suppressors. *Nat Genet* **39**: 582–83.

Hao QL, Smogorzewska EM, Barsky LW, Crooks GM. (1998) *In vitro* identification of single CD34+CD38− cells with both lymphoid and myeloid potential. *Blood* **91**: 4145–51.

Hao QL, Thiemann FT, Petersen D, *et al*. (1996) Extended long-term culture reveals a highly quiescent and primitive human hematopoietic progenitor population. *Blood* **88**: 3306–13.

干细胞之家www.stemcell8.cn ←点击进入

Hatfield SD, Shcherbata HR, Fischer KA, *et al*. (2005) Stem cell division is regulated by the microRNA pathway. *Nature* **435**: 974–78.

Hayette S, Thomas X, Bertrand Y, *et al*. (1997) Molecular analysis of cyclin-dependent kinase inhibitors in human leukemias. *Leukemia* **11**: 1696–99.

Haylock DN, Nilsson SK. (2005) Stem cell regulation by the hematopoietic stem cell niche. *Cell Cycle* **4**: 1353–55.

Heyworth C, Gale K, Dexter M, *et al*. (1999) A GATA-2/estrogen receptor chimera functions as a ligand-dependent negative regulator of self-renewal. *Genes Dev* **13**: 1847–60.

Hiramatsu H, Nishikomori R, Heike T, *et al*. (2003) Complete reconstitution of human lymphocytes from cord blood CD34+ cells using the NOD/SCID/gammacnull mice model. *Blood* **102**: 873–80.

Ho AD. (2005) Kinetics and symmetry of divisions of hematopoietic stem cells. *Exp Hematol* **33**: 1–8.

Hock H, Hamblen MJ, Rooke HM, *et al*. (2004) Gfi-1 restricts proliferation and preserves functional integrity of haematopoietic stem cells. *Nature* **431**: 1002–7.

Hogan CJ, Shpall EJ, Mcnulty O, *et al*. (1997) Engraftment and development of human CD34(+)-enriched cells from umbilical cord blood in NOD/LtSz-scid/scid mice. *Blood* **90**: 85–96.

Hope KJ, Jin L, Dick JE. (2004) Acute myeloid leukemia originates from a hierarchy of leukemic stem cell classes that differ in self-renewal capacity. *Nat Immunol* **5**: 738–43.

Hsieh JJ, Ernst P, Erdjument-Bromage H, *et al*. (2003) Proteolytic cleavage of MLL generates a complex of N- and C-terminal fragments that confers protein stability and subnuclear localization. *Mol Cell Biol* **23**: 186–94.

Hu M, Krause D, Greaves M, *et al*. (1997) Multilineage gene expression precedes commitment in the hemopoietic system. *Genes Dev* **11**: 774–85.

Huang G, Shigesada K, Ito K, *et al*. (2001) Dimerization with PEBP2beta protects RUNX1/AML1 from ubiquitin-proteasome-mediated degradation. *EMBO J* **20**: 723–33.

Huang Y, Du KM, Xue ZH, *et al*. (2003) Cobalt chloride and low oxygen tension trigger differentiation of acute myeloid leukemic cells: possible mediation of hypoxia-inducible factor-1alpha. *Leukemia* **17**: 2065–73.

Ichikawa M, Asai T, Saito T, *et al*. (2004) AML-1 is required for megakaryocytic maturation and lymphocytic differentiation, but not for maintenance of hematopoietic stem cells in adult hematopoiesis. *Nat Med* **10**: 299–304.

Ishikawa F, Yasukawa M, Lyons B, *et al*. (2005) Development of functional human blood and immune systems in NOD/SCID/IL2 receptor {gamma} chain(null) mice. *Blood* **106**: 1565–73.

Iwama A, Oguro H, Negishi M, *et al*. (2004) Enhanced self-renewal of hematopoietic stem cells mediated by the polycomb gene product Bmi-1. *Immunity* **21**: 843–51.

干细胞之家www.stemcell8.cn ←点击进入

Iwasaki H, Somoza C, Shigematsu H, *et al*. (2005) Distinctive and indispensable roles of PU.1 in maintenance of hematopoietic stem cells and their differentiation. *Blood* **106**: 1590–600.

Jimenez G, Griffiths SD, Ford AM, *et al*. (1992) Activation of the beta-globin locus control region precedes commitment to the erythroid lineage. *Proc Natl Acad Sci USA* **89**: 10618–22.

Jones RJ, Barber JP, Vala MS, *et al*. (1995) Assessment of aldehyde dehydrogenase in viable cells. *Blood* **85**: 2742–46.

Jordan CT, Upchurch D, Szilvassy SJ, *et al*. (2000) The interleukin-3 receptor alpha chain is a unique marker for human acute myelogenous leukemia stem cells. *Leukemia* **14**: 1777–84.

Kamminga LM, Bystrykh LV, De Boer A, *et al*. (2006) The polycomb group gene Ezh2 prevents hematopoietic stem cell exhaustion. *Blood* **107**: 2170–79.

Kappen C. (2000) Disruption of the homeobox gene Hoxb-6 in mice results in increased numbers of early erythrocyte progenitors. *Am J Hematol* **65**: 111–18.

Karanu FN, Murdoch B, Gallacher L, *et al*. (2000) The notch ligand jagged-1 represents a novel growth factor of human hematopoietic stem cells. *J Exp Med* **192**: 1365–72.

Kastan MB, Schlaffer E, Russo JE, *et al*. (1990) Direct demonstration of elevated aldehyde dehydrogenase in human hematopoietic progenitor cells. *Blood* **75**: 1947–50.

Kiel MJ, Yilmaz OH, Iwashita T, *et al*. (2005) SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell* **121**: 1109–21.

Kim M, Turnquist H, Jackson J, *et al*. (2002) The multidrug resistance transporter ABCG2 (breast cancer resistance protein 1) effluxes Hoechst 33342 and is overexpressed in hematopoietic stem cells. *Clin Cancer Res* **8**: 22–28.

Kim-Rouille MH, Macgregor A, Wiedemann LM, *et al*. (1999) MLL-AF4 gene fusions in normal newborns. *Blood* **93**: 1107–108.

Klein J, Nikolaidis N. (2005) The descent of the antibody-based immune system by gradual evolution. *Proc Natl Acad Sci USA* **102**: 169–74.

Klemsz MJ, Mckercher SR, Celada A, *et al*. (1990) The macrophage and B cell-specific transcription factor PU.1 is related to the ets oncogene. *Cell* **61**: 113–24.

Kollet O, Peled A, Byk T, *et al*. (2000) Beta2 microglobulin-deficient (B2m(null)) NOD/SCID mice are excellent recipients for studying human stem cell function. *Blood* **95**: 3102–105.

Krosl J, Austin P, Beslu N, *et al*. (2003) *In vitro* expansion of hematopoietic stem cells by recombinant TAT-HOXB4 protein. *Nat Med* **9**: 1428–32.

Lapidot T, Pflumio F, Doedens M, *et al*. (1992) Cytokine stimulation of multilineage hematopoiesis from immature human cells engrafted in SCID mice. *Science* **255**: 1137–41.

Lapidot T, Sirard C, Vormoor J, *et al*. (1994) A cell initiating human acute myeloid leukemia after transplantation into SCID mice. *Nature* **367**: 645–48.

干细胞之家www.stemcell8.cn ←点击进入

Larochelle A, Vormoor J, Hanenberg H, *et al*. (1996) Identification of primitive human hematopoietic cells capable of repopulating NOD/SCID mouse bone marrow: implications for gene therapy. *Nat Med* **2**: 1329–37.

Lawrence HJ, Christensen J, Fong S, *et al*. (2005) Loss of expression of the Hoxa-9 homeobox gene impairs the proliferation and repopulating ability of hematopoietic stem cells. *Blood* **106**: 3988–94.

Lawrence HJ, Helgason CD, Sauvageau G, *et al*. (1997) Mice bearing a targeted interruption of the homeobox gene HOXA9 have defects in myeloid, erythroid, and lymphoid hematopoiesis. *Blood* **89**: 1922–30.

Leonard M, Brice M, Engel JD, Papayannopoulou T. (1993) Dynamics of GATA transcription factor expression during erythroid differentiation. *Blood* **82**: 1071–79.

Leroy H, Roumier C, Huyghe P, *et al*. (2005) CEBPA point mutations in hematological malignancies. *Leukemia* **19**: 329–34.

Lessard J, Sauvageau G. (2003) Bmi-1 determines the proliferative capacity of normal and leukemic stem cells. *Nature* **423**: 255–60.

Lin FT, Macdougald OA, Diehl AM, Lane MD. (1993) A 30-kDa alternative translation product of the CCAAT/enhancer binding protein alpha message: transcriptional activator lacking antimitotic activity. *Proc Natl Acad Sci USA* **90**: 9606–10.

Liu P, Tarle SA, Hajra A, *et al*. (1993) Fusion between transcription factor CBF beta/PEBP2 beta and a myosin heavy chain in acute myeloid leukemia. *Science* **261**: 1041–44.

Lowenberg B, Downing JR, Burnett A. (1999) Acute myeloid leukemia. *N Engl J Med* **341**: 1051–62.

Lu Q, Kamps MP. (1997) Heterodimerization of Hox proteins with Pbx1 and oncoprotein E2a-Pbx1 generates unique DNA-binding specifities at nucleotides predicted to contact the N-terminal arm of the Hox homeodomain-demonstration of Hox-dependent targeting of E2a-Pbx1 *in vivo*. *Oncogene* **14**: 75–83.

Lukasik SM, Zhang L, Corpora T, *et al*. (2002) Altered affinity of CBF beta-SMMHC for Runx1 explains its role in leukemogenesis. *Nat Struct Biol* **9**: 674–79.

Lutterbach B, Westendorf JJ, Linggi B, *et al*. (2000) A mechanism of repression by acute myeloid leukemia-1, the target of multiple chromosomal translocations in acute leukemia. *J Biol Chem* **275**: 651–56.

Ma Y, Zeng S, Metcalfe DD, *et al*. (2002) The c-KIT mutation causing human mastocytosis is resistant to STI571 and other KIT kinase inhibitors; kinases with enzymatic site mutations show different inhibitor sensitivity profiles than wild-type kinases and those with regulatory-type mutations. *Blood* **99**: 1741–44.

Mann RS, Affolter M. (1998) Hox proteins meet more partners. *Curr Opin Genet Dev* **8**: 423–29.

Masuda A, Yoshikai Y, Kume H, Matsuguchi T. (2004) The interaction between GATA proteins and activator protein-1 promotes the transcription of IL-13 in mast cells. *J Immunol* **173**: 5564–73.

干细胞之家www.stemcell8.cn ←点击进入

Matsubara A, Iwama A, Yamazaki S, *et al*. (2005) Endomucin, a CD34-like sialomucin, marks hematopoietic stem cells throughout development. *J Exp Med* **202**: 1483–92.

Matsuzaki Y, Kinjo K, Mulligan RC, Okano H. (2004) Unexpectedly efficient homing capacity of purified murine hematopoietic stem cells. *Immunity* **20**: 87–93.

Mcarthur GA, Foley KP, Fero ML, *et al*. (2002) MAD1 and p27(KIP1) cooperate to promote terminal differentiation of granulocytes and to inhibit Myc expression and cyclin E-CDK2 activity. *Mol Cell Biol* **22**: 3014–23.

Mckercher SR, Torbett BE, Anderson KL, *et al*. (1996) Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. *EMBO J* **15**: 5647–58.

Mcmanus MT. (2003) MicroRNAs and cancer. *Semin Cancer Biol* **13**: 253–58.

Mikkola HK, Klintman J, Yang H, *et al*. (2003) Haematopoietic stem cells retain long-term repopulating activity and multipotency in the absence of stem-cell leukemia SCL/tal-1 gene. *Nature* **421**: 547–51.

Miller JS, Mccullar V, Punzel M, *et al*. (1999) Single adult human CD34(+)/Lin-/CD38(−) progenitors give rise to natural killer cells, B-lineage cells, dendritic cells, and myeloid cells. *Blood* **93**: 96–106.

Milne TA, Briggs SD, Brock HW, *et al*. (2002) MLL targets SET domain methyltransferase activity to Hox gene promoters. *Mol Cell* **10**: 1107–17.

Miyamoto T, Iwasaki H, Reizis B, *et al*. (2002) Myeloid or lymphoid promiscuity as a critical step in hematopoietic lineage commitment. *Dev Cell* **3**: 137–47.

Molofsky AV, He S, Bydon M, *et al*. (2005) Bmi-1 promotes neural stem cell self-renewal and neural development but not mouse growth and survival by repressing the p16Ink4a and p19Arf senescence pathways. *Genes Dev* **19**: 1432–37.

Moreno I, Martin G, Bolufer P, *et al*. (2003) Incidence and prognostic value of FLT3 internal tandem duplication and D835 mutations in acute myeloid leukemia. *Haematologica* **88**: 19–24.

Nakamura T, Mori T, Tada S, *et al*. (2002) ALL-1 is a histone methyltransferase that assembles a supercomplex of proteins involved in transcriptional regulation. *Mol Cell* **10**: 1119–28.

Nakao M, Yokota S, Iwai T, *et al*. (1996) Internal tandem duplication of the flt3 gene found in acute myeloid leukemia. *Leukemia* **10**: 1911–18.

Nakayama K, Nakayama K. (1998) Cip/Kip cyclin-dependent kinase inhibitors: brakes of the cell cycle engine during development. *Bioessays* **20**: 1020–29.

Nerlov C. (2004) C/EBPalpha mutations in acute myeloid leukemias. *Nat Rev Cancer* **4**: 394–400.

Nerlov C, Querfurth E, Kulessa H, Graf T. (2000) GATA-1 interacts with the myeloid PU.1 transcription factor and represses PU.1-dependent transcription. *Blood* **95**: 2543–51.

Newton RA, Raftos DA, Raison RL, Geczy CL. (1994) Chemotactic responses of hagfish (Vertebrata, Agnatha) leucocytes. *Dev Comp Immunol* **18**: 295–303.

干细胞之家www.stemcell8.cn ←点击进入

Nilsson L, Astrand-Grundstrom I, Arvidsson I, *et al*. (2000) Isolation and characterization of hematopoietic progenitor/stem cells in 5q-deleted myelodysplastic syndromes: evidence for involvement at the hematopoietic stem cell level. *Blood* **96**: 2012–21.

Nilsson SK, Haylock DN, Johnston HM, *et al*. (2003) Hyaluronan is synthesized by primitive hemopoietic cells, participates in their lodgment at the endosteum following transplantation, and is involved in the regulation of their proliferation and differentiation *in vitro*. *Blood* **101**: 856–62.

Nilsson SK, Johnston HM, Whitty GA, *et al*. (2005) Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells. *Blood* **106**: 1232–39.

Ohta H, Sawada A, Kim JY, *et al*. (2002) Polycomb group gene rae28 is required for sustaining activity of hematopoietic stem cells. *J Exp Med* **195**: 759–70.

Okuda T, Van Deursen J, Hiebert SW, *et al*. (1996) AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. *Cell* **84**: 321–30.

Orkin SH. (2003) Priming the hematopoietic pump. *Immunity* **19**: 633–34.

Orlic D, Anderson S, Biesecker LG, *et al*. (1995) Pluripotent hematopoietic stem cells contain high levels of mRNA for c-kit, GATA-2, p45 NF-E2, and c-myb and low levels or no mRNA for c-fms and the receptors for granulocyte colony-stimulating factor and interleukins 5 and 7. *Proc Natl Acad Sci USA* **92**: 4601–5.

Osawa M, Nakamura K, Nishi N, *et al*. (1996) *In vivo* self-renewal of c-Kit+ Sca-1+ Lin(low/−) hemopoietic stem cells. *J Immunol* **156**: 3207–14.

Pabst T, Mueller BU, Zhang P, *et al*. (2001) Dominant-negative mutations of CEBPA, encoding CCAAT/enhancer binding protein-alpha (C/EBPalpha), in acute myeloid leukemia. *Nat Genet* **27**: 263–70.

Park IK, Qian D, Kiel M, *et al*. (2003) Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. *Nature* **423**: 302–305.

Parmar K, Mauch P, Vergilio JA, *et al*. (2007) Distribution of hematopoietic stem cells in the bone marrow according to regional hypoxia. *Proc Natl Acad Sci USA* **104**: 5431–36.

Passegue E, Jamieson CH, Ailles LE, Weissman IL. (2003) Normal and leukemic hematopoiesis: are leukemias a stem cell disorder or a reacquisition of stem cell characteristics? *Proc Natl Acad Sci USA* **100**(Suppl 1): 11842–49.

Patient RK, Mcghee JD. (2002) The GATA family (vertebrates and invertebrates). *Curr Opin Genet Dev* **12**: 416–22.

Pearce DJ, Ridler CM, Simpson C, Bonnet D. (2004) Multiparameter analysis of murine bone marrow side population cells. *Blood* **103**: 2541–46.

Pearce DJ, Taussig D, Simpson C, *et al*. (2005) Characterization of cells with a high aldehyde dehydrogenase activity from cord blood and acute myeloid leukemia samples. *Stem Cells* **23**: 752–60.

干细胞之家www.stemcell8.cn ←点击进入

Pearce DJ, Taussig D, Zibara K, *et al*. (2006) AML engraftment in the NOD/SCID assay reflects the outcome of AML: implications for our understanding of the heterogeneity of AML. *Blood* **107**: 1166–73.

Pevny L, Simon MC, Robertson E, *et al*. (1991) Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. *Nature* **349**: 257–60.

Ploemacher RE, Van Der Sluijs JP, Voerman JS, Brons NH. (1989) An *in vitro* limiting-dilution assay of long-term repopulating hematopoietic stem cells in the mouse. *Blood* **74**: 2755–63.

Polakis P. (2000) Wnt signaling and cancer. *Genes Dev* **14**: 1837–51.

Popovic R, Zeleznik-Le NJ. (2005) MLL: how complex does it get? *J Cell Biochem* **95**: 234–42.

Potocnik AJ, Brakebusch C, Fassler R. (2000) Fetal and adult hematopoietic stem cells require beta1 integrin function for colonizing fetal liver, spleen, and bone marrow. *Immunity* **12**: 653–63.

Prasad R, Leshkowitz D, Gu Y, *et al*. (1994) Leucine-zipper dimerization motif encoded by the AF17 gene fused to ALL-1 (MLL) in acute leukemia. *Proc Natl Acad Sci USA* **91**: 8107–11.

Punzel M, Wissink SD, Miller JS, *et al*. (1999) The myeloid-lymphoid initiating cell (ML-IC) assay assesses the fate of multipotent human progenitors *in vitro*. *Blood* **93**: 3750–56.

Rabbitts TH, Appert A, Chung G, *et al*. (2001) Mouse models of human chromosomal translocations and approaches to cancer therapy. *Blood Cells Mol Dis* **27**: 249–59.

Rafii S, Mohle R, Shapiro F, *et al*. (1997) Regulation of hematopoiesis by microvascular endothelium. *Leuk Lymphoma* **27**: 375–86.

Reya T, Duncan AW, Ailles L, *et al*. (2003) A role for Wnt signalling in self-renewal of hematopoietic stem cells. *Nature* **423**: 409–14.

Reya T, Morrison SJ, Clarke MF, Weissman IL. (2001) Stem cells, cancer, and cancer stem cells. *Nature* **414**: 105–11.

Reynaud D, Ravet E, Titeux M, *et al*. (2005) SCL/TAL1 expression level regulates human hematopoietic stem cell self-renewal and engraftment. *Blood* **106**: 2318–28.

Robb L, Elwood NJ, Elefanty AG, *et al*. (1996) The scl gene product is required for the generation of all hematopoietic lineages in the adult mouse. *EMBO J* **15**: 4123–29.

Rombouts WJ, Martens AC, Ploemacher RE. (2000) Identification of variables determining the engraftment potential of human acute myeloid leukemia in the immunodeficient NOD/SCID human chimera model. *Leukemia* **14**: 889–97.

Rosenbauer F, Koschmieder S, Steidl U, Tenen DG. (2005) Effect of transcription-factor concentrations on leukemic stem cells. *Blood* **106**: 1519–24.

Rosenbauer F, Owens BM, Yu L, *et al*. (2006) Lymphoid cell growth and transformation are suppressed by a key regulatory element of the gene encoding PU.1. *Nat Genet* **38**: 27–37.

干细胞之家www.stemcell8.cn ←点击进入

Rosnet O, Marchetto S, Delapeyriere O, Birnbaum D. (1991) Murine Flt3, a gene encoding a novel tyrosine kinase receptor of the PDGFR/CSF1R family. *Oncogene* **6**: 1641–50.

Ruiz I Altaba A, Sanchez P, Dahmane N. (2002) Gli and hedgehog in cancer: tumours, embryos and stem cells. *Nat Rev Cancer* **2**: 361–72.

Sandberg ML, Sutton SE, Pletcher MT, *et al.* (2005) c-Myb and p300 regulate hematopoietic stem cell proliferation and differentiation. *Dev Cell* **8**: 153–66.

Santos-Rosa H, Schneider R, Bannister AJ, *et al*. (2002) Active genes are tri-methylated at K4 of histone H3. *Nature* **419**: 407–11.

Sato T, Laver JH, Ogawa M. (1999) Reversible expression of CD34 by murine hematopoietic stem cells. *Blood* **94**: 2548–54.

Sauvageau G, Thorsteinsdottir U, Eaves CJ, *et al*. (1995) Overexpression of HOXB4 in hematopoietic cells causes the selective expansion of more primitive populations *in vitro* and *in vivo*. *Genes Dev* **9**: 1753–65.

Schessl C, Rawat VP, Cusan M, *et al*. (2005) The AML1-ETO fusion gene and the FLT3 length mutation collaborate in inducing acute leukemia in mice. *J Clin Invest* **115**: 2159–68.

Schnittger S, Kohl TM, Haferlach T, *et al*. (2006) KIT-D816 mutations in AML1-ETO-positive AML are associated with impaired event-free and overall survival. *Blood* **107**: 1791–99.

Scholl C, Bansal D, Dohner K, *et al*. (2007) The homeobox gene CDX2 is aberrantly expressed in most cases of acute myeloid leukemia and promotes leukemogenesis. *J Clin Invest* **117**: 1037–48.

Scott EW, Simon MC, Anastasi J, Singh H. (1994) Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science* **265**: 1573–77.

Scott MP. (1992) Vertebrate homeobox gene nomenclature. *Cell* **71**: 551–53.

Serrano M, Lee H, Chin L, *et al*. (1996) Role of the INK4a locus in tumor suppression and cell mortality. *Cell* **85**: 27–37.

Shen W, Chrobak D, Krishnan K, *et al*. (2004) HOXB6 protein is bound to CREB-binding protein and represses globin expression in a DNA binding-dependent, PBX interaction-independent process. *J Biol Chem* **279**: 39895–904.

Shen WF, Detmer K, Mathews CH, *et al*. (1992) Modulation of homeobox gene expression alters the phenotype of human hematopoietic cell lines. *EMBO J* **11**: 983–89.

Shigesada K, Van De Sluis B, Liu PP. (2004) Mechanism of leukemogenesis by the inv(16) chimeric gene CBFB/PEBP2B-MHY11. *Oncogene* **23**: 4297–307.

Shivdasani RA, Fujiwara Y, Mcdevitt MA, Orkin SH. (1997) A lineage-selective knockout establishes the critical role of transcription factor GATA-1 in megakaryocyte growth and platelet development. *EMBO J* **16**: 3965–73.

Shivdasani RA, Mayer EL, Orkin SH. (1995) Absence of blood formation in mice lacking the T-cell leukemia oncoprotein tal-1/SCL. *Nature* **373**: 432–34.

干细胞之家www.stemcell8.cn ←点击进入

Shultz LD, Lyons BL, Burzenski LM, *et al*. (2005) Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells. *J Immunol* **174**: 6477–89.

Shultz LD, Schweitzer PA, Christianson SW, *et al*. (1995) Multiple defects in innate and adaptive immunologic function in NOD/LtSz-scid mice. *J Immunol* **154**: 180–91.

Siminovitch L, Mcculloch EA, Till JE. (1963) The distribution of colony- orming cells among spleen colonies. *J Cell Physiol* **62**: 327–36.

Singh SK, Hawkins C, Clarke ID, *et al*. (2004) Identification of human brain tumour initiating cells. *Nature* **432**: 396–401.

Slanicka Krieger M, Nissen C, Manz CY, *et al*. (1998) The membrane-bound isoform of stem cell factor synergizes with soluble flt3 ligand in supporting early hematopoietic cells in long-term cultures of normal and aplastic anemia bone marrow. *Exp Hematol* **26**: 365–73.

Smith ML, Cavenagh JD, Lister TA, Fitzgibbon J. (2004) Mutation of CEBPA in familial acute myeloid leukemia. *N Engl J Med* **351**: 2403–7.

So CW, Karsunky H, Passegue E, *et al*. (2003) MLL-GAS7 transforms multipotent hematopoietic progenitors and induces mixed lineage leukemias in mice. *Cancer Cell* **3**: 161–71.

So CW, Karsunky H, Wong P, *et al*. (2004) Leukemic transformation of hematopoietic progenitors by MLL-GAS7 in the absence of HOXA7 or HOXA9. *Blood* **103**: 3192–99.

Sophos NA, Vasiliou V. (2003) Aldehyde dehydrogenase gene superfamily: the 2002 update. *Chem Biol Interact* **143–144**: 5–22.

Spangrude GJ, Heimfeld S, Weissman IL. (1988) Purification and characterization of mouse hematopoietic stem cells. *Science* **241**: 58–62.

Spiekermann K, Bagrintseva K, Schoch C, *et al*. (2002) A new and recurrent activating length mutation in exon 20 of the FLT3 gene in acute myeloid leukemia. *Blood* **100**: 3423–25.

Stepanova L, Sorrentino BP. (2005) A limited role for p16Ink4a and p19Arf in the loss of hematopoietic stem cells during proliferative stress. *Blood* **106**: 827–32.

Stier S, Ko Y, Forkert R, *et al*. (2005) Osteopontin is a hematopoietic stem cell niche component that negatively regulates stem cell pool size. *J Exp Med* **201**: 1781–91.

Storms RW, Trujillo AP, Springer JB, *et al*. (1999) Isolation of primitive human hematopoietic progenitors on the basis of aldehyde dehydrogenase activity. *Proc Natl Acad Sci USA* **96**: 9118–23.

Sutherland HJ, Eaves CJ, Eaves AC, *et al*. (1989) Characterization and partial purification of human marrow cells capable of initiating long-term hematopoiesis *in vitro*. *Blood* **74**: 1563–70.

Sutherland HJ, Lansdorp PM, Henkelman DH, *et al*. (1990) Functional characterization of individual human hematopoietic stem cells cultured at limiting dilution on supportive marrow stromal layers. *Proc Natl Acad Sci USA* **87**: 3584–88.

干细胞之家www.stemcell8.cn ←点击进入

Suzuki N, Ohneda O, Minegishi N, *et al*. (2006) Combinatorial Gata2 and Sca1 expression defines hematopoietic stem cells in the bone marrow niche. *Proc Natl Acad Sci USA* **103**: 2202–7.

Suzuki T, Chiba S. (2005) Notch signaling in hematopoietic stem cells. *Int J Hematol* **82**: 285–94.

Takahashi S, Onodera K, Motohashi H, *et al*. (1997) Arrest in primitive erythroid cell development caused by promoter-specific disruption of the GATA-1 gene. *J Biol Chem* **272**: 12611–15.

Tallman MS, Gilliland DG, Rowe JM. (2005) Drug therapy for acute myeloid leukemia. *Blood* **106**: 1154–63.

Taniguchi T, Endo H, Chikatsu N, *et al*. (1999) Expression of p21(Cip1/Waf1/Sdi1) and p27(Kip1) cyclin-dependent kinase inhibitors during human hematopoiesis. *Blood* **93**: 4167–78.

Taussig DC, Pearce DJ, Simpson C, *et al*. (2005) Hematopoietic stem cells express multiple myeloid markers: implications for the origin and targeted therapy of acute myeloid leukemia. *Blood* **106**: 4086–92.

Tavor S, Petit I, Porozov S, *et al*. (2005) Motility, proliferation, and egress to the circulation of human AML cells are elastase dependent in NOD/SCID chimeric mice. *Blood* **106**: 2120–27.

Tenen DG. (2003) Disruption of differentiation in human cancer: AML shows the way. *Nat Rev Cancer* **3**: 89–101.

Theunissen K, Verfaillie CM. (2005) A multifactorial analysis of umbilical cord blood, adult bone marrow and mobilized peripheral blood progenitors using the improved ML-IC assay. *Exp Hematol* **33**: 165–72.

Thorsteinsdottir U, Sauvageau G, Hough MR, *et al*. (1997) Overexpression of HOXA10 in murine hematopoietic cells perturbs both myeloid and lymphoid differentiation and leads to acute myeloid leukemia. *Mol Cell Biol* **17**: 495–505.

Till JE, Mc CE. (1961) A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat Res* **14**: 213–22.

Tothova Z, Kollipara R, Huntly BJ, *et al*. (2007) FoxOs are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress. *Cell* **128**: 325–39.

Traggiai E, Chicha L, Mazzucchelli L, *et al*. (2004) Development of a human adaptive immune system in cord blood cell-transplanted mice. *Science* **304**: 104–107.

Trainor CD, Omichinski JG, Vandergon TL, *et al*. (1996) A palindromic regulatory site within vertebrate GATA-1 promoters requires both zinc fingers of the GATA-1 DNA-binding domain for high-affinity interaction. *Mol Cell Biol* **16**: 2238–47.

Trowbridge JJ, Xenocostas A, Moon RT, Bhatia M. (2006) Glycogen synthase kinase-3 is an *in vivo* regulator of hematopoietic stem cell repopulation. *Nat Med* **12** 89–98.

Tsai FY, Keller G, Kuo FC, *et al*. (1994) An early haematopoietic defect in mice lacking the transcription factor GATA-2. *Nature* **371**: 221–26.

干细胞之家www.stemcell8.cn ←点击进入

Uchida N, Combs J, Chen S, *et al*. (1996) Primitive human hematopoietic cells displaying differential efflux of the rhodamine 123 dye have distinct biological activities. *Blood* **88**: 1297–305.

Uchida N, Fujisaki T, Eaves A, Eaves CJ. (2001) Transplantable hematopoietic stem cells in human fetal liver have a CD34(+) side population (SP)phenotype. *J Clin Invest* **108**: 1071–77.

Van Der Vlag J, Otte AP. (1999) Transcriptional repression mediated by the human polycomb-group protein EED involves histone deacetylation. *Nat Genet* **23**: 474–78.

Vangala RK, Heiss-Neumann MS, Rangatia JS, *et al*. (2003) The myeloid master regulator transcription factor PU.1 is inactivated by AML1-ETO in t(8;21) myeloid leukemia. *Blood* **101**: 270–77.

Varnum-Finney B, Xu L, Brashem-Stein C, *et al*. (2000) Pluripotent, cytokine-dependent, hematopoietic stem cells are immortalized by constitutive Notch1 signaling. *Nat Med* **6**: 1278–81.

Verfaillie CM. (1998) Adhesion receptors as regulators of the hematopoietic process. *Blood* **92**: 2609–12.

Walkley CR, Fero ML, Chien WM, *et al*. (2005) Negative cell-cycle regulators cooperatively control self-renewal and differentiation of haematopoietic stem cells. *Nat Cell Biol* **7**: 172–78.

Wang JS, Fang Q, Sun DJ, *et al*. (2001) Genetic modification of hematopoietic progenitor cells for combined resistance to 4-hydroperoxycyclophosphamide, vincristine, and daunorubicin. *Acta Pharmacol Sin* **22**: 949–55.

Wang Q, Stacy T, Binder M, *et al*. (1996a) Disruption of the Cbfa2 gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis. *Proc Natl Acad Sci USA* **93**: 3444–49.

Wang Q, Stacy T, Miller JD, *et al*. (1996b) The CBFbeta subunit is essential for CBFalpha2 (AML1) function *in vivo*. *Cell* **87**: 697–708.

Wiemels JL, Ford AM, Van Wering ER, *et al*. (1999) Protracted and variable latency of acute lymphoblastic leukemia after TEL-AML1 gene fusion in utero. *Blood* **94**: 1057–62.

Wiemels JL, Xiao Z, Buffler PA, *et al*. (2002) *In utero* origin of t(8; 21) AML1-ETO translocations in childhood acute myeloid leukemia. *Blood* **99**: 3801–5.

Willert K, Brown JD, Danenberg E, *et al*. (2003) Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature* **423**: 448–52.

Wilson A, Murphy MJ, Oskarsson T, *et al*. (2004) c-Myc controls the balance between hematopoietic stem cell self-renewal and differentiation. *Genes Dev* **18**: 2747–63.

Wolffe AP, Hayes JJ. (1999) Chromatin disruption and modification. *Nucl Acids Res* **27**: 711–20.

Wulf G, Wang RY, Kuehnle I, *et al*. (2001) A leukemic stem cell with instrinsic drug efflux capacity in acute myelod leukemia. *Blood* **98**: 1166–73.

Xia ZB, Anderson M, Diaz MO, Zeleznik-Le NJ. (2003) MLL repression domain interacts with histone deacetylases, the polycomb group proteins HPC2 and

干细胞之家www.stemcell8.cn ←点击进入

BMI-1, and the corepressor C-terminal-binding protein. *Proc Natl Acad Sci USA* **100**: 8342–47.

Yahata T, Ando K, Nakamura Y, *et al*. (2002) Functional human T lymphocyte development from cord blood CD34+ cells in nonobese diabetic/Shi-scid, IL-2 receptor gamma null mice. *J Immunol* **169**: 204–209.

Yahata T, Ando K, Sato T, *et al*. (2003) A highly sensitive strategy for SCID-repopulating cell assay by direct injection of primitive human hematopoietic cells into NOD/SCID mice bone marrow. *Blood* **101**: 2905–13.

Yamamoto Y, Kiyoi H, Nakano Y, *et al*. (2001) Activating mutation of D835 within the activation loop of FLT3 in human hematologic malignancies. *Blood* **97**: 2434–39.

Yang L, Wang L, Geiger H, *et al*. (2007) Rho GTPase Cdc42 coordinates hematopoietic stem cell quiescence and niche interaction in the bone marrow. *Proc Natl Acad Sci USA* **104**: 5091–96.

Ye M, Iwasaki H, Laiosa CV, *et al*. (2003) Hematopoietic stem cells expressing the myeloid lysozyme gene retain long-term, multilineage repopulation potential. *Immunity* **19**: 689–99.

Yilmaz OH, Kiel MJ, Morrison SJ. (2006) SLAM family markers are conserved among hematopoietic stem cells from old and reconstituted mice and markedly increase their purity. *Blood* **107**: 924–30.

Yin AH, Miraglia S, Zanjani ED, *et al*. (1997) AC133, a novel marker for human hematopoietic stem and progenitor cells. *Blood* **90**: 5002–12.

Yokoyama A, Somervaille TC, Smith KS, *et al*. (2005) The menin tumor suppressor protein is an essential oncogenic cofactor for MLL-associated leukemogenesis. *Cell* **123**: 207–18.

Yoshida A, Rzhetsky A, Hsu LC, Chang C. (1998) Human aldehyde dehydrogenase gene family. *Eur J Biochem* **251**: 549–57.

Yu BD, Hanson RD, Hess JL, *et al*. (1998) MLL, a mammalian trithorax-group gene, functions as a transcriptional maintenance factor in morphogenesis. *Proc Natl Acad Sci USA* **95**: 10632–36.

Yu BD, Hess JL, Horning SE, *et al*. (1995) Altered Hox expression and segmental identity in Mll-mutant mice. *Nature* **378**: 505–508.

Yu H, Yuan Y, Shen H, Cheng T. (2005) Hematopoietic stem cell exhaustion impacted by p18INK4C and p21Cip1/Waf1 in opposite manners. *Blood* **107**: 1200–6.

Yuan Y, Shen H, Franklin DS, *et al*. (2004) *In vivo* self-renewing divisions of haematopoietic stem cells are increased in the absence of the early G1-phase inhibitor, p18INK4C. *Nat Cell Biol* **6**: 436–42.

Zanjani ED, Almeida-Porada G, Ascensao JL, *et al*. (1997) Transplantation of hematopoietic stem cells in utero. *Stem Cells* **15**(Suppl 1): 79–92; discussion 93.

Zanjani ED, Almeida-Porada G, Livingston AG, *et al*. (1999) Engraftment and multilineage expression of human bone marrow CD34- cells *in vivo*. *Ann N Y Acad Sci* **872**: 220–31; discussion 231–32.

Zeleznik-Le NJ, Harden AM, Rowley JD. (1994) 11q23 translocations split the "AT-hook" cruciform DNA-binding region and the transcriptional repression domain

干细胞之家www.stemcell8.cn ←点击进入

from the activation domain of the mixed-lineage leukemia (MLL) gene. *Proc Natl Acad Sci USA* **91**: 10610–14.

Zeng H, Yucel R, Kosan C, *et al*. (2004) Transcription factor Gfi1 regulates self-renewal and engraftment of hematopoietic stem cells. *EMBO J* **23**: 4116–25.

Zhang DE, Zhang P, Wang ND, *et al*. (1997) Absence of granulocyte colony-stimulating factor signaling and neutrophil development in CCAAT enhancer binding protein alpha-deficient mice. *Proc Natl Acad Sci USA* **94**: 569–74.

Zhang J, Niu C, Ye L, *et al*. (2003) Identification of the haematopoietic stem cell niche and control of the niche size. *Nature* **425**: 836–41.

Zhang P, Zhang X, Iwama A, *et al*. (2000) PU.1 inhibits GATA-1 function and erythroid differentiation by blocking GATA-1 DNA binding. *Blood* **96**: 2641–48.

Zhang Y, Payne KJ, Zhu Y, *et al*. (2005) SCL expression at critical points in human hematopoietic lineage commitment. *Stem Cells* **23**: 852–60.

Zhou S, Schuetz JD, Bunting KD, *et al*. (2001) The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. *Nat Med* **7**: 1028–34.

干细胞之家www.stemcell8.cn ←点击进入

Chapter 10

Mammary Stem Cells

Mark Shackleton†, Marie-Liesse Asselin-Labat, François Vaillant, Geoffrey J. Lindeman and Jane E. Visvader*

The defining anatomical characteristic of mammals is the mammary gland. Comprising of a ductal epithelial network and surrounding stroma, it is a remarkably adaptive organ whose development is closely related to physiological requirement. During pregnancy, the number of mammary epithelial cells increases dramatically and these undergo terminal differentiation to enable lactation after birth. After weaning, the number and differentiation status of these cells returns to their pre-pregnant state, maintaining a similar developmental potential for future pregnancies. Strong evidence now exists to support the reliance of this adaptability on mammary stem cells (MaSCs).

Through *in vivo* mammary reconstitution experiments, primarily using the mouse as a model system, it has been demonstrated that MaSCs reside within mature mammary epithelium as single cells that can be prospectively identified. This discovery, along with the identification of other progenitors with limited proliferation capacity, has indicated that mammary epithelium is organized in a hierarchical fashion, in which MaSCs give rise to mature, functional epithelium via intermediate, lineage-restricted progenitors. The ability to prospectively identify and isolate functionally distinct mammary epithelial subpopulations has been an important development in mammary biology and opened the way for a more detailed understanding of the molecular regulation of normal and neoplastic development of this organ. In addition, it should enable therapeutic strategies that target specific mammary cell subtypes to be explored.

Keywords: Breast cancer; cancer stem cell; CD24, CD29/β1-integrin, CD49f/α6-integrin, CD61/β3-integrin, estrogen receptor; mammary stem cell; MMTV-wnt-1, neu, wnt-1.

†Present address: Life Sciences Institute, University of Michigan, Ann Arbor, MI, USA
*Corresponding author. visvader@wehi.edu.au
The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria 3050, Australia

干细胞之家www.stemcell8.cn ←点击进入

1. Introduction

Most adult tissues are thought to harbor a tissue-specific stem cell population. In many cases, putative somatic stem cells have been identified through association with certain marker expression patterns and/or *in vitro* characteristics, as technical limitations have generally prevented their prospective isolation and direct evaluation *in vivo* (Kim *et al.*, 2005). Until recently, hematopoietic stem cells (HSCs) were the only type of somatic stem cell in which the defining properties of *in vivo* self-renewal and multi-lineage differentiation had been demonstrated at the single cell level (Krause *et al.*, 2001; Uchida *et al.*, 2003; Matsuzaki *et al.*, 2004; Takano *et al.*, 2004). In the mammary gland biology field, development and optimization of tissue dissociation and *in vivo* mammary reconstitution techniques over 50 years (De Ome *et al.*, 1959; Hoshino and Gardner, 1967; Daniel *et al.*, 1968; Smith, 1996) have recently allowed the isolation of single mammary stem cells (MaSCs) that display the requisite properties of self-renewal and multi-lineage differentiation *in vivo* (Shackleton *et al.*, 2006; Stingl *et al.*, 2006). This report will review the current understanding of mammary epithelial development, with particular emphasis on the nature of MaSCs, their relationships with other mammary cell types, their molecular regulation and potential role in breast cancer development.

2. Normal Mammary Development

2.1. *Mammary Gland Structure and Function*

The basic epithelial structure of the mammary gland comprises a branching network of hollow ducts that emanate from the nipple and end in lobules (Fig. 1). In the fully developed, lactating mammary gland, the ducts function to transport milk to the offspring during suckling, and the lobules, present as expanded clusters of alveolar structures, are the sites of milk production by specialized, differentiated epithelial cells. These epithelial structures reside within a stromal matrix comprising stromal cells such as fibroblasts and adipocytes as well as extracellular components (Neville *et al.*, 1998). Also resident within the mammary gland are hematopoietic cells, blood vessels and neuronal cells. In the human breast, the epithelium is located within a dense fibroblast-rich connective tissue, surrounded by adipose tissue. Conversely, the mouse mammary gland has significantly more adipocytes but less fibrous connective tissue. The non-epithelial elements in the mouse mammary gland are collectively referred to as the mammary fat pad (MFP) (Neville *et al.*, 1998) (Fig. 1).

干细胞之家www.stemcell8.cn ←点击进入

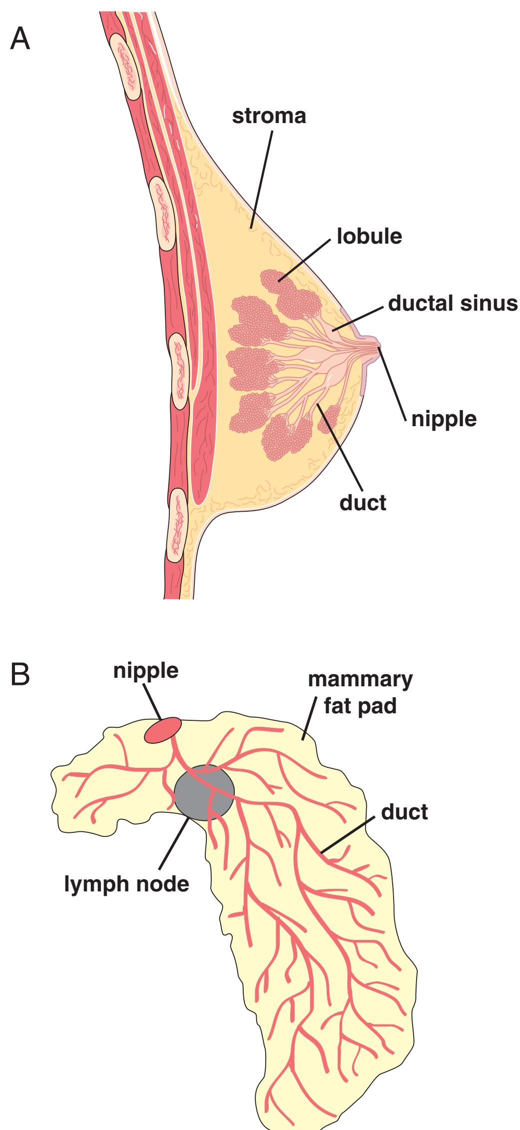


Fig. 1. Structure of the human and mouse mammary gland. **(A)** Human breast. **(B)** Mouse mammary gland (Ref. 4).

2.2. *Mammary Development in the Mouse*

Development of the mouse mammary epithelium has been well characterized and comprises distinct phases that include embryogenesis, puberty, pregnancy, lactation and involution [Fig. 2(A)]. At birth, the mouse mammary gland exists as a small, rudimentary ductal structure and remains

干细胞之家www.stemcell8.cn ←点击进入

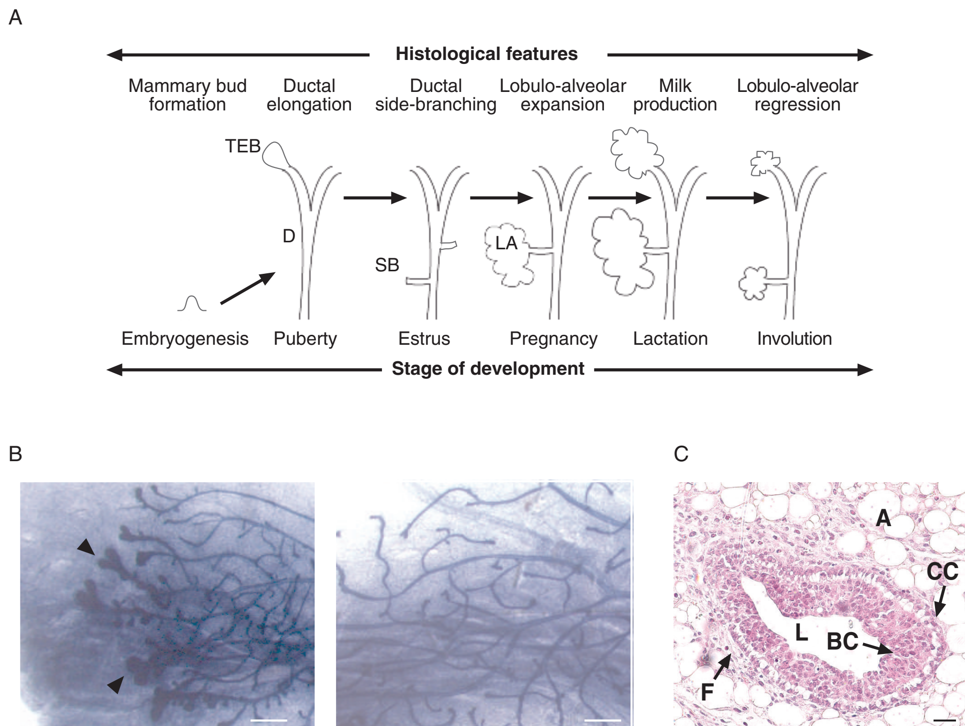


Fig. 2. Mammary epithelial development. **(A)** Schematic diagram of the anatomical changes in mammary epithelium associated with the different stages of development; D — duct; TEB — terminal end bud; SB — side-branch; LA — lobuloalveolar unit. The developmental changes of estrus, pregnancy and lactation overlap to varying degrees. Adapted from http://ccm.ucdavis.edu/bcancercd/22/development.html. **(B)** Whole-mount analyses of epithelial ductal trees in a developing 6-week-old mouse (left panel; TEBs are apparent at the tips of extending ducts, arrowheads) and a mature 12-week-old mouse (right panel; note fully filled fat pad and disappearance of TEBs). Scale bars, 1 mm. **(C)** H&E-stained section of a TEB showing different cell types; original magnification × 300. L — lumen; BC — body cells; CC — cap cells; F — fibroblasts; A — adipocytes. (Adapted from Hinck and Silberstein, *Breast Cancer Res* **7**: 245–251).

relatively dormant until the onset of puberty at approximately three weeks of age. Pubertal development is characterized by the elongation and branching of ducts that are subtended at their ends by structures called terminal end-buds (TEBs) (Richert *et al.*, 2000). TEBs are bulbous-shaped, multi-layered epithelial structures, contiguous with the developing ducts [Fig. 2(B)]. The tip of the TEB is composed of a single layer of undifferentiated, proliferating cells, called cap cells. The cap cell region is thought to contain progenitors that give rise to both the central body cells of the TEB, which themselves give rise to differentiated luminal epithelium, as well as a

干细胞之家www.stemcell8.cn ←点击进入

lateral, basally-located cell monolayer that becomes mature myoepithelium [Fig. 2(C)]. (Williams and Daniel, 1983) (see "Mammary epithelial cell lineages"). The TEB is replaced by a simple end-bud structure when the duct reaches the margin of the MFP (Silberstein, 2001) and further elongation of major mammary ducts ceases [Fig. 2(B)].

Two additional features of mammary epithelial development occur after puberty: ductal side-branching and lobuloalveolar proliferation and differentiation. Ductal side-branching is regulated by systemic hormones, beginning with the onset of estrus cycling during puberty and continuing during pregnancy [Fig. 2(A)]. It has been hypothesized to develop from sites in the major ducts that are enriched with mammary stem and progenitor cells (Smalley and Ashworth, 2003). Alveolar buds develop in tandem with the ductal side-branches during estrus and early pregnancy, and are the sub-structures from which milk-producing, lobuloalveolar epithelia are presumed to arise. The stage of mammary development from early- to mid-pregnancy is characterized by dramatic proliferation of lobuloalveolar epithelium (Kordon and Smith, 1998), such that by late pregnancy, the alveoli comprise the majority of the fat pad, effacing stromal elements (Richert *et al.*, 2000).

Differentiation of lobuloalveolar structures becomes evident in mid-pregnancy as milk protein and lipid droplets appear in alveolar epithelial cells and lumens (Richert *et al.*, 2000) and continues after parturition as suckling is established. After weaning, regression of lobuloalveolar structures occurs through apoptosis and remodeling of both the epithelium and stroma. By 14–21 days of involution, the mouse mammary gland has regressed to a near virginal state, though differences in virgin and parous non-pregnant mammary glands have been noted (Richert *et al.*, 2000). Nevertheless, the parous, non-pregnant mammary gland retains the functional capacity of its virgin antecedent, capable of repeated lobuloalveolar development in subsequent rounds of pregnancy. It is thus implied that a cell subpopulation with enormous regenerative capability resides within the mammary gland.

3. Mammary Epithelial Cell Lineages

The mammary epithelium is composed of two main cell lineages: luminal epithelium, located in the luminal aspect, and myoepithelium, located in a basal position adjacent to the basement membrane [Figs. 3(A) and (B)]. Luminal epithelium is composed of glandular cells with specialized apical and basolateral membrane domains that express sialomucins and adhesion

干细胞之家www.stemcell8.cn ←点击进入

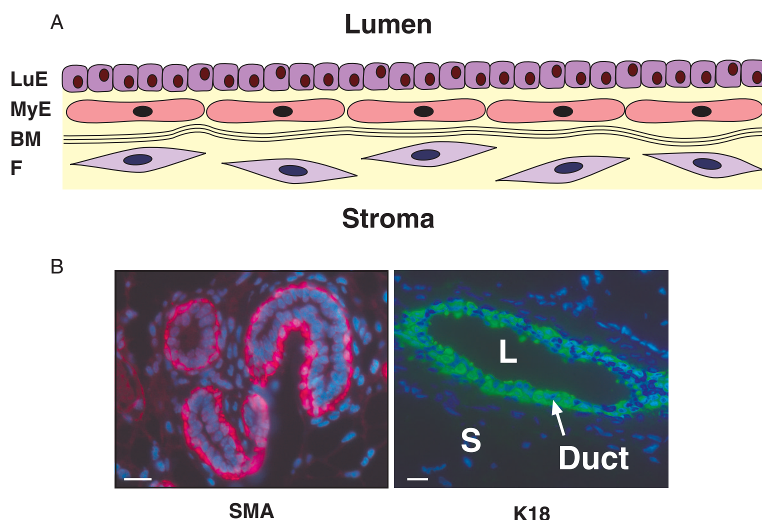


Fig. 3. Mammary epithelial cell lineages. **(A)** Schematic diagram depicting the two primary mammary epithelial cell lineages and their relationship with the surrounding stroma. LuE — luminal epithelium; MyE — myoepithelium; BM — basement membrane, F — fibroblasts. **(B)** Smooth muscle actin (left panel) and keratin 18 (right panel) immunofluorescent staining of myoepithelium and luminal epithelium, respectively, in mouse mammary ducts. L — lumen; S — stroma. Scale bars, 25 μm.

molecules, respectively (Ronnov-Jessen *et al.*, 1996). It is characterized by the cytoskeletal expression of cytokeratins 7, 8, 18 and 19 (Taylor-Papadimitriou *et al.*, 1992), as well as the surface marker proteins, MUC1 (Petersen and van Deurs, 1986) and ESA (Latza *et al.*, 1990).

Luminal mammary epithelium is further classified according to the substructure in which it resides. The ductal luminal epithelium, characterized by expression of the Na-K-Cl co-transporter NKCC1 (Shillingford *et al.*, 2002), lines tubular ducts in tightly associated sheets. The milk-producing alveolar luminal epithelium, which develops primarily during pregnancy, comprises a single layer of cells arranged spherically around a hollow lumen, and lines the lobuloalveolar structures.

It is likely that both the ductal and alveolar luminal epithelial cells arise from their own lineage-restricted progenitor cell types. This was suggested by the observation that transplantation of mammary cells into epithelium-free MFPs could produce epithelial outgrowths with a ductal-only or a lobule-only morphology (Smith, 1996). In addition, the ability to prospectively select cells that form colonies with luminal-only features *in vitro* suggests the presence of lineage-restricted luminal epithelial progenitors (Smalley *et al.*, 1998; Stingl *et al.*, 1998 and 2001).

干细胞之家www.stemcell8.cn ←点击进入

The myoepithelium provides contractile properties to the ductal tree to aid milk expulsion (Murrell, 1995), intercellular signals that affect mammary gland morphogenesis (Coleman *et al.*, 1988; Deugnier *et al.*, 1999; Gomm *et al.*, 1997; Buhler *et al.*, 1993; Liu *et al.*, 1996; Pierce *et al.*, 1993) and secreted components for maintenance of basement membrane integrity (Williams and Daniel, 1983; Dulbecco *et al.*, 1986). Differentiated myoepithelium expresses cytokeratins 5 and 14 (Taylor-Papadimitriou *et al.*, 1989), and smooth muscle actin (SMA) (Gugliotta *et al.*, 1988), as well as the surface marker proteins P-cadherin (Deugnier *et al.*, 2002), CD10/CALLA (Gusterson *et al.*, 1986) and CD90/Thy-1 (Gudjonsson *et al.*, 2002a). Although alveolar-associated and ductal-associated myoepithelia differ in their morphological characteristics (Emerman and Vogl, 1986), it may be that the observed differences in myoepithelial size and shape reflect the number of myofilaments and therefore differentiation status, rather than distinct cell lineages. These differences suggest that myoepithelial cells are responsive to hormones and growth factors that act at specific phases of their development. Indeed, myoepithelial cell bodies have also been noted to change in shape during involution (Emerman and Vogl, 1986).

Putative myoepithelial precursor cells have been identified in the cap cell region of TEBs in the pubertal mammary gland (Williams and Daniel, 1983) [Fig. 2(C)]. However, it is likely that they also reside in developed adult mammary epithelium, as mammary epithelial transplantation studies indicate the presence of precursor cells throughout the ductal tree (Hoshino, 1967; Hoshino and Gardner, 1967; Smith, 1996; Daniel *et al.*, 1968; Daniel and Young, 1971; Daniel *et al.*, 1971; Young *et al.*, 1971). Moreover, as for luminal epithelial progenitors, cells with an ability to form myoepithelium-restricted colonies *in vitro* can be prospectively isolated from fresh mammary cell preparations (Smalley *et al.*, 1998; Stingl *et al.*, 2001 and 1998; Asselin-Labat *et al.*, 2007; Sleeman *et al.*, 2007).

4. Evidence for the Existence of Mammary Stem Cells

Many aspects of mammary gland development imply the existence of MaSCs. The massive expansion of mammary epithelium during puberty and pregnancy (Kordon and Smith, 1998), together with the remarkable regenerative capacity apparent during successive reproductive cycles, implicates stem and/or progenitor cell activity. Moreover, classical transplantation studies in the mouse support the existence of a mammary epithelial cell subpopulation with the capacity for multi-lineage differentiation and self-renewal. With the use of the cleared MFP transplantation method

干细胞之家www.stemcell8.cn ←点击进入

pioneered by De Ome *et al*. (De Ome *et al*., 1959) and refined by others (Smith, 1996) (see "Identification of mouse mammary stem cells"), it has been demonstrated that small fragments (explants) of normal mouse mammary epithelium (Hoshino and Gardner, 1967; Daniel *et al*., 1968) or cell suspensions prepared from whole mammary tissues (Smith, 1996) can reconstitute normal, functioning mammary epithelial structures. Evidence that a self-renewing cell in the mammary gland could confer this property was first provided by the serial transplantation of explants derived from such structures that showed maintenance of mammary-reconstituting activity for up to seven generations of transplantation (Hoshino and Gardner, 1967; Daniel *et al*., 1968).

The identification of identical chromosomal alterations in all cell types of contiguous regions of human breast epithelium suggests MaSCs as the common, clonal origins of those regions (Tsai *et al*., 1996; Diallo *et al*., 2001; Lakhani *et al*., 1996; Deng *et al*., 1996; Lakhani *et al*., 1999). Based on these studies, candidate MaSC populations in the human breast have been identified. Experimental evaluation of these populations has relied upon *in vitro* studies, as mouse mammary stroma does not permit the normal growth of human mammary epithelium. The recent development of a method to "humanize" the mouse MFP (Kuperwasser *et al*., 2004), thereby facilitating growth of human cells, offers hope of a robust *in vivo* model for the evaluation of human MaSCs. In lieu of such, several investigators have evaluated freshly isolated and immortalized human mammary cell populations for the presence of cells with the potential for multi-lineage differentiation *in vitro*.

Three types of epithelial colony-forming cells within the human mammary gland were demonstrated by Stingl *et al*. (Stingl *et al*., 1998 and 2001). Whereas two of the cell types formed either pure luminal or pure myoepithelial colonies, a third cell type formed colonies containing both luminal and myoepithelial cells. In addition, only the latter cell type formed morphologically complex branching structures in three-dimensional collagen gel cultures. Other investigators have used *in vitro* cell culture to demonstrate that mammary epithelial cells with a luminal phenotype may acquire myoepithelial characteristics under specific conditions, whereas myoepithelium was not demonstrated to transdifferentiate in this way (Pechoux *et al*., 1999). Extending this concept, Gudjonsson *et al*. immortalized a subtype of luminal epithelial cells and demonstrated its capacity for multi-lineage differentiation *in vitro* (Gudjonsson *et al*., 2002b).

Further *in vitro* evidence for the existence of human MaSCs was offered by Wicha and colleagues through adaptation of the non-adherent cell culture

干细胞之家www.stemcell8.cn ←点击进入

system to mammary epithelium (Dontu *et al.*, 2003). Three-dimensional colonies, termed mammospheres, were produced from human reduction mammoplasty specimens by culture on non-adherent substrata. Cells within these mammospheres demonstrated *in vitro* self-renewal and multi-lineage differentiation.

While these studies provide a significant body of evidence for the existence of human MaSCs, direct proof of the ability of human mammary epithelial cells to undergo *in vivo* self-renewal and multi-lineage differentiation is lacking. However, recent observations in the mouse that unequivocally demonstrate the existence of mammary cells with these properties show that it is very likely that the human mammary gland also contains MaSCs.

5. Identification of Mouse Mammary Stem Cells

The methods used in the identification of mouse MaSCs have been adapted from those used in hematopoietic stem cell (HSC) research. Work in this field over a number of years, facilitated by the development of an *in vivo* assay of HSC function — that is, long-term hematopoietic reconstitution by transplantation of donor HSCs into bone marrow-depleted hosts — has defined the "gold standard" method for determination of somatic stem cell identity. With the use of the method of limiting dilution (Bonnefoix *et al.*, 1996) to allow evaluation of stem cell function at the clonal level, it has been possible to quantitate the frequency of individual cells with *in vivo* stem cell characteristics in phenotypically defined hematopoietic cell populations. As the most biologically relevant definitions of stem cells — that is, single cells with the capacity for self-renewal and multi-lineage differentiation — relate to *in vivo* function, such an approach to their evaluation is generally regarded as ideal.

There have been two major challenges in adapting this rigorous approach to solid organ systems. The first has been the requirement to first obtain single cells in suspension from solid tissues. Many cells in solid organs, particularly epithelial cells, exist tightly associated with one another and/or the extracellular matrix, and indeed depend on close interactions for their function and survival (Meredith *et al.*, 1993; Frisch and Francis, 1994; Gilmore, 2005). It is thus highly likely that dissociation of solid tissues into single cells will result in anoikis of some cells. In addition, the removal of cells from their normal microenvironment may affect their phenotype and/or function (Moir *et al.*, 2003). Although these caveats need to be considered when interpreting data from purification of specific cells types that

干细胞之家www.stemcell8.cn ←点击进入

normally reside within a solid organ, such approaches may yield valuable insights into solid organ biology and regulation.

A second challenge for solid tissue biologists to adapt the "gold standard" hematopoietic method of somatic stem cell identification has been the availability of robust, organ-specific *in vivo* organ reconstitution assays. Such assays present particular challenges when applied to stem cells of solid organs because of the close associations between different cell types and their relationships with the extracellular matrix that may be required to allow engraftment and proliferation of stem cells in these organs (Kim *et al.*, 2005). Fortunately for mammary biologists, an *in vivo* reconstitution assay exists to allow rigorous evaluation of MaSC activity in defined cell populations, akin to that used in the HSC field.

The mouse is an excellent model for the *in vivo* study of mammary development. In particular, the limited pre-pubertal development of the mouse mammary gland allows for its surgical manipulation and the combination of epithelial and stromal tissues from different sources. For the *in vivo* study of MaSC activity, host epithelium is first removed using defined anatomical landmarks (Fig. 4) (De Ome *et al.*, 1959). This procedure leaves intact the MFP, into which donor mammary cells may be transplanted. The host MFP is then evaluated for ductal outgrowths of mammary epithelium, the donor origin of which can be confirmed using genetically-tagged cells (Smith, 1996; Brisken *et al.*, 1998; Boulanger *et al.*, 2005; Shackleton *et al.*, 2006).

The MFP reconstitution assay has provided powerful evidence that all mammary epithelial cell types derive from a common cellular origin — the MaSC. Serial transplantation of retrovirally-tagged mouse mammary

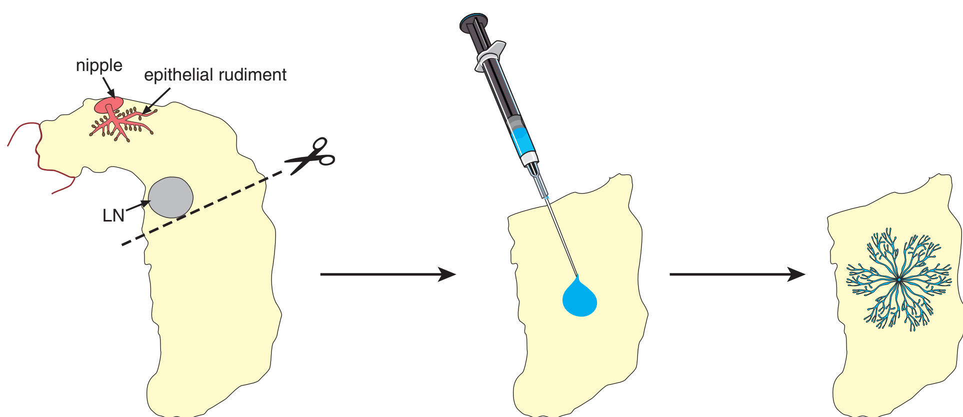


Fig. 4. Method of mammary fat pad (MFP) clearance and transplantation of cells to generate mammary outgrowths.

干细胞之家www.stemcell8.cn ←点击进入

epithelial fragments demonstrated increasing homogeneity of viral insertion site with transplant generation, consistent with the notion that a single progenitor cell can repopulate an entire mammary gland (Kordon and Smith, 1998). More recently, limiting dilution analysis of decreasing numbers of transplanted mammary cells from an MaSC-enriched population demonstrated a negative linear relationship between the number of cells transplanted and the proportion of negative fat pads obtained (Stingl *et al.*, 2006), consistent with a "single-hit" model (Bonnefoix *et al.*, 1996). Furthermore, mixing of freshly isolated, genetically tagged (carrying the *LacZ* transgene) cells with wild-type cells prior to transplantation at limiting numbers produced very few (2/97) and limited chimeric structures. Notably in these experiments, separate pure $LacZ^+$ and pure wild-type outgrowths were observed in 18 recipient MFPs, indicating the injection of at least one MaSC from each genotype into these MFPs, none of which formed a chimeric structure (Shackleton *et al.*, 2006) [Fig. 5(A)]. Finally, even more compelling evidence has been provided in the engraftment of MFPs by the direct transplantation of very low numbers of cells, and even single, prospectively visualized cells, isolated from MaSC-enriched populations (Stingl *et al.*, 2006; Shackleton *et al.*, 2006) [Fig. 5(B)]. It thus appears that the model of organ ontogeny defined in the hematopoietic system, in which single, pluripotent stem cells give rise to all cell types of a particular organ, is applicable to the mammary epithelium.

6. Multi-Lineage Differentiation and Self-Renewal of Mammary Stem Cells

Are single MaSCs really stem cells according to the strictest definitional criteria? Do they display the defining stem cell characteristics of *in vivo* multi-lineage differentiation and self-renewal? The answer to these questions is an unequivocal "yes". In the mouse MFP transplant experiments described above, clonally-derived mammary epithelial outgrowths were shown to contain both luminal and myoepithelial cell lineages (Shackleton *et al.*, 2006; Stingl *et al.*, 2006). Moreover, features of terminal differentiation, including milk production, were evident when outgrowths were analyzed during pregnancy or at parturition [Fig. 5(C)]. These findings provide direct evidence for multi-lineage differentiation, in that all the mammary epithelial cell types may be derived from single MaSCs.

Although the self-renewing capability of MaSCs had been suggested by earlier work in which serial transplantation of fragments of mammary epithelium was performed for several generations (Hoshino and

干细胞之家www.stemcell8.cn ←点击进入

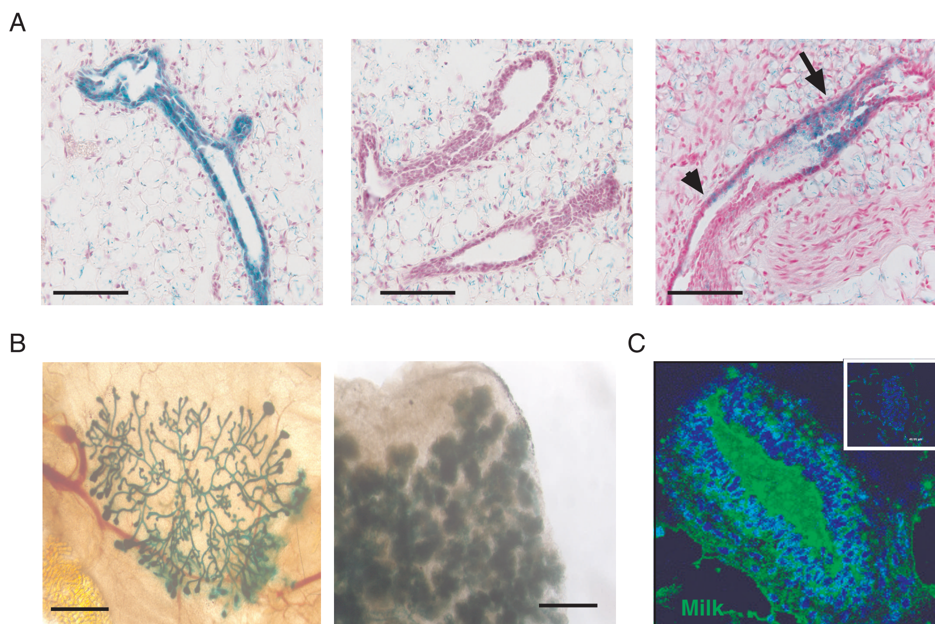


Fig. 5. Evidence for reconstitution of a mammary gland by a single stem cell. **(A)** X-gal- and nuclear fast red-stained sections of mammary ductal outgrowths that resulted from transplantation of mixed wild-type and genetically-tagged (Rosa26) mammary epithelial cells. The vast majority of outgrowths were either pure wild-type or pure Rosa26 (left and central panels, respectively). Only two chimeric outgrowths (right panel, arrow: Rosa26 region, arrowhead: wild type region) were produced. **(B)** X-gal stained outgrowths derived from single transplanted cells in virgin (left panel) and pregnant (right panel) recipients. **(C)** Immunofluorescent staining for milk proteins in a mammary duct derived from a single cell and harvested during pregnancy. (Based on supplementary Fig. 3(d–f) (A) and on Fig. 3 a (B) & c (C) of: Shackleton *et al.*, *Nature* **439**: 84–88 (2006), with permission from *Nature*).

Gardner, 1967; Daniel *et al.*, 1968), recent serial transplantation studies performed with very low numbers of transplanted cells provide powerful evidence for *in vivo* MaSC self-renewal (Shackleton *et al.*, 2006; Stingl *et al.*, 2006). In these experiments, cell suspensions prepared from clonally-derived primary mammary epithelial outgrowths were divided and secondarily transplanted into multiple recipient MFPs. For each clonal primary outgrowth, more than one secondary outgrowth was produced, an occurrence that could only be explained by self-renewal of the primarily transplanted MaSC (Fig. 6). Indeed, most clonally-derived primary outgrowths were able to generate more than 10 secondary outgrowths, and calculation of MaSC frequencies in primary and secondary cell suspensions indicated that at least 10 symmetrical, self-renewing MaSC divisions occurred during formation of the primary outgrowths (Stingl *et al.*, 2006). We have continued to serially

干细胞之家www.stemcell8.cn ←点击进入

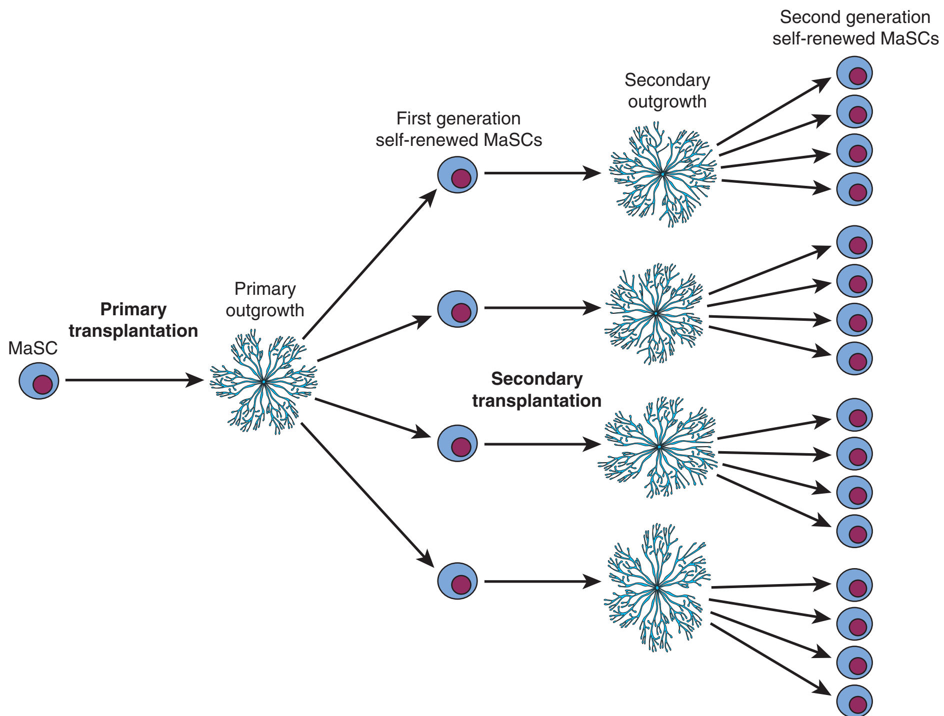


Fig. 6. Evidence for self-renewal of mammary stem cells from serial transplantation assays. The derivation of multiple secondary mammary outgrowths from a clonal primary outgrowth can only occur through self-renewal of the primarily transplanted MaSC.

transplant MaSCs in this way and demonstrated the formation of robust epithelial outgrowths for several generations (FV, unpublished data), highlighting the remarkable proliferative and degenerative capacities of MaSCs.

7. Characterization of Mammary Stem Cells

7.1. *Cell Markers Enabling Enrichment of Mammary Stem Cells*

The identification of specific cell markers that can prospectively identify MaSCs, thus allowing their purification, is an important goal in mammary biology. In the human mammary gland, several cell surface markers have been associated with candidate MaSC populations. The bipotent mammary epithelial progenitor cell identified prospectively in studies by Stingl *et al.* had the surface marker phenotype MUC1^{-}CALLA$^{lo/hi}$ESA^{+}EpCAM^{+}CD49f^{+} (Stingl *et al.*, 1998 and 2001). In support of these studies, an immortalized human mammary epithelial cell

干细胞之家www.stemcell8.cn ←点击进入

line that displayed *in vitro* multi-lineage differentiation was derived from the MUC1$^-$ESA$^+$ subpopulation (Gudjonsson *et al.*, 2002b).

In the mouse, an even more detailed picture of the phenotype of MaSCs has emerged. CD24 (HSA, heat stable antigen) has been identified as a pan-epithelial cell surface marker in the mouse mammary gland (Sleeman *et al.*, 2006; Stingl *et al.*, 2006; Shackleton *et al.*, 2006). Transplantation of limiting numbers of cells from subpopulations of CD24$^+$ mammary cells defined by CD29 (β1 integrin) (Shackleton *et al.*, 2006) and CD49f (α6 integrin) expression (Stingl *et al.*, 2006) (Fig. 7) has shown significant enrichment of MaSC activity in the CD29^{hi} and CD49f^{hi} fractions. Indeed, almost all the CD24$^+$CD29^{hi} mammary cells express high levels of CD49f (unpublished data), indicating that high levels of expression of the CD29/CD49f heterodimer may be a feature of mouse MaSCs. Other reports have indicated that MaSCs may be distinguished from luminal epithelium by lower (but not negative) levels of CD24 expression (Sleeman *et al.*, 2006 and 2007).

It is important to note that the MaSC-enriched CD24$^+$CD29^{hi}/CD49f^{hi} cell population identified above contains other mammary cell types in addition to MaSCs. Indeed, limiting dilution analysis of cells purified from these populations indicated MaSC frequencies of only 1/64 (Shackleton *et al.*, 2006) and 1/62 (Stingl *et al.*, 2006), respectively. Specifically, characterization of these populations for lineage-associated marker expression (Shackleton *et al.*, 2006; Asselin-Labat *et al.*, 2007) and gene expression (Stingl *et al.*, 2006) indicated the presence of a significant myoepithelial cell population. The heterogeneity of the CD24$^+$CD29^{hi}/CD49f^{hi} population currently inhibits attempts to further characterize MaSCs, except when findings are unequivocal (see below). The CD24$^+$CD29^{hi}/CD49f^{hi} phenotype is thus not specific

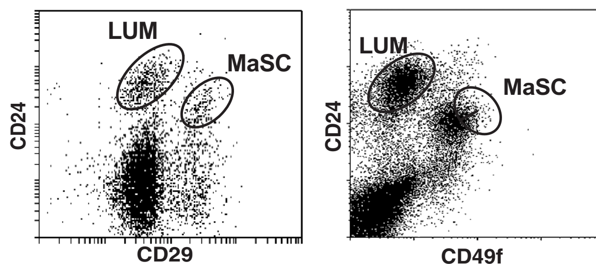


Fig. 7. Cell surface markers of mammary stem cells. Flow cytometric analysis of CD31$^-$, CD45$^-$ mammary epithelial cell suspensions co-stained with CD24 and CD29 (left panel) or CD24 and CD49f (right panel). LUM — luminal epithelial population.

干细胞之家www.stemcell8.cn ←点击进入

to mouse MaSCs and additional markers that define a more enriched MaSC population are being sought.

Previous reports suggested that high Sca-1 expression and Hoechst33342 (Ho) exclusion, features of HSCs, were also features of MaSCs (Welm *et al.*, 2002; Alvi *et al.*, 2003). However, when freshly isolated cells with these features were transplanted in limiting numbers, no enrichment in MaSC activity was found (Shackleton *et al.*, 2006; Stingl *et al.*, 2006). In support of these latter findings, recent observations by Sleeman *et al*. have characterized the epithelial Sca-1^{hi} population as being highly enriched for differentiated, estrogen-receptor (ER)-expressing luminal epithelium (Sleeman *et al.*, 2007). The most likely explanation for the discrepancy in reported Sca-1 expression levels of MaSCs lies in the culturing of cells that was performed by Welm *et al*. prior to flow cytometric analysis, as *in vitro* culture increases the expression of Sca-1 in primary mammary epithelial cells (Stingl *et al.*, 2006 and unpublished data), an effect that may also occur in HSCs (Rebel *et al.*, 1994).

The ability to efflux vital dyes such as Ho is a feature of quiescent murine HSCs that is lost when these cells enter the cell cycle (Uchida *et al.*, 2004; Spangrude and Johnson, 1990). The observation that MaSCs are not enriched in the Ho-excluding mammary "side-population" thus raises the possibility that they may not be as quiescent as stem cells of other tissues. In support of this, Stingl *et al.* showed that the majority of MaSCs were in the G_1 and $S/G_2/M$ phases of the cell cycle by transplantation of cells fractionated according to Pyronin Y and Ho labeling (Stingl *et al.*, 2006). The expression and/or activity of transporter pumps may also be intrinsically reduced in MaSCs irrespective of cell cycle status. The observation that expression of the ABCG2 transmembrane protein pump, a determinant of the Ho side population, is prominent in differentiated luminal epithelium (Jonker *et al.*, 2005) further indicates that Ho exclusion is not a specific property of MaSCs.

7.2. *Expression of Hormone Receptors in Mammary Stem Cells*

Despite the fact that estrogen and progesterone are mammary epithelial mitogens, the mouse MaSC-enriched population defined by either $CD24^{+}CD29^{hi}$ or $CD24^{+}CD49f^{hi}$ has been demonstrated to lack expression of receptors for these hormones (Asselin-Labat *et al.*, 2006). In support of this, Sleeman *et al*. found that MaSC activity resided within a basal mammary epithelial population that did not express estrogen receptor (ER) (Sleeman *et al.*, 2007). However, using DNA-label retention as a marker of MaSCs, other groups have identified subpopulations of putative MaSCs in both mouse (Booth and Smith, 2006) and human (Clarke *et al.*, 2005) mammary tissue

干细胞之家www.stemcell8.cn ←点击进入

that express hormone receptors. These cells are distinct from the mouse MaSC defined by using *in vivo* repopulation assays (Asselin-Labat *et al.*, 2006; Sleeman *et al.*, 2007) but may represent a different type of stem cell (such as a short-term repopulating cell) within the epithelial hierarchy. It is notable that ER expression is generally highest in the most differentiated and least proliferative cells of mouse mammary epithelium (Zeps *et al.*, 1998; Clarke, 2003; Asselin-Labat *et al.*, 2007). Thus, estrogen may influence MaSC function indirectly through paracrine factors that are yet to be identified.

7.3. *Expression of Lineage-Associated Markers in Mammary Stem Cells*

Expression of specific cytokeratins has been shown to be associated with certain mammary epithelial lineages (see "Mammary epithelial cell lineages"). In the mouse, basally located myoepithelium expresses high levels of CD29 and CD49f (Shackleton *et al.*, 2006; Stingl *et al.*, 2006). Flow cytometric analysis of K18 and K14 expression in the MaSC-enriched $CD24^{+}CD29^{hi}$ population showed that it is predominantly characterized by strong expression of K14, although small populations of $K14^{lo}$- and $K18^{+}$-expressing cells could also be detected (Shackleton *et al.*, 2006). Interestingly, almost all the cells within this population also expressed SMA (MS, unpublished data). Consistent with these observations, Stingl *et al.* performed gene expression studies of the MaSC-enriched $CD24^{+}CD49f^{hi}$ population and showed high levels of expression of K14 and SMA. Indeed, it was found unexpectedly in these studies that the gene expression profile of the MaSC population was not significantly different from the functionally-distinct "MYO" population ($CD24^{lo}CD49f^{lo}$), enriched for myoepithelial cells and their colony-forming progenitors (Stingl *et al.*, 2006). While these findings raise questions about the relationships between the myoepithelial cells and MaSCs, it is also possible that significant contamination of the myoepithelial cells in the MaSC-enriched $CD24^{+}CD49f^{hi}$ population (see above) obscured the distinct gene profile of MaSCs.

Although production of milk proteins, a feature of differentiation in the luminal mammary epithelium, would not be expected to be a feature of MaSCs, Wagner and colleagues have made an intriguing observation regarding the possibility that MaSCs may express whey acidic protein (WAP), a milk constituent, during pregnancy and lactation (Wagner *et al.*, 2002; Boulanger *et al.*, 2005). Using Wap-driven Cre recombinase to activate constitutive LacZ expression, a population of $LacZ^{+}$ mammary epithelial cells was found to persist following mammary gland involution. These

干细胞之家www.stemcell8.cn ←点击进入

cells, termed pregnancy-induced mammary epithelial cells (PI-MECs), were located mainly in lobuloalveolar regions and a proportion of them displayed evidence of multi-lineage differentiation and self-renewal in serial transplantation studies. Although these observations raise the possibility that a subclass of MaSCs may arise during pregnancy (Wagner and Smith, 2005), it may be that WAP expression occurs transiently in MaSCs, particularly those located distally in the lobuloalveolar regions, during the hormonal milieu of pregnancy and lactation, resulting in sufficient levels of Cre recombinase to effect loxP-targeted recombination and constitutive LacZ expression in the model used in these studies.

It would not necessarily be surprising to find that MaSCs express lineage-associated markers of differentiation (see "Mammary epithelial cell lineages") and even milk proteins in appropriate circumstances. Promiscuous expression of lineage-associated markers by HSCs has been described and termed "lineage-priming" (Jimenez *et al.*, 1992; Hu *et al.*, 1997; Delassus *et al.*, 1999; Ye *et al.*, 2003; Orkin, 2000). The "lineage priming" hypothesis proposes that genes normally expressed in various differentiated cell lineages may be expressed in stem cells just prior to commitment down a particular lineage-restricted developmental path (Orkin, 2003). While the mechanism and reason for this phenomenon are not clear, the active cycling status of MaSCs (Stingl *et al.*, 2006) compared to HSCs would be consistent with the idea that MaSCs are continually "primed" for lineage commitment. The definition of a more pure MaSC population and the prospective isolation of functionally distinct progenitor populations will provide significant insights into this area.

8. Identification of Progenitor Populations in Mammary Epithelium

In the mouse, low levels of CD29 and CD49f expression have been shown to characterize the mammary luminal epithelium (Fig. 7) (Shackleton *et al.*, 2006; Stingl *et al.*, 2006). A subpopulation of luminal epithelium expressing either high levels of CD61 (Asselin-Labat *et al.*, 2007) or low levels of prominin-1/CD133 or Sca-1 (Sleeman *et al.*, 2007) has recently been shown to be highly enriched for colony-forming ability *in vitro*, suggesting a model of luminal epithelial cell development in which increasing differentiation and loss of proliferative capability is associated with decreased CD61 and increased prominin-1 and Sca-1 expression.

Interestingly, the identification of CD61 as a luminal progenitor marker has recently revealed that the zinc finger transcription factor Gata-3 is a

干细胞之家www.stemcell8.cn ←点击进入

key regulator of differentiation along both the ductal and alveolar luminal cell lineages (Asselin-Labat *et al.*, 2007). CD61 appears to mark a common luminal progenitor that can either commit to a ductal or alveolar cell fate, dependent on the developmental stage. It is also possible that ductal- and alveolar-restricted progenitors exist.

9. The Mammary Stem Cell Niche

The regulation of a stem cell's fate and function is dependent on interactions within its microenvironment, or niche, comprising other cell types and extracellular factors. The derivation of normal mammary epithelium from the transplantation of very low numbers of mammary cells (Shackleton *et al.*, 2006; Stingl *et al.*, 2006) has significant implications for understanding the nature of MaSC niches. Notably, the lack of effect of co-injection of supporting mammary cells on the ability of single MaSCs to produce an epithelial outgrowth (Shackleton *et al.*, 2006) indicates the importance of non-epithelial factors in MaSC regulation. Such an observation is consistent with the demonstrated roles of mammary stroma in ductal development (Silberstein *et al.*, 1992; Pierce *et al.*, 1993; Soriano *et al.*, 1998; Joseph *et al.*, 1999; Kamalati *et al.*, 1999; Cunha *et al.*, 1997; Walden *et al.*, 1998; Wiesen *et al.*, 1999; Silberstein, 2001) and the basal orientation of MaSCs within the mammary epithelium (Chepko and Dickson, 2003; Shackleton *et al.*, 2006; Stingl *et al.*, 2006). In fact, a dynamic interplay between the epithelium and stroma is likely to provide tight control over MaSC proliferation. This may involve interactions with different cell types as well as regulation by stromal-derived factors. Little is known, however, about the cellular components that constitute the MaSC niche.

The open, branching mammary ductal structure that develops during puberty results from a combination of ductal elongation via TEBs (see "Mammary development in the mouse") as well as inhibition of lateral branching (Silberstein, 2001). A model to explain this patterning during pubertal development is that MaSCs exist primarily within TEBs and distribute more MaSCs (via self-renewing cell divisions) at sites from which lateral branching will subsequently occur along the elongating duct (Silberstein, 2001; Smalley and Ashworth, 2003) (Fig. 8). This process is in part mediated by stromal factors, as mammary mitogens such as estrogen, epidermal growth factor and growth hormone influence ductal growth in a stroma-associated manner (Cunha *et al.*, 1997; Wiesen *et al.*, 1999; Kleinberg, 1997; Walden *et al.*, 1998; Ruan and Kleinberg, 1999), although estrogen also acts directly on mammary epithelium (Mallepell *et al.*, 2006). In addition,

干细胞之家www.stemcell8.cn ←点击进入

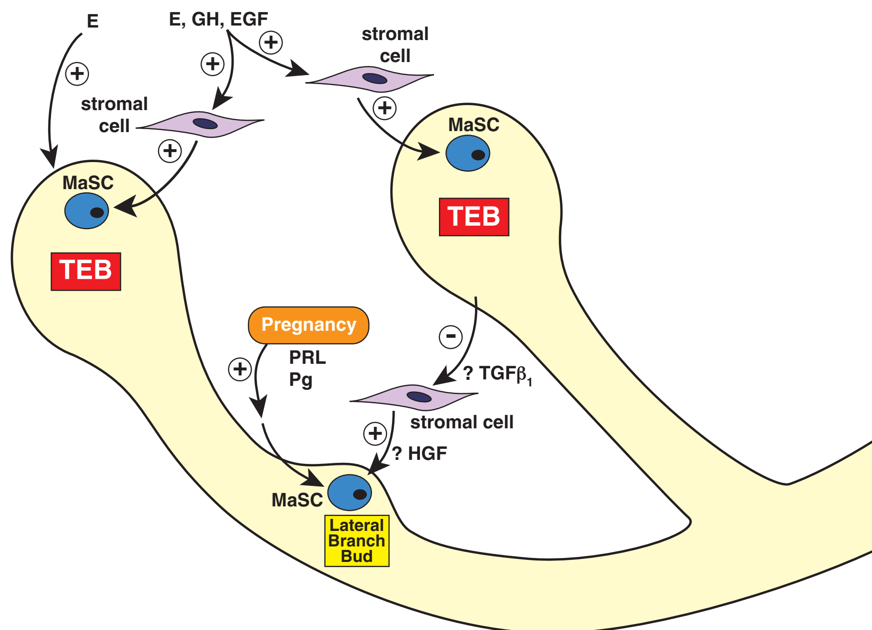


Fig. 8. Proposed roles of non-epithelial factors in the mammary stem cell niche. E — estrogen, GH — growth hormone, EGF — epidermal growth factor, PRL — prolactin, Pg — progesterone, TGFβ1 — transforming growth factor beta 1, HGF — hepatocyte growth factor, TEB — terminal end bud.

the propagation of TEBs and ductal elongation is limited by the extent of the MFP.

Concurrent with the activation of MaSCs in TEBs, it is likely that proliferation is inhibited in MaSCs that have been distributed along mature ducts, preventing the development of lateral branching (Fig. 8) during puberty. It has been proposed that the inhibition of lateral branching during puberty is caused by secretion of factors from other nearby ducts that are themselves mediated by stromal elements (see "Molecular regulation of mammary stem cells"). Histological and immunochemical studies of developing mammary epithelium have noted alterations in the clustering and gene expression patterns of stromal factors at sites of lateral branching that may be related to MaSC activity within mature ducts (Silberstein *et al.*, 1992).

The inhibition of MaSC activity in this context is apparently overcome during pregnancy, when lateral branching of ducts and development of lobuloalveolar structures predominate under the influence of pregnancy-associated hormones such as progesterone and prolactin (Fig. 8). The direct activation of MaSCs during pregnancy is supported by observations that

干细胞之家www.stemcell8.cn ←点击进入

the number of MaSCs expands dramatically during pregnancy (MA, unpublished data). The absence of steroid hormone receptor expression in MaSCs (Asselin-Labat *et al.*, 2006; Sleeman *et al.*, 2007) indicates that the activation of MaSCs by estrogen or progesterone is likely to be mediated by paracrine factors.

The MaSC niche thus comprises a variety of cellular and biochemical microenvironments that change during development to co-ordinate the activation status and fate of MaSC proliferation. Evidence suggests a significant role for stromal factors as key elements of the MaSC niche and central mediators of MaSC growth and inhibitory signals.

10. Molecular Regulation of Mammary Stem Cells

Interest in the identification and purification of MaSCs is in part stimulated by observations that the molecular pathways regulating stem cell function are frequently perturbed in cancer development (Pardal *et al.*, 2003 and 2005; Morrison and Kimble, 2006). It is hoped that an understanding of normal MaSC regulation will provide insights into breast cancer pathogenesis and suggest novel therapeutic strategies. Toward this end, emerging evidence now indicates a role for several molecules in the regulation of MaSCs.

In the mouse, direct evidence implicates Wnt-1 in the promotion of MaSC self-renewal. Wnt ligands are secreted molecules that induce self-renewal in a variety of stem cell types (Reya and Clevers, 2005). In the mammary gland, *wnt-1* is a frequent site of integration by the mouse mammary tumor virus (Nusse and Varmus, 1982; Nusse *et al.*, 1984) and over-expression of Wnt-1 causes mammary hyperplasia and tumors (Tsukamoto *et al.*, 1988; Bocchinfuso *et al.*, 1999). Evaluation of pre-neoplastic mammary tissue in these mice revealed an expanded MaSC-enriched $CD24^{+}CD29^{hi}$ population and a 6.4-fold increase in the absolute number of MaSCs compared to controls (Shackleton *et al.*, 2006). Other studies have also suggested that the oncogenic effects of Wnt-1 on mammary epithelium are initiated in mammary progenitor cell populations (Li *et al.*, 2003; Liu *et al.*, 2004).

Observations regarding the pubertal development of mouse mammary epithelium suggest a role for TGF-β1 signaling in MaSC regulation (Silberstein, 2001). Mammary glands from transgenic mice over-expressing an activated form of TGF-β1 displayed reduced lateral branching (Pierce *et al.*, 1993), consistent with inhibition of MaSCs located along ducts. In support of this, Smith and colleagues have reported that mammary expression of TGF-β1 results in senescence of MaSCs (Kordon *et al.*, 1995a; Boulanger and Smith, 2001, Boulanger *et al.*, 2005). Interestingly, the inhibitory effects

干细胞之家www.stemcell8.cn ←点击进入

of TGF-β1 on MaSC proliferation appear to be mediated by stromal intermediates. Expression of a hypomorphic TGF-β receptor mutant in the periductal stroma increased lateral branching and was associated with increased expression of hepatocyte growth factor (HGF) (Joseph *et al.*, 1999). The observation that HGF promotes branching morphogenesis of mammary epithelium *in vitro* (Kamalati *et al.*, 1999; Soriano *et al.*, 1998) suggests a model in which TGF-β1 inhibits MaSC proliferation by suppression of HGF production in mammary stroma (Fig. 8). Direct evaluation of MaSC number and function in these models is warranted.

In the absence of a robust *in vivo* assay for human MaSC function, the mammosphere assay has been used to evaluate candidate molecular regulators of human MaSCs. Wicha and colleagues found that activation of Notch signaling increased the number of self-renewing cells within the mammospheres (Dontu *et al.*, 2004). Interestingly, potentiation of Notch signaling also promoted apparent myoepithelial differentiation and branching morphogenesis, suggesting pleiotropic roles for this signaling pathway in mammary development. Similar effects on the self-renewal of mammosphere-initiating cells were observed with the Hedgehog (Hh) ligands Sonic Hh and Indian Hh, or their downstream signaling mediators Gli1 and Gli2 (Liu *et al.*, 2006). In addition, the Polycomb group oncogene Bmi-1 was found to be over-expressed in mammospheres treated with Hh ligands (Liu *et al.*, 2006).

The finding that established molecular regulators of stem cells such as Wnt- and Hedgehog-signaling components also affect MaSC function provides further evidence that important regulatory pathways are conserved between different adult stem cell types. By implication, if cancers arising in different tissues are characterized by dysregulation of cellular self-renewal (Pardal *et al.*, 2005), an increased understanding of self-renewal may reveal therapeutic targets common to different cancers.

11. Mammary Stem Cells and Breast Cancer

The malignant transformation of mammary epithelium is thought to occur through a step-wise accumulation of genetic and epigenetic changes. Evidence for the multi-step model of breast cancer progression is found in studies that showed increasing allelic imbalance with the stage of disease development (O'Connell *et al.*, 1998; Radford *et al.*, 1995; Stratton *et al.*, 1995; Zhuang *et al.*, 1995; Fujii *et al.*, 1996; Ahmadian *et al.*, 1997; Dillon *et al.*, 1997). In addition, perturbations of specific molecular signaling pathways have

干细胞之家www.stemcell8.cn ←点击进入

been identified to occur at morphologically distinct stages. These molecular changes result in a phenotypic transformation of the mammary epithelium through benign and atypical proliferative phases, to carcinoma *in situ* and overtly invasive disease (Allred *et al.*, 2001), analogous to the classical multi-step mutation model that has been described for colorectal cancer development (Vogelstein *et al.*, 1988).

11.1. *Cell Self-Renewal and Cancer*

It is notable that the metastatic disease associated with breast cancer in humans (Weigelt *et al.*, 2003; Carlsson *et al.*, 2004) and model systems (Di Carlo *et al.*, 1999) frequently resembles that of the primary tumor. In order to be able to spread and establish new sites of disease, some cells within primary cancers must be able to self-renew. Direct evidence for the role of self-renewal in malignant transformation has been provided by *Drosophila* mutants that have aberrant regulation of asymmetric cell division (Caussinus and Gonzalez, 2005). In support of the importance of cell self-renewal in mammalian tumorigenesis, animal models have revealed that disruption of this process can be tumorigenic. Examples of self-renewal-associated genes whose disruption can been tumorigenic include the Hedgehog (Berman *et al.*, 2003; Thayer *et al.*, 2003; Watkins *et al.*, 2003; Taipale and Beachy, 2001), Wnt (Reya *et al.*, 2001; Reya and Clevers, 2005; Taipale and Beachy, 2001), Bmi-1 (Haupt *et al.*, 1991; Lessard and Sauvageau, 2003; Leung *et al.*, 2004), Notch (van Es *et al.*, 2005; Ellisen *et al.*, 1991), and Hox genes (Lawrence *et al.*, 1996). In addition, loss of normal APC function in intestinal epithelium, which is required to disrupt stem cell self-renewal via Wnt-mediated accumulation of β-catenin, predisposes to malignant transformation (Miyoshi *et al.*, 1992; Radtke and Clevers, 2005). As self-renewal is a defining and distinctive property of normal stem cells, it is likely that stem cells, or at least the molecular machinery that regulates their self-renewal, are involved in the process of oncogenic transformation.

11.2. *The Origins of Breast Cancer*

In the mouse, evidence exists to support the notion that mammary tumors are clonal, arising from a single cell (Cohen *et al.*, 1979; Cardiff *et al.*, 1983; Kordon *et al.*, 1995b). Similar conclusions have been reached for a number of other malignancies, including human tumors (Fearon *et al.*, 1987; Wainscoat and Fey, 1990; Sidransky *et al.*, 1992; Junker *et al.*, 2002). These observations raise important questions about the nature of the cell of origin of breast

干细胞之家www.stemcell8.cn ←点击进入

cancer and whether its relative susceptibility to malignant transformation may be exploitable in a therapeutic sense.

There is evidence in non-mammary tissues that cancers can originate in stem cells. In acute myeloid leukemia (AML), evidence for HSCs as the direct origins of this cancer was provided in studies that demonstrated the tumorigenic fraction of leukemic cells had a similar phenotype ($CD34^{+}CD38^{-}$) to normal HSCs and harbored AML-associated genetic mutations (Lapidot *et al.*, 1994; Bonnet and Dick, 1997; Hope *et al.*, 2004). In addition, it has been observed that bronchoalveolar stem cells in mouse lung epithelium are primarily expanded in a model of lung adenocarcinoma (Kim *et al.*, 2005). However, there is also evidence in both AML (Cozzio *et al.*, 2003) and retinoblastoma (Chen *et al.*, 2004) that these cancers can arise in lineage-restricted progenitor cells that may mutate to acquire a self-renewing capability. A model of carcinogenesis that unifies these two ideas proposes that genetic mutations arising in the stem cell component of an organ confer a transforming predisposition to downstream committed progenitor cells. These progenitors then develop further mutations, including the acquisition of stem cell characteristics such as self-renewal, and eventually undergo malignant transformation (Passegue *et al.*, 2003). This model is supported by the finding that the HSCs of AML patients in remission can express the leukemia-associated AML1-ETO translocation fusion product without being overtly leukemic (Miyamoto *et al.*, 2000 and 1996).

Breast cancer is a heterogeneous disease, and it may be that differences in tumor subtypes reflect not only different mutation profiles (Perou *et al.*, 2000; Sorlie *et al.*, 2001), but also differences in cellular origin (Gusterson *et al.*, 2005). Most breast cancers, however, including the common types of invasive ductal carcinoma and invasive lobular carcinoma, display evidence of luminal cell differentiation in the form of secretory acinar structures, implicating a cell committed to the luminal lineage as the primary target of transformation in most cases. Consistent with this notion, gene-profiling studies (Perou *et al.*, 2000; Sorlie *et al.*, 2001) indicate that the majority of breast cancers are characterized by gene expression patterns consistent with a luminal cell origin (Fan *et al.*, 2006; Brenton *et al.*, 2005). In addition, over-expression of the *neu* oncogene in mouse mammary epithelium was not found to perturb the MaSC pool (Shackleton *et al.*, 2006) and produced mammary cancers with luminal features (Di Carlo *et al.*, 1999).

Neverthless, it is likely that genetic mutations in MaSCs occur as harbingers of breast cancer, either directly or indirectly. For example, unimpeded cell self-renewal may be important in the establishment of

干细胞之家www.stemcell8.cn ←点击进入

the pre-neoplastic phenotype in the mammary gland, as suggested by the markedly enhanced serial transplantability of hyperplastic compared to normal mammary epithelium (Daniel *et al.*, 1968). Also, the increased risk of breast cancer imposed by ionizing radiation exposure in teenage women is not evident for many years following exposure (Land and McGregor, 1979), suggesting the persistence of a mutated, long-lived cell type over many years in these women. The multi-hit model of carcinogenesis (Vogelstein *et al.*, 1988; Hanahan and Weinberg, 2000) implies that cells that are long-lived and mitotically active (so as to enhance the probability of mutation) are at increased risk for transformation compared to those that do not have these characteristics. Thus, the prolonged lifespan, self-renewing capacity and proliferative potential of stem cells in some organ systems, make them targets for malignant transformation (Sell, 2004; Passegue *et al.*, 2003).

The discovery that gene-expression profiles in breast cancers are associated with clinical features of the disease (Perou *et al.*, 2000; Sorlie *et al.*, 2001) has led to the hypothesis that the basal subtype of breast cancer, which is characterized by an association with germline *BRCA1* mutations, insensitivity to estrogen-receptor modulation and a poor prognosis, may arise from MaSCs (Behbod and Rosen, 2005; Yehiely *et al.*, 2006). This idea is supported by observations in the mouse (Asselin-Labat *et al.*, 2006) that MaSCs have a similar phenotype (absence of the estrogen, progesterone and ErbB2 receptors) to basal breast cancers (Nielsen *et al.*, 2004). In addition, mouse models that over-express Wnt-1 to promote self-renewal in MaSCs (see "Molecular regulation of mammary stem cells") display marked mammary epithelial hyperplasia and develop moderately differentiated mammary tumors with a median latency of six months (Tsukamoto *et al.*, 1988; Bocchinfuso *et al.*, 1999). In consideration of the possible effects of TGF-β1 and HGF signaling on MaSC regulation, it is notable that increased TGF-β1 expression is tumor-suppressing (Boulanger and Smith, 2001) and increased HGF expression is oncogenic (Takayama *et al.*, 1997) in the mouse mammary gland.

While some breast cancers may arise in MaSCs, evidence that mutations in MaSCs are required for human mammary tumorigenesis is lacking, largely because these cells have not been identified. In the same way that identification of HSCs has allowed the role of these cells in the development of leukemia to be studied (Passegue *et al.*, 2003), it is hoped that elucidation of the nature and regulation of human MaSCs will provide insights into breast cancer development.

千细胞之家www.stemcell8.cn ←点击进入

11.3. *Breast Cancer Stem Cells*

Although established breast cancers probably arise from single cells, they usually demonstrate cellular heterogeneity. This phenomenon of cellular heterogeneity in cancer is demonstrated most dramatically in teratocarcinoma, in which a transformed germ cell may give rise to a breadth of differentiated cell lineages, producing teeth, hair and other cell types within the tumor mass (O'Hare, 1978). The best-characterized example of cellular heterogeneity within cancer is found in human AML, in which multiple leukemic cell lineages, distinguishable by surface phenotype and functional capability, have been identified (Lapidot *et al.*, 1994; Bonnet and Dick, 1997; Hope *et al.*, 2004). Although such heterogeneity could arise as a result of varied random mutations in a mass of rapidly dividing cancer cells, an alternative — and not necessarily exclusive — explanation invokes the existence of "stem-like" cells within the cancer that are capable of multi-lineage differentiation.

Evidence now exists to support the idea that not only are some cells within cancer capable of differentiation into other cancer cell types, they are also capable of forming new cancers. This critical property of tumorigenicity has been ascribed to only a minority of cancer cells in the cancers studied thus far (Lapidot *et al.*, 1994; Bonnet and Dick, 1997; Singh *et al.*, 2004; O'Brien *et al.*, 2007; Ricci-Vitiani *et al.*, 2007; Li *et al.*, 2007; Prince *et al.*, 2007), including breast cancer (Al-Hajj *et al.*, 2003). Notably, the tumorigenic fractions of cancer cells display the properties of self-renewal as well as multi-lineage differentiation, in that they are serially transplantable through immunocompromised mice. They have thus become known colloquially as "cancer stem cells." At present, it remains to be determined whether the cancer stem cell model will be applicable across the broad spectrum of malignant disease, or whether there will be significant numbers of cancers in which most cancer cells have a similar tumorigenic capacity.

It is important to note in the context of this discussion that the cancer stem cell model does not necessitate that cancer stem cells arise from normal tissue stem cells. Human breast cancer stem cells have been identified and enriched by their expression of low levels of CD24 and high levels of CD44 (Al-Hajj *et al.*, 2003). Although the phenotype of normal human MaSCs has yet to be identified, it was surprising that normal mouse mammary epithelium was $CD24^{+}$ (Sleeman *et al.*, 2006; Shackleton *et al.*, 2006; Stingl *et al.*, 2006). In addition, analysis of expression of CD24 and CD29 in tumor cells isolated from different mouse mammary cancer models has not generally revealed enlargement of the MaSC-associated $CD24^{+}CD29^{hi}$

干细胞之家www.stemcell8.cn ←点击进入

population (unpublished data). For example, in *MMTV-wnt-1* mice the expanded CD24^{+}CD29^{hi} population seen in preneoplastic mammary tissue (see "Molecular regulation of mammary stem cells") was lost in cells derived from *MMTV-wnt-1* tumors, indicating that down-regulation of CD29 may be a feature of malignant progression in this model. Notably, recent evidence suggests that normal human MaSCs and human breast cancer stem cells share increased levels of expression of several self-renewal regulators (Liu *et al.*, 2006). Careful experimentation will be required to discern the relationships between breast cancer stem cells and their normal tissue counterparts.

12. Conclusions

The therapeutic targeting of breast cancer stem cells offers hope of better and more specific treatments for established disease (Al-Hajj, 2007). However, answers to questions concerning the cellular origins of breast cancer have the potential to reduce the burden of breast cancer even more profoundly by facilitating early diagnosis and prevention. In both instances, defining the normal developmental cellular hierarchy of mammary epithelium will be important.

Recent work in the mouse has confirmed that the mammary epithelium develops from single MaSCs that have the capacity for *in vivo* self-renewal and multi-lineage differentiation. The phenotype of mouse MaSCs, CD24^{+}CD29^{hi}/CD49f^{hi}, indicates that they reside basally within the mammary epithelial layer, in proximity to the myoepithelium and stromal factors. The identification of additional markers that define more enriched populations of MaSCs is a high priority in mammary research. This will enable a more detailed characterization of MaSCs and investigation of their molecular regulation.

Analogous to hematopoiesis, it is apparent that differentiated mammary epithelium develops from MaSCs via intermediate, proliferating progenitors with a limited differentiation capacity. Although the detailed cell lineage hierarchy of mammary epithelium has yet to be revealed, it is apparent that unipotent progenitors exist for both the myoepithelium and luminal epithelium (see Fig. 9, Model A). The identification of CD61 as a marker of luminal epithelial progenitor cells, together with the analysis of Gata-3-deficient mammary glands, has revealed that this cell population contains precursors for both ductal and alveolar luminal cell lineages (Asselin-Labat *et al.*, 2007). The CD61^{+} luminal progenitor population presumably commits to either a ductal or an alveolar cell-fate dependent on the hormonal milieu and developmental stage. Further committed progenitors along this lineage

干细胞之家www.stemcell8.cn ←点击进入

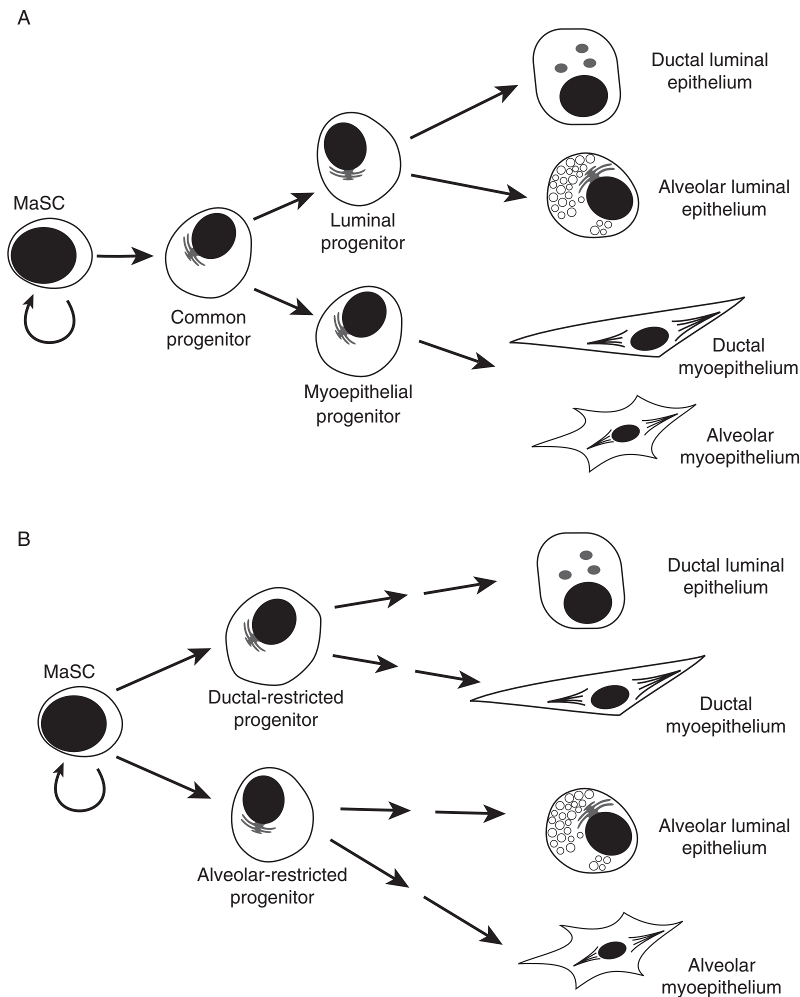


Fig. 9. Schematic models of the developmental hierarchy of mammary epithelium. **(A)** Model in which a common progenitor commits to either a luminal or myoepithelial cell fate. **(B)** Model in which commitment to either a ductal or alveolar cell fate occurs prior to commitment along either the luminal or myoepithelial lineage. For each model, intermediate progenitors may exist.

may also exist. This model also postulates the existence of a single progenitor that can give rise to the differently shaped myoepithelial cells that surround the ducts and alveoli. Markers of the myoepithelial progenitor are yet to be identified, although the availability of *in vitro* culture assays that favour myoepithelial colony formation (Petersen and van Deurs, 1988) indicates it should be possible to do so.

干细胞之家www.stemcell8.cn ←点击进入

An alternative model of mammary development holds that the decision to commit to a ductal or alveolar fate occurs prior to the decision to commit to a luminal or myoepithelial fate. This invokes the existence of bipotent progenitors capable of producing both myoepithelial and luminal cells and committed to either alveolar or ductal differentiation (Fig. 9, Model B). This model is based on the findings of Smith and colleagues that transplantation of mammary epithelial cells can produce epithelial outgrowths with either ductal-only or alveolar-only characteristics that contain both luminal and myoepithelial elements (Smith, 1996). It will be important to characterize these observations further through transplantation of freshly isolated cells at clonal dilutions. In addition, the identification of molecular markers specific for phenotypically and functionally distinct cell populations will enable lineage-tracking studies to determine the exact relationships between mammary cells and a robust model of mammary epithelial development.

Collectively, the significant progress made in correlating cell surface marker expression and functional characteristics of subtypes of mammary epithelium has confirmed the validity of adapting methods of cell separation used in hematopoiesis research to mammary biology. Moreover, it has indicated that mammary epithelium develops through a series of defined cellular stages characterized by increasing lineage-specificity and decreasing proliferative capacity. The race is now on to similarly characterize all mammary epithelial subpopulations so their relationships and molecular regulation can be studied in detail. The age of cell-type specific therapy for diseases of the breast is not upon us yet, but its dawn could be just over the horizon.

References

Ahmadian M, Wistuba II, Fong KM, *et al*. (1997) Analysis of the FHIT gene and FRA3B region in sporadic breast cancer, preneoplastic lesions, and familial breast cancer probands. *Cancer Res* **57**: 3664–68.

Al-Hajj M. (2007) Cancer stem cells and oncology therapeutics. *Curr Opin Oncol* **19**: 61–64.

Al-Hajj M, Wicha MS, Benito-Hernandez A, *et al*. (2003) Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci USA* **100**: 3983–88.

Allred DC, Mohsin SK, Fuqua SA. (2001) Histological and biological evolution of human premalignant breast disease. *Endocr Relat Cancer* **8**: 47–61.

Alvi AJ, Clayton H, Joshi C, *et al*. (2003) Functional and molecular characterisation of mammary side population cells. *Breast Cancer Res* **5**: R1–8.

Asselin-Labat ML, Shackleton M, Stingl J, *et al*. (2006) Steroid hormone receptor status of mouse mammary stem cells. *J Natl Cancer Inst* **98**: 1011–14.

干细胞之家www.stemcell8.cn ←点击进入

Asselin-Labat ML, Sutherland KD, Barker H, *et al*. (2007) Gata-3 is an essential regulator of mammary-gland morphogenesis and luminal-cell differentiation. *Nat Cell Biol* **9**: 201–209.

Behbod F, Rosen JM. (2005) Will cancer stem cells provide new therapeutic targets? *Carcinogenesis* **26**: 703–11.

Berman DM, Karhadkar SS, Maitra A, *et al*. (2003) Widespread requirement for Hedgehog ligand stimulation in growth of digestive tract tumours. *Nature* **425**: 846–51.

Bocchinfuso WP, Hively WP, Couse JF, *et al*. (1999) A mouse mammary tumor virus-Wnt-1 transgene induces mammary gland hyperplasia and tumorigenesis in mice lacking estrogen receptor-alpha. *Cancer Res* **59**: 1869–76.

Bonnefoix T, Bonnefoix P, Verdiel P, Sotto JJ. (1996) Fitting limiting dilution experiments with generalized linear models results in a test of the single-hit Poisson assumption. *J Immunol Meth* **194**: 113–19.

Bonnet D, Dick JE. (1997) Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med* **3**: 730–37.

Booth BW, Smith GH. (2006) Estrogen receptor-alpha and progesterone receptor are expressed in label-retaining mammary epithelial cells that divide asymmetrically and retain their template DNA strands. *Breast Cancer Res* **8**: R49.

Boulanger CA, Smith GH. (2001) Reducing mammary cancer risk through premature stem cell senescence. *Oncogene* **20**: 2264–72.

Boulanger CA, Wagner KU, Smith GH. (2005) Parity-induced mouse mammary epithelial cells are pluripotent, self-renewing and sensitive to TGF-beta1 expression. *Oncogene* **24**: 552–60.

Brenton JD, Carey LA, Ahmed AA, Caldas C. (2005) Molecular classification and molecular forecasting of breast cancer: ready for clinical application? *J Clin Oncol* **23**: 7350–60.

Brisken C, Park S, Vass T, *et al*. (1998) A paracrine role for the epithelial progesterone receptor in mammary gland development. *Proc Natl Acad Sci USA* **95**: 5076–81.

Buhler TA, Dale TC, Kieback C, *et al*. (1993) Localization and quantification of Wnt-2 gene expression in mouse mammary development. *Dev Biol* **155**: 87–96.

Cardiff RD, Morris DW, Young LJ. (1983) Alterations of acquired mouse mammary tumor virus DNA during mammary tumorigenesis in BALB/cfC3H mice. *J Natl Cancer Inst* **71**: 1011–19.

Carlsson J, Nordgren H, Sjostrom J, *et al*. (2004) HER2 expression in breast cancer primary tumours and corresponding metastases. Original data and literature review. *Br J Cancer* **90**: 2344–48.

Caussinus E, Gonzalez C. (2005) Induction of tumor growth by altered stem-cell asymmetric division in *Drosophila melanogaster*. *Nat Genet* **37**: 1125–29.

Chen D, Livne-bar I, Vanderluit JL, *et al*. (2004) Cell-specific effects of RB or RB/p107 loss on retinal development implicate an intrinsically death-resistant cell-of-origin in retinoblastoma. *Cancer Cell* **5**: 539–51.

干细胞之家www.stemcell8.cn ←点击进入

Chepko G, Dickson RB. (2003) Ultrastructure of the putative stem cell niche in rat mammary epithelium. *Tissue Cell* **35**: 83–93.

Clarke RB. (2003) Steroid receptors and proliferation in the human breast. *Steroids* **68**: 789–94.

Clarke RB, Spence K, Anderson E, *et al*. (2005) A putative human breast stem cell population is enriched for steroid receptor-positive cells. *Dev Biol* **277**: 443–56.

Cohen JC, Shank PR, Morris VL, *et al*. (1979) Integration of the DNA of mouse mammary tumor virus in virus-infected normal and neoplastic tissue of the mouse. *Cell* **16**: 333–45.

Coleman S, Silberstein GB, Daniel CW. (1988) Ductal morphogenesis in the mouse mammary gland: evidence supporting a role for epidermal growth factor. *Dev Biol* **127**: 304–15.

Cozzio A, Passegue E, Ayton PM, *et al*. (2003) Similar MLL-associated leukemias arising from self-renewing stem cells and short-lived myeloid progenitors. *Genes Dev* **17**: 3029–35.

Cunha GR, Young P, Hom YK, *et al*. (1997) Elucidation of a role for stromal steroid hormone receptors in mammary gland growth and development using tissue recombinants. *J Mammary Gland Biol Neoplasia* **2**: 393–402.

Daniel CW, De Ome KB, Young JT, *et al*. (1968) The *in vivo* life span of normal and preneoplastic mouse mammary glands: a serial transplantation study. *Proc Natl Acad Sci USA* **61**: 53–60.

Daniel CW, Young LJ. (1971) Influence of cell division on an aging process. Life span of mouse mammary epithelium during serial propagation *in vivo*. *Exp Cell Res* **65**: 27–32.

Daniel CW, Young LJ, Medina D, DeOme KB. (1971) The influence of mammogenic hormones on serially transplanted mouse mammary gland. *Exp Gerontol* **6**: 95–101.

De Ome KB, Faulkin LJ Jr, Bern HA, Blair PB. (1959) Development of mammary tumors from hyperplastic alveolar nodules transplanted into gland-free mammary fat pads of female C3H mice. *Cancer Res* **19**: 515–20.

Delassus S, Titley I, Enver T. (1999) Functional and molecular analysis of hematopoietic progenitors derived from the aorta-gonad-mesonephros region of the mouse embryo. *Blood* **94**: 1495–503.

Deng G, Lu Y, Zlotnikov G, *et al*. (1996) Loss of heterozygosity in normal tissue adjacent to breast carcinomas. *Science* **274**: 2057–59.

Deugnier MA, Faraldo MM, Rousselle P, *et al*. (1999) Cell-extracellular matrix interactions and EGF are important regulators of the basal mammary epithelial cell phenotype. *J Cell Sci* **112** (Pt 7): 1035–44.

Deugnier MA, Teuliere J, Faraldo MM, *et al*. (2002) The importance of being a myoepithelial cell. *Breast Cancer Res* **4**: 224–30.

Di Carlo E, Diodoro MG, Boggio K, *et al*. (1999) Analysis of mammary carcinoma onset and progression in HER-2/neu oncogene transgenic mice reveals a lobular origin. *Lab Invest* **79**: 1261–69.

干细胞之家www.stemcell8.cn ←点击进入

Diallo R, Schaefer KL, Poremba C, *et al*. (2001) Monoclonality in normal epithelium and in hyperplastic and neoplastic lesions of the breast. *J Pathol* **193**: 27–32.

Dillon EK, de Boer WB, Papadimitriou JM, Turbett GR. (1997) Microsatellite instability and loss of heterozygosity in mammary carcinoma and its probable precursors. *Br J Cancer* **76**: 156–62.

Dontu G, Abdallah WM, Foley JM, *et al*. (2003) *In vitro* propagation and transcriptional profiling of human mammary stem/progenitor cells. *Genes Dev* **17**: 1253–70.

Dontu G, Jackson KW, McNicholas E, *et al*. (2004) Role of Notch signaling in cell-fate determination of human mammary stem/progenitor cells. *Breast Cancer Res* **6**: R605–15.

Dulbecco R, Allen WR, Bologna M, Bowman M. (1986) Marker evolution during the development of the rat mammary gland: stem cells identified by markers and the role of myoepithelial cells. *Cancer Res* **46**: 2449–56.

Ellisen LW, Bird J, West DC, *et al*. (1991) TAN-1, the human homolog of the *Drosophila* notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. *Cell* **66**: 649–61.

Emerman JT, Vogl AW. (1986) Cell size and shape changes in the myoepithelium of the mammary gland during differentiation. *Anat Rec* **216**: 405–15.

Fan C, Oh DS, Wessels L, *et al*. (2006) Concordance among gene-expression-based predictors for breast cancer. *N Engl J Med* **355**: 560–69.

Fearon ER, Hamilton SR, Vogelstein B. (1987) Clonal analysis of human colorectal tumors. *Science* **238**: 193–97.

Frisch SM, Francis H. (1994) Disruption of epithelial cell-matrix interactions induces apoptosis. *J Cell Biol* **124**: 619–26.

Fujii H, Marsh C, Cairns P, *et al*. (1996) Genetic divergence in the clonal evolution of breast cancer. *Cancer Res* **56**: 1493–97.

Gilmore AP. (2005) Anoikis. *Cell Death Differ* **12** (Suppl 2): 1473–77.

Gomm JJ, Browne PJ, Coope RC, *et al*. (1997) A paracrine role for myoepithelial cell-derived FGF2 in the normal human breast. *Exp Cell Res* **234**: 165–73.

Gudjonsson T, Ronnov-Jessen L, Villadsen R, *et al*. (2002a) Normal and tumor-derived myoepithelial cells differ in their ability to interact with luminal breast epithelial cells for polarity and basement membrane deposition. *J Cell Sci* **115**: 39–50.

Gudjonsson T, Villadsen R, Nielsen HL, *et al*. (2002b) Isolation, immortalization, and characterization of a human breast epithelial cell line with stem cell properties. *Genes Dev* **16**: 693–706.

Gugliotta P, Sapino A, Macri L, *et al*. (1988) Specific demonstration of myoepithelial cells by anti-alpha smooth muscle actin antibody. *J Histochem Cytochem* **36**: 659–63.

Gusterson BA, Monaghan P, Mahendran R, *et al*. (1986) Identification of myoepithelial cells in human and rat breasts by anti-common acute lymphoblastic leukemia antigen antibody A12. *J Natl Cancer Inst* **77**: 343–49.

干细胞之家www.stemcell8.cn ←点击进入

Gusterson BA, Ross DT, Heath VJ, Stein T. (2005) Basal cytokeratins and their relationship to the cellular origin and functional classification of breast cancer. *Breast Cancer Res* **7**: 143–48.

Hanahan D, Weinberg RA. (2000) The hallmarks of cancer. *Cell* **100**: 57–70.

Haupt Y, Alexander WS, Barri G, *et al*. (1991) Novel zinc finger gene implicated as myc collaborator by retrovirally accelerated lymphomagenesis in E mu-myc transgenic mice. *Cell* **65**: 753–63.

Hinck L, Silberstein GB. (2005) Key stages in mammary gland development: the mammary end bud as a motile organ. *Breast Cancer Res* **7**: 245–51.

Hope KJ, Jin L, Dick JE. (2004) Acute myeloid leukemia originates from a hierarchy of leukemic stem cell classes that differ in self-renewal capacity. *Nat Immunol* **5**: 738–43.

Hoshino K. (1967) Transplantability of mammary gland in brown fat pads of mice. *Nature* **213**: 194–95.

Hoshino K, Gardner WU. (1967) Transplantability and life span of mammary gland during serial transplantation in mice. *Nature* **213**: 193–94.

Hu M, Krause D, Greaves M, *et al*. (1997) Multilineage gene expression precedes commitment in the hemopoietic system. *Genes Dev* **11**: 774–85.

Jimenez G, Griffiths SD, Ford AM, *et al*. (1992) Activation of the beta-globin locus control region precedes commitment to the erythroid lineage. *Proc Natl Acad Sci USA* **89**: 10618–22.

Jonker JW, Merino G, Musters S, *et al*. (2005) The breast cancer resistance protein BCRP (ABCG2) concentrates drugs and carcinogenic xenotoxins into milk. *Nat Med* **11**: 127–29.

Joseph H, Gorska AE, Sohn P, *et al*. (1999) Overexpression of a kinase-deficient transforming growth factor-beta type II receptor in mouse mammary stroma results in increased epithelial branching. *Mol Biol Cell* **10**: 1221–34.

Junker K, Thrum K, Schlichter A, *et al*. (2002) Clonal origin of multifocal renal cell carcinoma as determined by microsatellite analysis. *J Urol* **168**: 2632–36.

Kamalati T, Niranjan B, Yant J, Buluwela L. (1999) HGF/SF in mammary epithelial growth and morphogenesis: *in vitro* and *in vivo* models. *J Mammary Gland Biol Neoplasia* **4**: 69–77.

Kim CF, Jackson EL, Woolfenden AE, *et al*. (2005) Identification of bronchioalveolar stem cells in normal lung and lung cancer. *Cell* **121**: 823–35.

Kleinberg DL. (1997) Early mammary development: growth hormone and IGF-1. *J Mammary Gland Biol Neoplasia* **2**: 49–57.

Kordon EC, McKnight RA, Jhappan C, *et al*. (1995a) Ectopic TGF beta 1 expression in the secretory mammary epithelium induces early senescence of the epithelial stem cell population. *Dev Biol* **168**: 47–61.

Kordon EC, Smith GH. (1998) An entire functional mammary gland may comprise the progeny from a single cell. *Development* **125**: 1921–30.

干细胞之家www.stemcell8.cn ←点击进入

Kordon EC, Smith GH, Callahan R, Gallahan D. (1995b) A novel non-mouse mammary tumor virus activation of the Int-3 gene in a spontaneous mouse mammary tumor. *J Virol* **69**: 8066–69.

Krause DS, Theise ND, Collector MI, *et al*. (2001) Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. *Cell* **105**: 369–77.

Kuperwasser C, Chavarria T, Wu M, *et al*. (2004) Reconstruction of functionally normal and malignant human breast tissues in mice. *Proc Natl Acad Sci USA* **101**: 4966–71.

Lakhani SR, Chaggar R, Davies S, *et al*. (1999) Genetic alterations in 'normal' luminal and myoepithelial cells of the breast. *J Pathol* **189**: 496–503.

Lakhani SR, Slack DN, Hamoudi RA, *et al*. (1996) Detection of allelic imbalance indicates that a proportion of mammary hyperplasia of usual type are clonal, neoplastic proliferations. *Lab Invest* **74**: 129–35.

Land CE, McGregor DH. (1979) Breast cancer incidence among atomic bomb survivors: implications for radiobiologic risk at low doses. *J Natl Cancer Inst* **62**: 17–21.

Lapidot T, Sirard C, Vormoor J, *et al*. (1994) A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature* **367**: 645–48.

Latza U, Niedobitek G, Schwarting R, *et al*. (1990) Ber-EP4: new monoclonal antibody which distinguishes epithelia from mesothelial. *J Clin Pathol* **43**: 213–19.

Lawrence HJ, Sauvageau G, Humphries RK, Largman C. (1996) The role of HOX homeobox genes in normal and leukemic hematopoiesis. *Stem Cells* **14**: 281–91.

Lessard J, Sauvageau G. (2003) Bmi-1 determines the proliferative capacity of normal and leukaemic stem cells. *Nature* **423**: 255–60.

Leung C, Lingbeek M, Shakhova O, *et al*. (2004) Bmi1 is essential for cerebellar development and is overexpressed in human medulloblastomas. *Nature* **428**: 337–41.

Li C, Heidt DG, Dalerba P, *et al*. (2007) Identification of pancreatic cancer stem cells. *Cancer Res* **67**: 1030–37.

Li Y, Welm B, Podsypanina K, *et al*. (2003) Evidence that transgenes encoding components of the Wnt signaling pathway preferentially induce mammary cancers from progenitor cells. *Proc Natl Acad Sci USA* **100**: 15853–58.

Liu BY, McDermott SP, Khwaja SS, Alexander CM. (2004) The transforming activity of Wnt effectors correlates with their ability to induce the accumulation of mammary progenitor cells. *Proc Natl Acad Sci USA* **101**: 4158–63.

Liu QY, Niranjan B, Gomes P, *et al*. (1996) Inhibitory effects of activin on the growth and morpholgenesis of primary and transformed mammary epithelial cells. *Cancer Res* **56**: 1155–63.

Liu S, Dontu G, Mantle ID, *et al*. (2006) Hedgehog signaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells. *Cancer Res* **66**: 6063–71.

干细胞之家www.stemcell8.cn ←点击进入

Mallepell S, Krust A, Chambon P, Brisken C. (2006) Paracrine signaling through the epithelial estrogen receptor alpha is required for proliferation and morphogenesis in the mammary gland. *Proc Natl Acad Sci USA* **103**: 2196–201.

Matsuzaki Y, Kinjo K, Mulligan RC, Okano H. (2004) Unexpectedly efficient homing capacity of purified murine hematopoietic stem cells. *Immunity* **20**: 87–93.

Meredith JE Jr, Fazeli B, Schwartz MA. (1993) The extracellular matrix as a cell survival factor. *Mol Biol Cell* **4**: 953–61.

Miyamoto T, Nagafuji K, Akashi K, *et al*. (1996) Persistence of multipotent progenitors expressing AML1/ETO transcripts in long-term remission patients with t(8;21) acute myelogenous leukemia. *Blood* **87**: 4789–96.

Miyamoto T, Weissman IL, Akashi K. (2000) AML1/ETO-expressing nonleukemic stem cells in acute myelogenous leukemia with 8;21 chromosomal translocation. *Proc Natl Acad Sci USA* **97**: 7521–26.

Miyoshi Y, Ando H, Nagase H, *et al*. (1992) Germ-line mutations of the APC gene in 53 familial adenomatous polyposis patients. *Proc Natl Acad Sci USA* **89**: 4452–56.

Moir LM, Ward JP, Hirst SJ. (2003) Contractility and phenotype of human bronchiole smooth muscle after prolonged fetal bovine serum exposure. *Exp Lung Res* **29**: 339–59.

Morrison SJ, Kimble J. (2006) Asymmetric and symmetric stem-cell divisions in development and cancer. *Nature* **441**: 1068–74.

Murrell TG. (1995) The potential for oxytocin (OT) to prevent breast cancer: a hypothesis. *Breast Cancer Res Treat* **35**: 225–29.

Neville MC, Medina D, Monks J, Hovey RC. (1998) The mammary fat pad. *J Mammary Gland Biol Neoplasia* **3**: 109–16.

Nielsen TO, Hsu FD, Jensen K, *et al*. (2004) Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. *Clin Cancer Res* **10**: 5367–74.

Nusse R, van Ooyen A, Cox D, *et al*. (1984) Mode of proviral activation of a putative mammary oncogene (int-1) on mouse chromosome 15. *Nature* **307**: 131–36.

Nusse R, Varmus HE. (1982) Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. *Cell* **31**: 99–109.

O'Brien CA, Pollett A, Gallinger S, Dick JE. (2007) A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature* **445**: 106–10.

O'Connell P, Pekkel V, Fuqua SA, *et al*. (1998) Analysis of loss of heterozygosity in 399 premalignant breast lesions at 15 genetic loci. *J Natl Cancer Inst* **90**: 697–703.

O'Hare MJ. (1978) Teratomas, neoplasia and differentiation: a biological overview. I. The natural history of teratomas. *Invest Cell Pathol* **1**: 39–63.

Orkin SH. (2000) Diversification of haematopoietic stem cells to specific lineages. *Nat Rev Genet* **1**: 57–64.

Orkin SH. (2003) Priming the hematopoietic pump. *Immunity* **19**: 633–34.

干细胞之家www.stemcell8.cn ←点击进入

Pardal R, Clarke MF, Morrison SJ. (2003) Applying the principles of stem-cell biology to cancer. *Nat Rev Cancer* **3**: 895–902.

Pardal R, Molofsky AV, He S, Morrison SJ. (2005) Stem cell self-renewal and cancer cell proliferation are regulated by common networks that balance the activation of proto-oncogenes and tumor suppressors. *Cold Spring Harb Symp Quant Biol* **70**: 177–85.

Passegue E, Jamieson CH, Ailles LE, Weissman IL. (2003) Normal and leukemic hematopoiesis: are leukemias a stem cell disorder or a reacquisition of stem cell characteristics? *Proc Natl Acad Sci USA* **100 (**Suppl 1**)**: 11842–49.

Pechoux C, Gudjonsson T, Ronnov-Jessen L, *et al*. (1999) Human mammary luminal epithelial cells contain progenitors to myoepithelial cells. *Dev Biol* **206**: 88–99.

Perou CM, Sorlie T, Eisen MB, *et al*. (2000) Molecular portraits of human breast tumours. *Nature* **406**: 747–52.

Petersen OW, van Deurs B. (1986) Characterization of epithelial membrane antigen expression in human mammary epithelium by ultrastructural immunoperoxidase cytochemistry. *J Histochem Cytochem* **34**: 801–809.

Petersen OW, van Deurs B. (1988) Growth factor control of myoepithelial-cell differentiation in cultures of human mammary gland. *Differentiation* **39**: 197–215.

Pierce DF Jr, Johnson MD, Matsui Y, *et al*. (1993) Inhibition of mammary duct development but not alveolar outgrowth during pregnancy in transgenic mice expressing active TGF-beta 1. *Genes Dev* **7**: 2308–17.

Prince ME, Sivanandan R, Kaczorowski A, *et al*. (2007) Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. *Proc Natl Acad Sci USA* **104**: 973–78.

Radford DM, Phillips NJ, Fair KL, *et al*. (1995) Allelic loss and the progression of breast cancer. *Cancer Res* **55**: 5180–83.

Radtke F, Clevers H. (2005) Self-renewal and cancer of the gut: two sides of a coin. *Science* **307**: 1904–909.

Rebel VI, Dragowska W, Eaves CJ, *et al*. (1994) Amplification of Sca-1+ Lin- WGA+ cells in serum-free cultures containing steel factor, interleukin-6, and erythropoietin with maintenance of cells with long-term *in vivo* reconstituting potential. *Blood* **83**: 128–36.

Reya T, Clevers H. (2005) Wnt signalling in stem cells and cancer. *Nature* **434**: 843–50.

Reya T, Morrison SJ, Clarke MF, Weissman IL. (2001) Stem cells, cancer, and cancer stem cells. *Nature* **414**: 105–11.

Ricci-Vitiani L, Lombardi DG, Pilozzi E, *et al*. (2007) Identification and expansion of human colon-cancer-initiating cells. *Nature* **445**: 111–15.

Richert MM, Schwertfeger KL, Ryder JW, Anderson SM. (2000) An atlas of mouse mammary gland development. *J Mammary Gland Biol Neoplasia* **5**: 227–41.

Ronnov-Jessen L, Petersen OW, Bissell MJ. (1996) Cellular changes involved in conversion of normal to malignant breast: importance of the stromal reaction. *Physiol Rev* **76**: 69–125.

干细胞之家www.stemcell8.cn ←点击进入

Ruan W, Kleinberg DL. (1999) Insulin-like growth factor I is essential for terminal end bud formation and ductal morphogenesis during mammary development. *Endocrinology* **140**: 5075–81.

Sell S. (2004) Stem cell origin of cancer and differentiation therapy. *Crit Rev Oncol Hematol* **51**: 1–28.

Shackleton M, Vaillant F, Simpson KJ, *et al*. (2006) Generation of a functional mammary gland from a single stem cell. *Nature* **439**: 84–88.

Shillingford JM, Miyoshi K, Flagella M, *et al*. (2002) Mouse mammary epithelial cells express the Na-K-Cl Cotransporter, NKCC1: characterization, localization, and involvement in ductal development and morphogenesis. *Mol Endocrinol* **16**: 1309–21.

Sidransky D, Frost P, Von Eschenbach A, *et al*. (1992) Clonal origin bladder cancer. *N Engl J Med* **326**: 737–40.

Silberstein GB. (2001) Postnatal mammary gland morphogenesis. *Microsc Res Tech* **52**: 155–62.

Silberstein GB, Flanders KC, Roberts AB, Daniel CW. (1992) Regulation of mammary morphogenesis: evidence for extracellular matrix-mediated inhibition of ductal budding by transforming growth factor-beta 1. *Dev Biol* **152**: 354–62.

Singh SK, Hawkins C, Clarke ID, *et al*. (2004) Identification of human brain tumour initiating cells. *Nature* **432**: 396–401.

Sleeman KE, Kendrick H, Ashworth A, *et al*. (2006) CD24 staining of mouse mammary gland cells defines luminal epithelial, myoepithelial/basal and non-epithelial cells. *Breast Cancer Res* **8**: R7.

Sleeman KE, Kendrick H, Robertson D, *et al*. (2007) Dissociation of estrogen receptor expression and *in vivo* stem cell activity in the mammary gland. *J Cell Biol* **176**: 19–26.

Smalley M, Ashworth A. (2003) Stem cells and breast cancer: a field in transit. *Nat Rev Cancer* **3**: 832–44.

Smalley MJ, Titley J, O'Hare MJ. (1998) Clonal characterization of mouse mammary luminal epithelial and myoepithelial cells separated by fluorescence-activated cell sorting. *In Vitro Cell Dev Biol Anim* **34**: 711–21.

Smith GH. (1996) Experimental mammary epithelial morphogenesis in an *in vivo* model: evidence for distinct cellular progenitors of the ductal and lobular phenotype. *Breast Cancer Res Treat* **39**: 21–31.

Soriano JV, Pepper MS, Orci L, Montesano R. (1998) Roles of hepatocyte growth factor/scatter factor and transforming growth factor-beta1 in mammary gland ductal morphogenesis. *J Mammary Gland Biol Neoplasia* **3**: 133–50.

Sorlie T, Perou CM, Tibshirani R, *et al*. (2001) Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci USA* **98**: 10869–74.

Spangrude GJ, Johnson GR. (1990) Resting and activated subsets of mouse multipotent hematopoietic stem cells. *Proc Natl Acad Sci USA* **87**: 7433–37.

干细胞之家www.stemcell8.cn ←点击进入

Stingl J, Eaves CJ, Kuusk U, Emerman JT. (1998) Phenotypic and functional characterization *in vitro* of a multipotent epithelial cell present in the normal adult human breast. *Differentiation* **63**: 201–13.

Stingl J, Eaves CJ, Zandieh I, Emerman JT. (2001) Characterization of bipotent mammary epithelial progenitor cells in normal adult human breast tissue. *Breast Cancer Res Treat* **67**: 93–109.

Stingl J, Eirew P, Ricketson I, *et al*. (2006) Purification and unique properties of mammary epithelial stem cells. *Nature* **439**: 993–97.

Stratton MR, Collins N, Lakhani SR, Sloane JP. (1995) Loss of heterozygosity in ductal carcinoma *in situ* of the breast. *J Pathol* **175**: 195–201.

Taipale J, Beachy PA. (2001) The Hedgehog and Wnt signalling pathways in cancer. *Nature* **411**: 349–54.

Takano H, Ema H, Sudo K, Nakauchi H. (2004) Asymmetric division and lineage commitment at the level of hematopoietic stem cells: inference from differentiation in daughter cell and granddaughter cell pairs. *J Exp Med* **199**: 295–302.

Takayama H, LaRochelle WJ, Sharp R, *et al*. (1997) Diverse tumorigenesis associated with aberrant development in mice overexpressing hepatocyte growth factor/scatter factor. *Proc Natl Acad Sci USA* **94**: 701–706.

Taylor-Papadimitriou J, Stampfer M, Bartek J, *et al*. (1989) Keratin expression in human mammary epithelial cells cultured from normal and malignant tissue: relation to *in vivo* phenotypes and influence of medium. *J Cell Sci* **94** (Pt 3): 403–13.

Taylor-Papadimitriou J, Wetzels R, Ramaekers F. (1992) Intermediate filament protein expression in normal and malignant human mammary epithelial cells. *Cancer Treat Res* **61**: 355–78.

Thayer SP, di Magliano MP, Heiser PW, *et al*. (2003) Hedgehog is an early and late mediator of pancreatic cancer tumorigenesis. *Nature* **425**: 851–56.

Tsai YC, Lu Y, Nichols PW, *et al*. (1996) Contiguous patches of normal human mammary epithelium derived from a single stem cell: implications for breast carcinogenesis. *Cancer Res* **56**: 402–404.

Tsukamoto AS, Grosschedl R, Guzman RC, *et al*. (1988) Expression of the int-1 gene in transgenic mice is associated with mammary gland hyperplasia and adenocarcinomas in male and female mice. *Cell* **55**: 619–25.

Uchida N, Dykstra B, Lyons K, *et al*. (2004) ABC transporter activities of murine hematopoietic stem cells vary according to their developmental and activation status. *Blood* **103**: 4487–95.

Uchida N, Dykstra B, Lyons KJ, *et al*. (2003) Different *in vivo* repopulating activities of purified hematopoietic stem cells before and after being stimulated to divide *in vitro* with the same kinetics. *Exp Hematol* **31**: 1338–47.

van Es JH, van Gijn ME, Riccio O, *et al*. (2005) Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. *Nature* **435**: 959–63.

干细胞之家www.stemcell8.cn ←点击进入

Vogelstein B, Fearon ER, Hamilton SR, *et al*. (1988) Genetic alterations during colorectal-tumor development. *N Engl J Med* **319**: 525–32.
Wagner KU, Boulanger CA, Henry MD, *et al*. (2002) An adjunct mammary epithelial cell population in parous females: its role in functional adaptation and tissue renewal. *Development* **129**: 1377–86.
Wagner KU, Smith GH. (2005) Pregnancy and stem cell behavior. *J Mammary Gland Biol Neoplasia* **10**: 25–36.
Wainscoat JS, Fey MF. (1990) Assessment of clonality in human tumors: a review. *Cancer Res* **50**: 1355–60.
Walden PD, Ruan W, Feldman M, Kleinberg DL. (1998) Evidence that the mammary fat pad mediates the action of growth hormone in mammary gland development. *Endocrinology* **139**: 659–62.
Watkins DN, Berman DM, Burkholder SG, *et al*. (2003) Hedgehog signalling within airway epithelial progenitors and in small-cell lung cancer. *Nature* **422**: 313–17.
Weigelt B, Glas AM, Wessels LF, *et al*. (2003) Gene expression profiles of primary breast tumors maintained in distant metastases. *Proc Natl Acad Sci USA* **100**: 15901–905.
Welm BE, Tepera SB, Venezia T, *et al*. (2002) Sca-1(pos) cells in the mouse mammary gland represent an enriched progenitor cell population. *Dev Biol* **245**: 42–56.
Wiesen JF, Young P, Werb Z, Cunha GR. (1999) Signaling through the stromal epidermal growth factor receptor is necessary for mammary ductal development. *Development* **126**: 335–44.
Williams JM, Daniel CW. (1983) Mammary ductal elongation: differentiation of myoepithelium and basal lamina during branching morphogenesis. *Dev Biol* **97**: 274–90.
Ye M, Iwasaki H, Laiosa CV, *et al*. (2003) Hematopoietic stem cells expressing the myeloid lysozyme gene retain long-term, multilineage repopulation potential. *Immunity* **19**: 689–99.
Yehiely F, Moyano JV, Evans JR, *et al*. (2006) Deconstructing the molecular portrait of basal-like breast cancer. *Trends Mol Med* **12**: 537–44.
Young LJ, Medina D, DeOme KB, Daniel CW. (1971) The influence of host and tissue age on life span and growth rate of serially transplanted mouse mammary gland. *Exp Gerontol* **6**: 49–56.
Zeps N, Bentel JM, Papadimitriou JM, *et al*. (1998) Estrogen receptor-negative epithelial cells in mouse mammary gland development and growth. *Differentiation* **62**: 221–26.
Zhuang Z, Merino MJ, Chuaqui R, *et al*. (1995) Identical allelic loss on chromosome 11q13 in microdissected *in situ* and invasive human breast cancer. *Cancer Res* **55**: 467–71.

干细胞之家www.stemcell8.cn ←点击进入

Chapter 11

Human Brain Tumor Stem Cells

Sara G. M. Piccirillo and Angelo L. Vescovi*

Recent findings in stem cell research have demonstrated the existence of cancer stem cells in leukemia and also in solid tumors such as those of the brain, mainly in glioblastoma multiforme (GBM) and perhaps in medulloblastomas (MDBs) and ependymomas (EPMs). These cells could be defined as cancer stem cells because they show a degree of similarity with their normal counterpart, such as the ability to self-renew and differentiate into the cell lineages typical of the tissue of origin. In particular, brain tumor stem cells isolated from human GBM satisfy all of the expected stem cell features: they self-renew under clonal conditions, proliferate *in vitro* for long periods of time and differentiate into neuron- and glia-like cells as well as into abnormal cells, with aberrant, mixed neuronal/astroglia phenotypes. The ability to generate all of the cell types of the tissue of origin *in vivo* results in the development of serially transplantable tumors — which are faithful phenocopies of the original human pathology — upon orthotopic injection into immunodeficient mice. From this standpoint, the first and most critical need in the newborn brain cancer stem cell field is the identification of selective markers and molecular mechanisms that underpin the tumorigenic potential of these cells. This would allow us to devise new therapeutic strategies for the treatment of malignant brain tumors, in particular GBM. The recent discovery that bone morphogenetic proteins inhibit the tumorigenic potential of cancer stem cells derived from GBMs, by promoting the acquisition of a more differentiated phenotype, suggests that new, noncytotoxic, pro-differentiation therapies may be established in the future. These treatments could be used in combination with conventional therapies to curb tumor progression and recurrence.

Keywords: Normal and cancer stem cells; brain tumors; tumorigenicity; bone morphogenetic proteins; neurosphere assay.

*Corresponding author. vescovi@tin.it
Department of Biotechnology and Biosciences, University of Milan-Bicocca, Milan, Italy
StemGen Spa, Milan, Italy

干细胞之家www.stemcell8.cn ←点击进入

1. Introduction

Most human cancers derive from a single cell, targeted by genetic and epigenetic alterations which eventually initiate malignant transformation. Progressively, the progeny of these early cancer cells accumulate additional alterations that, acting in concert, drive them into acquiring a full malignant phenotype (Hanahan and Weinberg, 2000). While we have identified many of the gene pathways disrupted or altered in many cancers, the nature of the normal cells that are more susceptible to transformation remains unsolved. Adult stem cells possess long-term replicative potential, together with the capacities of auto-maintenance (or self-renewal) and of the generation of all cell lineages found in their tissue of residence (defined as multipotency). Alteration in the mechanisms underlying stemness may be a critical event in the onset of tumorigenesis. The striking degree of similarity between somatic stem cells and cancer cells, including their fundamental ability to self-renew and their multipotency, has reinforced this concept. Hence, it has been proposed that cancers might be caused by the transformation of somatic stem cells (reviews: Reya *et al.*, 2001; Vescovi *et al.*, 2006). This hypothesis has been strengthened by the observation that, among all cancer cells in a given tumor, only a minute cell fraction possesses stem-like properties and can be defined as "cancer stem cells" (CSCs), particularly by virtue of their unique ability to establish and regenerate the whole tumor cell population in immunocompromised mice (e.g., Bonnet and Dick, 1997). Notably, CSCs might also derive from more differentiated progenitor cells or even mature cells that, upon mutation/epigenetic alteration, would become reprogrammed to acquire aberrant, stem-like functions, operating in a cell-autonomous tumorigenic context. Whichever the case, mutated genes can promote deregulated expansion of the stem/progenitor pool by expanding self-renewal and multipotency and by overcoming their normal tendency towards relative quiescence and normal differentiation (Vescovi *et al.*, 2006), predisposing them to accumulate even more mutations. The outcome of this situation would be the development of a tumor whose growth is sustained by a small subset of CSCs, the true tumor culprit.

2. Stem Cells and Malignant Transformation

Malignant transformation is known to occur in few or even single founder cells, resulting in an unlimited and deregulated proliferative advantage of these cells (Hanahan and Weinberg, 2000). Although usually clonal, only a few cells within the cancerous mass appear to be responsible for the growth

干细胞之家www.stemcell8.cn ←点击进入

and recurrence of many tumors. Two hypothetical explanations have been proposed to understand this phenomenon. The stochastic model postulates that every single cell residing in a tumor possesses the capacity to generate new tumors at a low frequency. On the contrary, the hierarchic model predicts that only a rare subset of cells bears significant proliferation capacity and, particularly, tumorigenic ability, with the rest of the tumor being constituted by differentiating, terminally differentiated or dying cells (Bonnet and Dick, 1997; Reya *et al.*, 2001).

The identity of the cell actually driving tumor formation, its growth and its progression remains controversial. New evidence suggests that one of the cell types undergoing malignant transformation is represented by tissue-specific (somatic) stem cells (SCs) (Frank and Nowak, 2003). In fact, SCs share important functional properties with tumor cells, such as long-term proliferative capacity, inherent self-renewal, a broad-range differentiation potential and the ability to regenerate their tissue of residence/origin. In addition, the same key molecular pathways that often underpin tumor initiation and progression (Reya *et al.*, 2001) regulate SCs (Sanai *et al.*, 2005). Notably, recent observations lend support to the involvement of somatic stem cells in the process of tumorigenesis; in particular, initial evidence came from studies on acute myeloid leukemia (Bonnet and Dick, 1997; Hope *et al.*, 2004) and breast cancer (Al-Hajj *et al.*, 2003), followed by studies on germ cell (Looijenga *et al.*, 2003) and brain tumors such as gliomas, medulloblastomas (MDBs) and ependymoma (EPs) (Ignatova *et al.*, 2002; Singh *et al.*, 2003; Hemmati *et al.*, 2003; Galli *et al.*, 2004; Singh *et al.*, 2004; Taylor *et al.*, 2005). Lately, similar results were obtained for prostate (Collins *et al.*, 2005), ovarian (Szotek *et al.*, 2006), colon (O'Brien *et al.*, 2007; Ricci-Vitiani *et al.*, 2007) and skin cancers (Monzani *et al.*, 2007).

3. Cancer Stem Cells and Brain Tumors

Glioblastoma multiforme (GBM) represents the most severe type of glioma. It is classified into two categories of highly aggressive brain tumors that develop either 1) *de novo* (primary GBMs) or 2) as a result of low-grade gliomas undergoing malignant progression (secondary GBMs). GBMs are characterized by genetic, morphological and phenotypic heterogeneity, and by extensive dissemination of the tumor cells within the brain tissue, resulting in the practical impossibility of removing the tumor mass completely (Holland, 2000).

It has been historically held that the presumptive cell of origin for this tumor was to be sought within the compartment of the few mitotically

干细胞之家www.stemcell8.cn ←点击进入

active adult brain cells, that is, the adult mature glial cell pool. However, the later identification of a neural stem cell (NSC) population in the adult mammalian central nervous system (CNS) (Reynolds and Weiss, 1992), including humans (Sanai *et al.*, 2004), has suggested that these proliferating, self-renewing cells might constitute an additional, perhaps most likely candidate for gliomagenesis. *In vivo*, cells displaying astroglial features, such as glial fibrillary acidic protein (GFAP) immunoreactivity, have now been acknowledged as the true neural stem cells (Doetsch *et al.*, 1999). The twofold nature of NSCs, sharing characteristics of both undifferentiated SCs and "committed" astrocytes, seems to reconcile the two hypotheses — glia versus neural stem cells — on the true identity of the cell of origin in gliomas. Moreover, selective deregulation of specific regulatory pathways as Ink4a/ARF, Ras, and p53/NF1 in both NSCs and mature astrocytes has been shown to determine tumorigenesis, confirming the idea that NSC "astroglia" could represent a source of glioma-founding cells (Holland *et al.*, 1998 and 2000; Zhu *et al.*, 2005; Sanai *et al.*, 2005).

4. Identification of *Bona Fide* Brain Tumor Stem Cells

4.1. *Neurosphere Assay*

Isolation of cancer stem cells from hematopoietic and breast malignancies has been carried out on the basis of a series of well-established stem cell-specific markers. On the contrary, the lack of reliable antigens specifically expressed on NSCs (Uchida *et al.*, 2000; Rietze *et al.*, 2001; Capela and Temple, 2002) forced the initial identification of neoplastic stem cells from brain tumors by an *in vitro* functional assay — originally developed to provide an estimation of the proliferative and self-renewal ability of NSCs — called "neurosphere" assay (NSA) (Reynolds and Weiss, 1992; Reynolds and Rietze, 2005). In particular, the NSA requires the exposure of neural cells to specific mitogens such as epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF2) which, by compelling stem/progenitor cells into an active proliferative status, allow the positive selection of these cells, while eliminating the more committed precursors and differentiated cells found in the primary tissue (Reynolds and Rietze, 2005; Fig. 1). Of note, since non-stem cells are endowed with limited proliferative capacity which, nevertheless, allow them to generate up to tertiary neurospheres, it is of the essence that the generation of neurospheres be monitored by both clonogenic and population analysis. This must be ensured over an extended period of time, defined not as the absolute time frame, but as the number of subculturing

干细胞之家www.stemcell8.cn ←点击进入

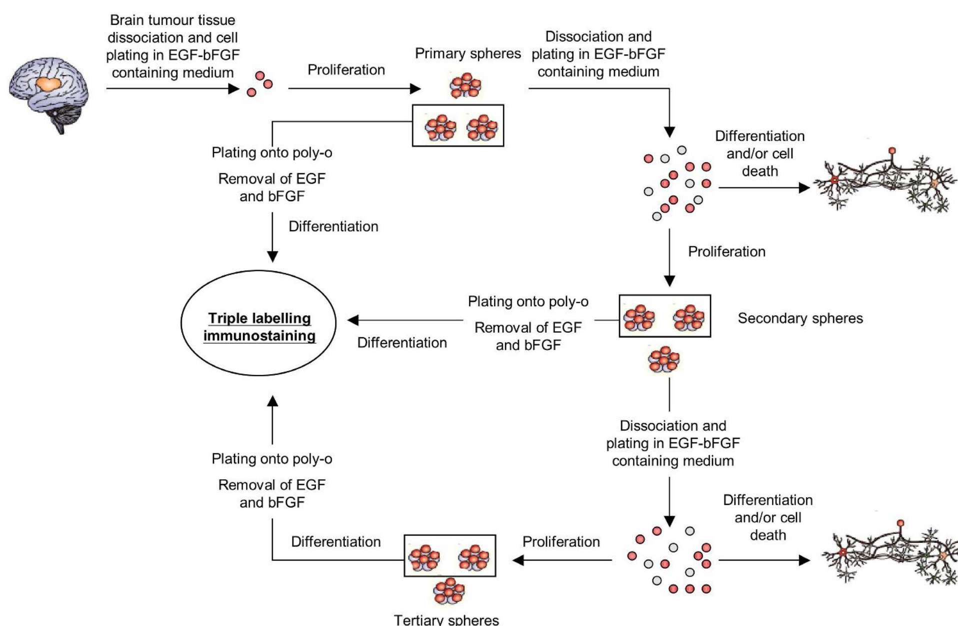


Fig. 1. Experimental protocol for assessing self-renewal capacity of cancer stem cells using the neurosphere assay. Individual cells from GBM brain tumor tissue proliferated in response to EGF and FGF2, and produced spheres under clonal culture conditions. These cells could differentiate into neurons, astroglia or oligodendroglia when grown on poly-O-coated glass coverslips without EGF and FGF2. When individual primary spheres were dissociated and replated as single cells under clonal conditions in EGF- and FGF2-containing medium, some cells either differentiated to acquire the typical morphology of neuronal or glial cells or died. However, a subset of cells proliferated and generated secondary spheres. Most of the secondary spheres were found to include neurons, oligodendroglia, and astroglia. Secondary spheres not processed for immunolabeling underwent a second round of subcloning in growth factors-containing medium. Cell proliferation still occurred, as did cell death and differentiation, causing the formation of tertiary spheres containing neurons and both major glia types. Adapted from Fig. 1 of: Gritti A, Parati EA, Cova L, Frolichsthal P, Galli R, Wanke E, Faravelli L, Morassutti DJ, Roisen F, Nickel DD and Vescovi AL, *J Neurosci.*, **16**: 1091–1100 (1996).

steps that cells can undergo while expanding in a steady fashion (Reynolds and Rietze, 2005). The extent of the minimum propagation requirement is not an absolute value in its own nature, but depends on cell-specific characteristics (i.e., age, species). Hence, it has been suggested that the minimum number of passages required to rule out the involvement/isolation of committed progenitors is reached when, throughout serial passaging, stable growth curves are established. Based on the slope of the growth curves, it is possible to infer the stem cell frequency within the cell culture and its progressive increase (population analysis) (Reynolds and Rietze, 2005).

干细胞之家www.stemcell8.cn ←点击进入

Through this approach, which somewhat parallels *in vitro* the serial transplantation procedure employed in the hematopoietic field to prove self-renewal in hematopoietic stem cells (HSCs), *bona fide* NSCs are traditionally identified, isolated and characterized (Reynolds and Rietze, 2005). Of note, the application of this methodology to neoplastic tissues has been possible since tumors can be considered as atypical and aberrant stem cell compartments, in which a small population of tumorigenic stem cells can be identified within a wider cell pool, also composed of non-stem transit amplifying progenitors and of their "differentiated" or dead progeny, the latter two being defined as end cells (Kummermehr and Trott, 1997).

4.2. *Discriminating True Cancer Stem Cells From Short-term Proliferating Progenitors*

The first suggestion of putative stem cells being found within malignant brain tumors came from Ignatova and colleagues (Ignatova *et al.*, 2002) who showed, using the NSA, that clonogenic precursors could be isolated from specimens of GBM, after exposure of dissociated cells to both EGF and FGF2. On the basis of these preliminary results, two additional papers confirmed the original hypothesis, proposing also that MDBs might contain a compartment of true stem cells (Singh *et al.*, 2003; Hemmati *et al.*, 2003). Although these studies paved the way for a more detailed identification of the cell hierarchy existing within tumors and of their significance in the context of glioma biology (Ignatova *et al.*, 2002; Singh *et al.*, 2003; Hemmati *et al.*, 2003), analysis was limited to the generation of neurospheres up to the third/fourth subculturing passage. This left the open possibility that the neurosphere-generating cells might be short-term proliferating progenitors (clonogens) rather than *bona fide* stem cells. Notwithstanding, it also remained unclear as to whether the cells identified until then possessed any tumor-initiating activity, preventing any conclusion as to their nature as cancer stem cells for brain tumors.

In 2004, our group showed that, whereas at the early culturing stages, GBMs and MDBs both contained cells able to give rise to neurospheres (Singh *et al.*, 2003; Hemmati *et al.*, 2003), only GBMs comprised neurosphere-forming cells which expanded in number exponentially, establishing long-term propagating tumor stem cell lines and demonstrating extensive self-renewing capability (Galli *et al.*, 2004), i.e., candidate cancer stem cells. Conversely, MDB cells were able to generate up to tertiary neurospheres, but then stopped dividing, progressively exhausting the cell population, thus behaving as transient dividing progenitors and not true stem

干细胞之家www.stemcell8.cn ←点击进入

cells (Galli *et al.*, 2004). These observations confirmed how the loose application of the NSA approach may lead to misleading conclusions and point to the concept that the formation of neurospheres *in vitro* does not necessarily result from the proliferation of a (cancer) stem cell (Reynolds and Rietze, 2005).

4.3. *Identification of Brain Tumor Stem Cells: Multipotency and Tumor-initiating Ability of Neurosphere-forming Cells*

Importantly, in order to prove that the long-term self-renewing, neurosphere-forming cells obtained from human brain tumors by means of the NSA were true cancer stem cells, we showed both their multipotency and tumorigenicity.

When neurospheres from MDBs were induced to differentiate, mostly neuron-like cells were generated, with very sporadic genesis of astrocytes and no signs of oligodendroglial differentiation (Singh *et al.*, 2003; Hemmati *et al.*, 2003; Galli *et al.*, 2004), thereby suggesting that they have the restricted differentation potential of committed progenitors and may thus be either true proliferation-restricted progenitors or cancer SCs originating from transformed progenitors as previously described for leukemic SCs (review: Pearce and Bonnet, 2007). Conversely, GBM-derived cells gave rise to a progeny composed of all the three major CNS cell types, demonstrating multipotency, as expected from true stem cells (Galli *et al.*, 2004).

A putative tumor SC has to demonstrate an ability to generate a new tumor. This tumor-initiating ability is critical and exclusive of tumor SCs and cannot be retrieved in normal SCs, thus providing researchers a ready-to-use way of validating the actual stemness of a candidate cancer cell (Bonnet and Dick, 1997; Kummermehr and Trott, 1997).

To assess this feature, putative human MDB stem cells obtained through standard NSA growth selection were injected intracranially in immunodeficient mice and after 6 weeks no tumor formation could be observed (Galli *et al.*, 2004). It should be observed that the finding that MDB-derived cancer stem cells could not be retrieved under the standard culture conditions of the NSA does not imply that they are not present at all within MDBs; it might be possible that specific factors need to be added to the culture medium in order to achieve isolation and long-term expansion of these cells. Such a hypothesis is consistent with the results published by Dirks and co-workers, showing that freshly dissociated AC133-positive cells from human MDB tumors have a clear-cut tumor-initiating ability when transplanted in immunodeficient mice (Singh *et al.*, 2004; see below).

干细胞之家www.stemcell8.cn ←点击进入

On the other hand, GBM-derived stem cell lines were able to develop highly infiltrative and malignant gliomas, showing to be endowed with the cardinal feature of tumor stem cells, that is, the tumorigenic ability, even when the transplanted cells were clonally derived (Galli *et al.*, 2004). Interestingly, these cells recapitulated the genetic and phenotypic signature of the tumors from which they were obtained, demonstrating that they could be considered as a reliable model of the original neoplasia (Galli *et al.*, 2004; Lee *et al.*, 2006).

4.4. *Cell Sorting of Brain Cancer Stem Cells: AC133-Positive Cells and Stem Cell Heterogeneity*

Subsequently, Singh and colleagues (Singh *et al.*, 2004) elegantly demonstrated that the AC133-positive cell fraction, obtained from freshly dissociated brain tumor tissues including both GBMs and MDBs, was the sole one able to form new tumors upon serial transplantation and, therefore, identified this specific cell population as containing *bona fide* cancer stem cells. This notion is now being challenged, however. Lately, some studies have demonstrated that the AC133-positive cell fraction from freshly dissociated GBM tumors could be considered different from the negative one in terms of angiogenic and radiotherapy-resistance properties, but not always on the basis of tumor-initiating ability (Bao *et al.*, 2006a and 2006b), thereby indicating that cancer stem cell heterogeneity first shown in leukemia (Hope *et al.*, 2004) does most likely apply to glioblastoma too. Hence, while separating cells based on their expression of the AC133 antigen may lead to the isolation of distinct populations, both of these may contain cancer stem cells.

4.5. *Establishment of Cancer Stem Cell Lines and Pitfalls of the Neurosphere Assay*

Importantly, the NSA, in addition to providing a reliable, first-line cancer stem cell assay, also provides an efficient technique to establish long-term proliferating cancer stem cell lines. These are relatively stable, propagate in chemically defined medium and can be exploited as selective cell target(s) for the development of novel, effective therapies against some of the most malignant brain cancers. In this view, the need to avoid any ambiguity in applying the term "neural cancer stem cell" to a candidate cell emerges as an imperative one.

The lack of unequivocal antigenic markers that allow us to identify candidate neural cells as brain stem cells once they are manipulated and

干细胞之家www.stemcell8.cn ←点击进入

expanded in culture has generated a somewhat confusing scenario. Accordingly, the same term "neural stem cell" is applied to precursors that, quite often, display very different functional properties and that, most likely, occupy quite distinct hierarchical positions within the normal neurogenic lineage. Unfortunately, this situation is now at risk of extending to brain cancer stem cells. The situation is aggravated by the fact that one of the most obvious properties of tumor cells is the loss of proliferation control. Hence, in cells derived from tumors, proliferation will inherently occur even in more mature cells that, under normal conditions, would extinguish quite rapidly in culture. This significantly increases the risk of false-positive identification of cancer stem cells in one's culture, when the NSA is applied loosely, on a too short-term basis, and the formation of neurospheres is construed as underlying the presence of stem cells in the absence of long-term population and growth kinetic analysis. We ought to resist this approach, for the price to pay would be to include within the cancer stem cell family a plethora of cells that, rather than being responsible for or contribute to tumor establishment and perpetuation, are simply a by- or end-product of the tumor growth. The consequences of such a scenario are, understandably, unacceptable.

5. Therapeutic Implications: Promoting Differentiation Using BMP Proteins

The cancer stem cell hypothesis suggests that these cells represent a minor population which, however, is the main culprit in fueling tumour growth and which can remain within the patient's tissue well after conventional therapy has been terminated (Reya *et al.*, 2001; Dean *et al.*, 2005). This hypothesis predicts that effective tumor eradication will require the identification of selective agents which can specifically target the cancer stem cell population. The discovery that brain tumor stem cells can be isolated, expanded *in vitro* and used to reproduce an *in vivo* phenocopy of the original human pathology provides an unprecedented tool to develop new therapeutic strategies for the treatment of incurable malignancies, such as GBM. There are preliminary indications that this approach can be successful.

5.1. *Differentiation Inhibits the Tumour-initiating Ability of the Cancer Stem Cell Population*

Very recently, we have demonstrated that *in vitro* treatment of brain tumor stem cells isolated from human GBMs with bone morphogenetic protein 4 (BMP4) resulted in the acquisition of a more differentiated

干细胞之家www.stemcell8.cn ←点击进入

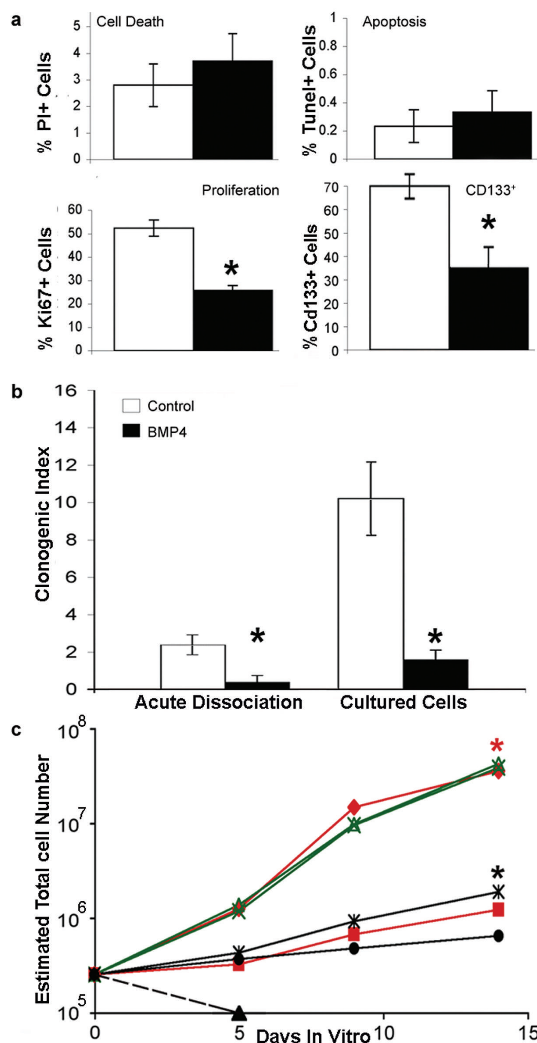


Fig. 2. BMP4 depletes the GBM tumor-initiating cell population *in vitro*. **(a)** BMP4 does not affect cell death or apoptosis, but decreases proliferation (Ki67 immunoreactivity) and the $CD133^+$ population in GBM cultures. Untreated control, empty column; BMP4-treated, solid column; mean ± s.e.m., $n = 3$; $^*P < 0.005$. **(b)** BMP4 reduces the clonogenic index in GBM tumor-initiating cells. Untreated control, empty column; BMP4-treated, solid column; mean ± s.e.m., $n = 3$; $^*P < 0.005$. **(c)** GBM cells could not be expanded in the presence of BMP4 (black dotted line, triangles). After brief expansion with mitogens alone (red rhombuses), cells received BMP4, resulting in the reduction of the growth kinetics (red squares), as for human fetal neural stem cells (black stars, untreated control; black circles, BMP4-treated) but unlike U87 human glioma lines (green triangles, untreated control; green stars, BMP4-treated). Mean ± s.e.m., $n = 3$; $^*P < 0.005$ for red asterisk and $^*P < 0.01$ for black asterisk). Based on Fig. 2 of: Piccirillo *et al.*, *Nature*, **444**, 761–765 (2006) with permission from Nature Publishing Group.

干细胞之家www.stemcell8.cn ←点击进入

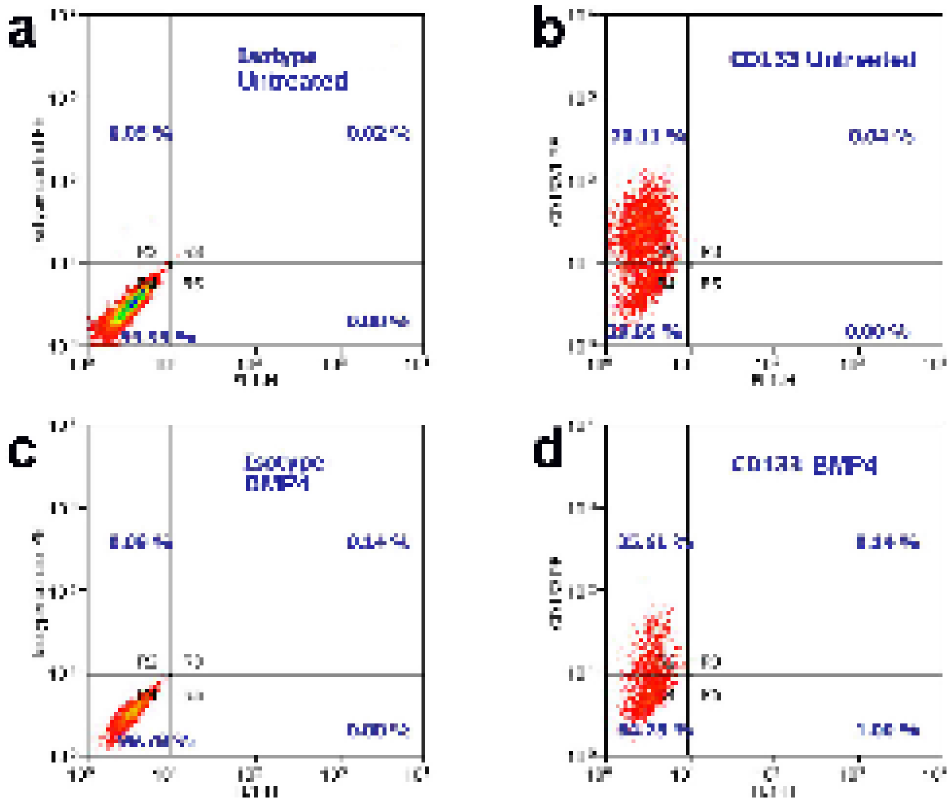


Fig. 3. Quantitative analysis of the expression of the CD133 antigen in dot plot graph performed by flow cytometry on control [(**b**) and isotype control in (**a**)] and BMP4-treated GBM tumor-initiating cells [(**d**) and isotype control in (**c**)]. BMP4 treatment results in a significant decrease of the actual overall percentage of CD133-immunoreactive cells. Based on supplementary Fig. 5 of: Piccirillo *et al.*, *Nature*, **444**, 761–765 (2006) with permission from Nature Publishing Group.

phenotype, depleting the stem cell population (Piccirillo *et al.*, 2006; Figs. 2 and 3). Accordingly, transient *ex vivo* exposure to BMP4 abolished the capacity of transplanted GBM cells to establish intracerebral tumors (Figs. 4, 5 and 6). Furthermore, *in vivo* delivery of BMP4 resulted in decreased tumor growth and mortality (Fig. 4); indeed, death occurred in 100% of the control mice in less than 12 weeks, following intracerebral grafting of GBM tumor stem cells (Piccirillo *et al.*, 2006).

5.2. *Differentiation as an Alternate to Drug-Targeting/Sensitization of CSCs*

This opens intriguing new therapeutic vistas. If, truly, resistance to conventional therapy is related to the undifferentiated state of brain tumor

干细胞之家www.stemcell8.cn ←点击进入

stem cells (Bao *et al.*, 2006a), innovative treatments promoting loss of stemness and differentiation of this pool may also result in radiosensitivity and chemosensitivity. Other therapeutic approaches involve drugs targeting specific features of brain cancer stem cells such as radioresistance (Bao *et al.*, 2006a). Thus, the combination of novel, cancer stem cell-directed and

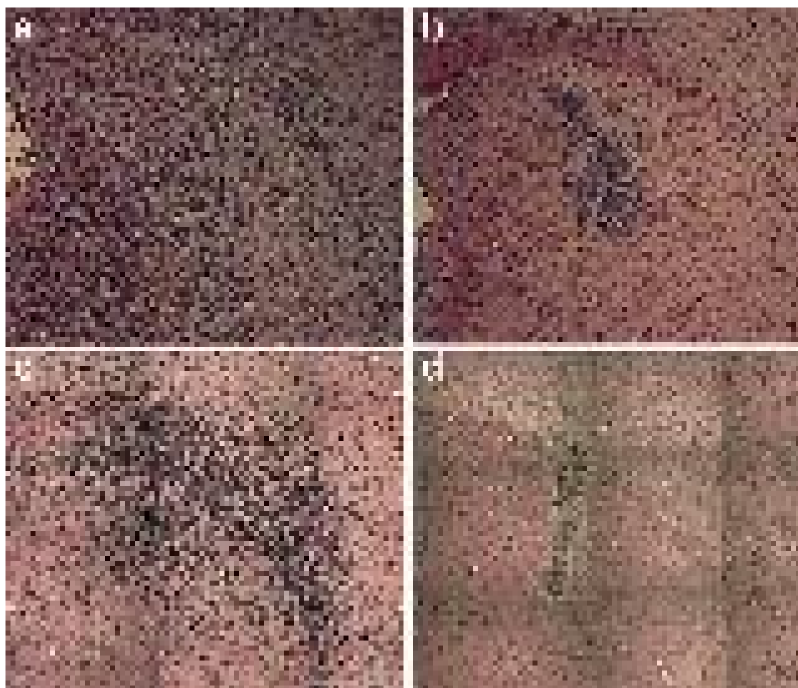
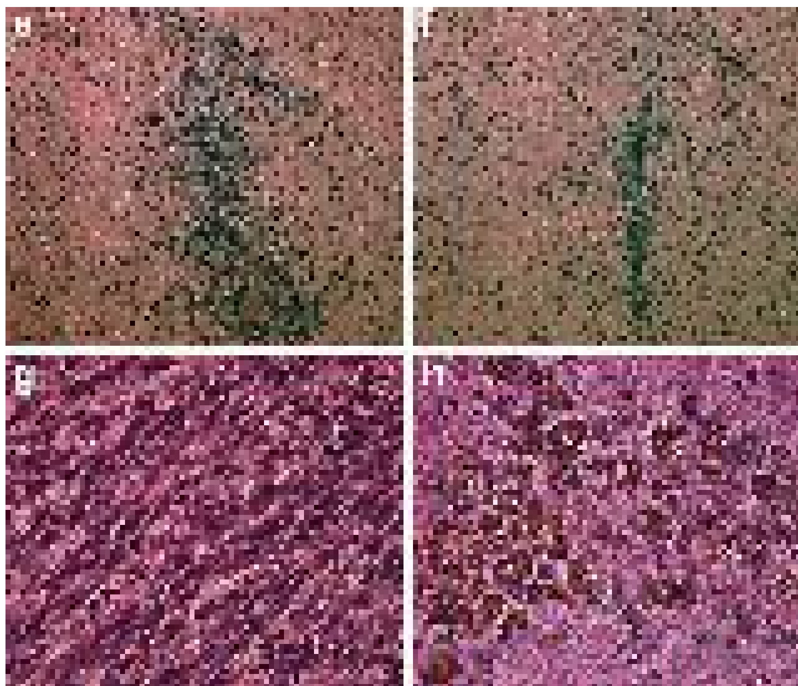


Fig. 4. BMP4 inhibits tumorigenicity of GBM-derived cells. **(a, b)** 48-hour exposure of GBM-derived cells to BMP4 *in vitro*, before transplantation into the brain of immunodeficient mice, dramatically inhibit tumor-initiating ability [(a) untreated control; (b) 5 weeks post-injection)]. **(c–f)** Polyacrylic beads, loaded with BMP4 and implanted at the cell injection site, inhibit tumor growth when co-injected at the same time as cells [(co-treatment (c) untreated control; (d) BMP4-treated; (c,d), GBM-derived cells, 4 weeks post-injection, $n = 14$) or 10 days thereafter [(post-treatment: (e), untreated control; (f), BMP4-treated; (e,f), GBM-derived cells, 4 weeks from cell injection, $n = 5$)]. The mitotic index was always significantly higher in untreated controls compared with BMP4-treated animals, in cotransplantation (P, 0.01; two-tailed Student's t-test) and post-transplantation experiments (P, 0.05; two-tailed Student's t-test). **(g)** Tumors in untreated control mice contain pleiomorphic, neoplastic and malignant, infiltrating cells. **(h)** Lesions in BMP4-treated animals contained differentiated cells and macrophages. Magnification was 5× in (a–f), and 40× in (g) and (h). **(i)** Survival of BMP4-treated animals was increased in pre- (left), co- (center) and post-transplantation (right) treatment paradigms (Log-rank test, $P < 0.001$, $P < 0.001$ and $P < 0.005$, respectively). Based on Fig. 4 of: Piccirillo *et al.*, *Nature*, **444**, 761–765 (2006) with permission from Nature Publishing Group.

干细胞之家www.stemcell8.cn ←点击进入



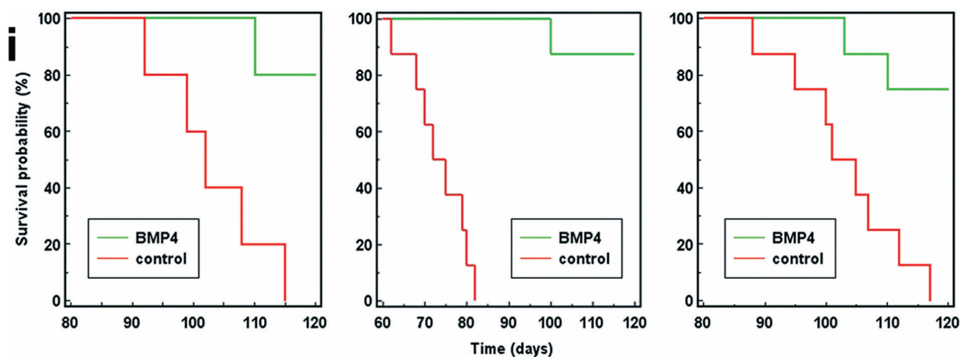


Fig. 4. (*Continued*).

conventional therapies may in the future be exploited to devise new and more effective cures for these and, perhaps, other lethal human brain tumors. In this respect, embryonal carcinomas stand as the very precursors, since induction of differentiation as an anti-proliferative and a drug-sensitization therapy has been an early therapeutic option for these tumors (see: Andrews *et al.*, 1994; Timmer-Bosscha *et al.*, 1998).

干细胞之家www.stemcell8.cn ←点击进入

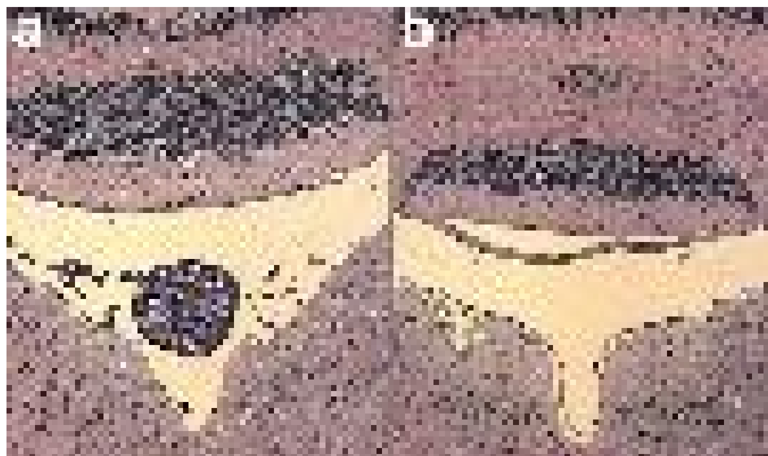


Fig. 5. *Ex vivo* pre-treatment of GBM-derived cells with BMP4 inhibits ventricular invasion following orthotopic injection. **(a)** 5 weeks after transplantation into the adult brain of immunodeficient mice, tumors from control GBM cells have invaded the ventricular system including the fourth ventricle **(b)**. Cells from cultures pretreated with BMP4 for 48 h showed no signs of ventricular invasion. Magnification: 5×. Based on supplementary Fig. 8 of: Piccirillo *et al*., *Nature*, **444**, 761–765 (2006) with permission from Nature Publishing Group.

6. Conclusions

The discovery that brain tumors, or at least some of them, comprise cancer stem cells presents us with new opportunities at both the experimental and clinical level. To achieve an effective implementation of new therapeutic strategies, scientists/clinicians will require new methods of determining the identity, nature, physiology and idiosyncratic properties of the cancer stem cells from a given patient's tumor. It is certainly reasonable to argue that tumors of the same family may also share common features at the level of cancer stem cell populations, which would facilitate diagnosis and the application of new, standard treatments. By the same token, cancer stem cells from different patients may bear distinctive, peculiar properties that might warrant the development of more specific, patient-selective treatments. This point, however, needs to be verified and refined by further study.

Unquestionably, to move the cancer stem cell field forward, a vital issue will be the discovery of unequivocal markers allowing for the reliable identification of brain tumor stem cells prior to any experimental manipulation. Furthermore, it is essential to generate novel drugs or strategies that could overcome the issue of putative drug resistance in cancer stem cells, improve therapeutic efficacy, and make cancer treatment more successful

干细胞之家www.stemcell8.cn ←点击进入

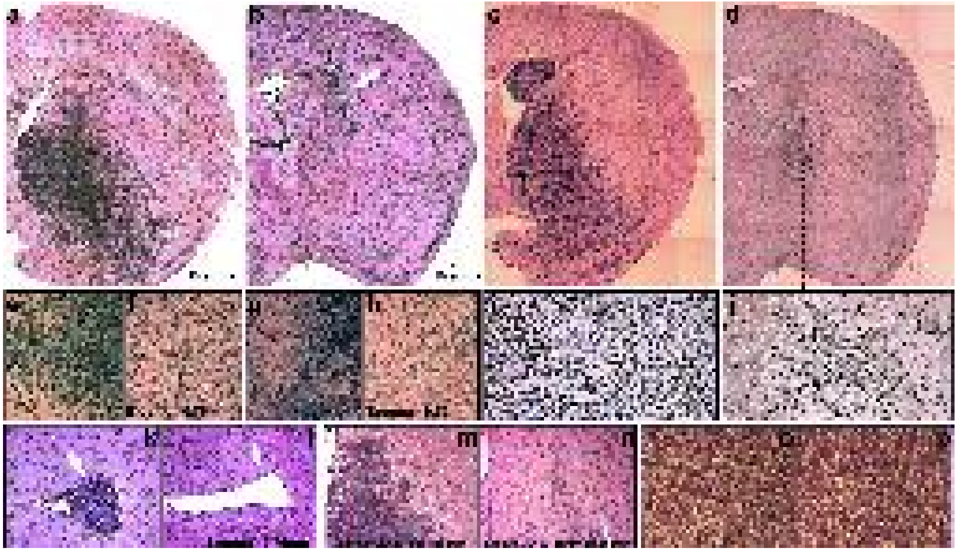


Fig. 6. BMP4 inhibits the tumorigenicity of GBM-derived cells. 48-hour exposure of GBM-derived cells to BMP4 *in vitro*, prior to transplantation into the brain of immunodeficient mice, caused a significant reduction in the ability of the injected cells to form tumors (b,d,f,h,j,l) as compared to controls (a,c,e,g,i,k). **(a–j)** Hematoxylin and eosin staining showed typical glioblastoma masses 5 weeks after injection of control cells (a,e,g), whereas BMP4-treated GBM-derived cells developed very small grafts (b,f,h). Tumors established from control cells displayed a much higher mitotic index, as revealed by immunohistochemistry for Ki67 (4.3 $\pm$ 0.3% control vs. 0.76 $\pm$ 0.5% BMP-treated, mean $\pm$ SE, $n = 3$, Student's t-test, $P < 0.05$) [(c), high power in (i)], than BMP4-treated cells [(d), high power in (j)]. **(k, l)** Hematoxylin and eosin staining showing invasion of the lumen of the right lateral ventricle at bregma point [(k), arrow] 5 weeks post-injection following implantation of control cells. This phenomenon was never observed with BMP4-pretreated GBM-derived cells [(l), arrow]. 3 months post-injection, all control animals died; on the contrary, all animals receiving BMP4-pretreated GBM-derived cells survived well beyond 5 months. **(m, n)** Following a 48-hour incubation in control conditions (control), $CD133^+$ cells were purified by FACS (purity 97.3%) and the ability of 3×10^5 of these cells to establish GBMs intracerebrally was compared to that of the same number of $CD133^+$ cells, purified from sister cultures (purity 98.7%) after incubation for 48 hours in the presence of BMP4 (treated). Animals injected with $CD133^+$ cells from control cultures developed large neoplastic formations by 5–7 weeks post-transplant (m, $n = 8$), whereas none of the animals ($n = 8$) receiving $CD133^+$ cells sorted from BMP4-treated cells developed any tumor (n). By 90 days post-injection, all of the control animals died whereas those receiving $CD133^+$ cells from BMP4-treated cultures were alive. This shows that the tumorigenic ability of the residual fraction of $CD133^+$ cells found in BMP4-treated cultures is virtually abolished by BMP4. **(o, p)** When $CD133^+$ cells, which were acutely isolated from primary tumors established in mice injected with $CD133^+$ acutely isolated control cells, were retransplanted into the brain of secondary recipients, they established secondary tumors in less than 4 months [example in (o)]. These same cells could also be cultured and expanded with mitogens. Neither culturing nor establishment of secondary tumors could be accomplished with cells extracted from mice, which were initially injected with BMP4-treated GBM-derived cells. Similar serial transplantation experiments yielded equivalent results when the primary tumor was generated by transplantation of 3×10^5 briefly cultured $CD133^+$ cells [(p), example of secondary tumor established from $CD133^+$ GBM-derived cells]. Magnification: (a–h) and (k–n), 5×; (i, j) and (o, p), 10×. Based on supplementary Fig. 7 of: Piccirillo *et al.*, *Nature*, **444**, 761–765 (2006) with permission from Nature Publishing Group.

干细胞之家www.stemcell8.cn ←点击进入

and perhaps even curative, using the cancer stem cell population as a specific target. The search for such anti-CSC agents/protocols harmless to normal stem cells should also broaden our understanding of normal stem cell biology as well as of the genetics/epigenetics of tumor progression, thereby unraveling the roots of the inherent resistance of CSCs to conventional therapies.

References

Al-Hajj M, Wicha MS, Benito-Hernandez A, *et al*. (2003) Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci USA* **100**: 3983–88.

Andrews PW, Damjanov I, Berends J, *et al*. (1994) Inhibition of proliferation and induction of differentiation of pluripotent human embryonal carcinoma cells by osteogenic protein-1 (or bone morphogenetic protein-7). *Lab Invest* **71**: 243–51.

Bao S, Wu Q, McLendon RE, *et al*. (2006a) Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature* **444**: 756–60.

Bao S, Wu Q, Sathornsumetee S, *et al*. (2006b) Stem cell-like glioma cells promote tumor angiogenesis through vascular endothelial growth factor. *Cancer Res* **66**: 7843–48.

Bonnet D, Dick JE. (1997) Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med* **3**: 730–37.

Capela A, Temple S. (2002) LeX/ssea-1 is expressed by adult mouse CNS stem cells, identifying them as nonependymal. *Neuron* **35**: 865–75.

Collins AT, Berry PA, Hyde C, *et al*. (2005) Prospective identification of tumorigenic prostate cancer stem cells *Cancer Res* **65**: 10946–51.

Dean M, Fojo T, Bates S. (2005) Tumour stem cells and drug resistance. *Nat Rev Cancer*. **5**: 275–84.

Doetsch F, Caille I, Lim DA, *et al*. (1999) Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* **97**: 703–16.

Frank SA, Nowak MA. (2003) Cell biology: developmental predisposition to cancer. *Nature* **422**: 494.

Galli R, Binda E, Orfanelli U, *et al*. (2004) Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. *Cancer Res* **64**: 7011–21.

Gritti A, Parati EA, Cova L, *et al*. (1996) Multipotential stem cells from the adult mouse brain proliferate and self-renew in response to basic fibroblast growth factor. *J Neurosci* **16**: 1091–100.

Hanahan D, Weinberg RA. (2000) The hallmarks of cancer. *Cell* **100**: 57–70.

Hemmati HD, Nakano I, Lazareff JA, *et al*. (2003) Cancerous stem cells can arise from pediatric brain tumors. *Proc Natl Acad Sci USA* **100**: 15178–83.

Holland EC. (2000) Glioblastoma multiforme: the terminator. *Proc Natl Acad Sci USA* **97**: 6242–44.

干细胞之家www.stemcell8.cn ←点击进入

Holland EC, Celestino J, Dai C, *et al*. (2000) Combined activation of Ras and Akt in neural progenitors induces glioblastoma formation in mice. *Nat Genet* **25**: 55–57.

Holland EC, Hively WP, Gallo V, Varmus HE. (1998) Modeling mutations in the G1 arrest pathway in human gliomas: overexpression of CDK4 but not loss of INK4a-ARF induces hyperploidy in cultured mouse astrocytes. *Genes Dev* **12**: 3644–49.

Hope KJ, Jin L, Dick JE. (2004) Acute myeloid leukemia originates from a hierarchy of leukemic stem cell classes that differ in self-renewal capacity. *Nat Immunol* **5**: 738–43.

Ignatova TN, Kukekov VG, Laywell ED, *et al*. (2002) Human cortical glial tumors contain neural stem-like cells expressing astroglial and neuronal markers *in vitro*. *Glia* **39**: 193–206.

Kummermehr J, Trott KR. (1997). Tumour stem cells. In: *Stem Cells*, Potten CS (ed.), Academic Press, London, UK, pp. 363–99.

Lee J, Kotliarova S, Kotliarov Y, *et al*. (2006) Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines *Cancer Cell* **9**: 391–403.

Looijenga LH, Stoop H, de Leeuw HP, *et al*. (2003) POU5F1 (OCT3/4) identifies cells with pluripotent potential in human germ cell tumors. *Cancer Res* **63**: 2244–50.

Monzani E, Facchetti F, Galmozzi E, *et al*. (2007) Melanoma contains CD133 and ABCG2 positive cells with enhanced tumourigenic potential. *Eur J Cancer* **43**: 935–46.

O'Brien CA, Pollett A, Gallinger S, Dick JE. (2007) A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature* **445**: 106–10.

Pearce DJ, Bonnet D. (2007) Leukemia stem cells: studying the root of leukemia. *Gene Ther Regul* **3**: 65–90.

Piccirillo SGM, Reynolds BA, Zanetti N, *et al*. (2006) Bone morphogenetic proteins inhibit the tumorigenic potential of human brain tumour-initiating cells *Nature* **444**: 761–65.

Reya T, Morrison SJ, Clarke MF, Weissman IL. (2001) Stem cells, cancer, and cancer stem cells. *Nature* **414**: 105–11.

Reynolds BA, Rietze RL. (2005) Neural stem cells and neurospheres — re-evaluating the relationship. *Nat Meth* **2**: 333–36.

Reynolds BA, Weiss S. (1992) Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* **255**: 1707–10.

Ricci-Vitiani L, Lombardi DG, Pilozzi E, *et al*. (2007) Identification and expansion of human colon-cancer-initiating cells. *Nature* **445**: 111–15.

Rietze RL, Valcanis H, Brooker GF, *et al*. (2001) Purification of a pluripotent neural stem cell from the adult mouse brain. *Nature* **412**: 736–39.

Sanai N, Alvarez-Buylla A, Berger MS. (2005) Neural stem cells and the origin of gliomas. *N Engl J Med* **353**: 811–22.

干细胞之家www.stemcell8.cn ←点击进入

Sanai N, Tramontin AD, Quinones-Hinojosa A, *et al*. (2004) Unique astrocyte ribbon in adult human brain contains neural stem cells but lacks chain migration. *Nature* **427**: 740–44.

Singh SK, Clarke ID, Terasaki M, *et al*. (2003) Identification of a cancer stem cell in human brain tumors *Cancer Res* **63**: 5821–28.

Singh SK, Hawkins C, Clarke ID, *et al*. (2004) Identification of human brain tumour initiating cells. *Nature* **432**: 396–401.

Szotek PP, Pieretti-Vanmarcke R, Masiakos PT, *et al*. (2006) Ovarian cancer side population defines cells with stem cell-like characteristics and Mullerian inhibiting substance responsiveness. *Proc Natl Acad Sci USA*, **103**: 11154–59.

Taylor MD, Poppleton H, Fuller C, *et al*. (2005) Radial glia cells are candidate stem cells of ependymoma. *Cancer Cell* **8**: 323–35.

Timmer-Bosscha H, de Vries EG, Meijer C, *et al*. (1998) Differential effects of all-trans-retinoicacid, docosahexaenoic acid, and hexadecylphosphocholine on cisplatin-induced cytotoxicity and apoptosis in a cisplantin-sensitive and resistant human embryonal carcinoma cell line. *Cancer Chemother Pharmacol* **41**: 469–76.

Uchida N, Buck DW, He D, *et al*. (2000) Direct isolation of human central nervous system stem cells. *Proc Natl Acad Sci USA* **97**: 14720–25.

Vescovi AL, Galli R, Reynolds BA. (2006) Brain tumour stem cells. *Nat Rev Cancer* **6**: 425–36.

Zhu Y, Guignard F, Zhao D, *et al*. (2005) Early inactivation of p53 tumor suppressor gene cooperating with NF1 loss induces malignant astrocytoma. *Cancer Cell* **8**: 119–30.

干细胞之家www.stemcell8.cn ←点击进入

Chapter 12

CD44 as a Functional Cancer Stem Cell Marker and a Potential Therapeutic Target

Lubna Patrawala[†] and Dean G. Tang[*,†,‡]

CD44 is a well-characterized adhesion molecule with multi-faceted biological functions. Earlier studies have implicated CD44 in the invasion and metastasis of multiple human cancers. Newly generated data suggest that tumor cells expressing CD44 may be intrinsically different from the bulk $CD44^{-}$ cells and that its expression may mark a population of tumor-initiating cells called cancer stem cells (CSCs). In this review, we succinctly summarize the evidence of CD44 as a CSC marker and also discuss the possibility of targeting CD44-expressing CSCs.

Keywords: CD44; tumor-initiating cells; cancer stem cells; tumor progenitors; metastasis; homing.

1. Introduction

In the past decade, there has been a major paradigm shift in our understanding of cancer. Rather than being viewed as a disease driven by random mutations, cancer is now viewed as a product of abnormal stem cell development. In other words, a mutated stem cell or its immediate progeny, still retaining the ability to both self-renew and partially differentiate, develops into a disorganized tumor mass comprising a kaleidoscope of cells with different phenotypic properties. Regardless of the origin, many cancers are now found to contain small subsets of cells, which have the exclusive ability

[*]Corresponding author. dtang@mdanderson.org
[†]Department of Carcinogenesis, The University of Texas MD Anderson Cancer Center, Science Park–Research Division, Smithville, TX 78957, USA
[‡]Program in Environmental & Molecular Carcinogenesis, Graduate School of Biomedical Sciences (GSBS), Houston, TX 77030, USA

干细胞之家www.stemcell8.cn ←点击进入

to reinitiate tumors and are therefore called tumor-initiating cells or cancer stem cells (CSCs) (Reya *et al.*, 2001; Clarke *et al.*, 2006; Dalerba *et al.*, 2007). These CSCs, at the cellular level, resemble normal stem cells in that they possess the ability to self-renew and differentiate. Identification and characterization of CSCs in every type of human cancer will not only facilitate our understanding of the etiology of that specific tumor, but also, more importantly, help develop novel therapeutics to eradicate the "roots" of the malignancy. Most putative CSCs so far reported have been identified using cell surface markers, among which CD44 and CD133 have emerged as the most common ones. In this review, we focus on CD44 as a tempting "universal" CSC marker and on its potential utility in the development of therapeutic regimens aimed specifically at CSCs.

2. The CD44 Family

The CD44 proteins are a family of transmembrane glycoproteins that are encoded by a highly conserved gene containing 20 exons (Screaton *et al.*, 1992; reviewed in Ponta *et al.*, 2003). Structural and functional heterogeneity of the proteins can be attributed to alternative splicing events and post-translational modifications leading to the generation of a number of isoforms. CD44s or CD44H, the standard form encoded by nine exons, is the most widely expressed isoform and is found in developing and normal adult tissues. Alternative splicing of at least 10 exons leads to the insertion of amino acids in the stem region of the CD44s protein, giving rise to the variant (v) isoforms. In contrast to the standard form, the v forms are more restricted in their expression, appearing mainly in proliferating cells of some epithelial tissues and in transformed cells (Ponta *et al.*, 2003). All isoforms of CD44 bind to hyaluronan, a glycosaminoglycan that is ubiquitously distributed in the extracellular matrix (ECM), although CD44 has also been shown to bind to a number of other ligands including fibronectin, collagen, laminin and osteopontin (Weber *et al.*, 1996; Ponta *et al.*, 2003). Interaction between CD44 and hyaluronan activates an array of downstream signaling pathways that regulates functions as diverse as proliferation, adhesion, apoptosis, migration, metastasis and hematopoiesis. The complex involvement of CD44 in multiple physiological and disease processes owes to its structural heterogeneity as well as to the diversity of its ligands (Stamenkovic *et al.*, 1991; Ponta *et al.*, 2003). Another level of CD44 regulation is post-transcriptional, i.e., its proteolysis at both extracellular and transmembrane domains. For example, CD44 is a substrate of the presenilin-dependent gamma-secretase, which induces intramembranous cleavage of CD44 (Murakami *et al.*, 2003). Similarly, two

干细胞之家www.stemcell8.cn ←点击进入

ADAM family metalloproteinases, ADAM10 and ADAM17 can induce CD44 ectodomain cleavage and consequently terminate the CD44-mediated cell–matrix adhesion (Nagano *et al.*, 2004).

3. CD44 and Normal Stem Cells

Surprisingly, CD44-null mice are viable and, with the exception of mild hematopoietic abnormalities, do not show any overt developmental deficiencies (Schmits *et al.*, 1997; Protin *et al.*, 1999). It has been suggested that in the early absence of CD44, compensatory mechanisms may take over resulting in only a subtle developmental phenotype. On the other hand, transgenic mice expressing antisense CD44 cDNA driven by the K5 promoter display a more severe phenotype such as decrease in skin elasticity, impaired inflammation and tissue repair, and delayed hair growth (Kaya *et al.*, 1997). These observations suggest that there is a small window of opportunity in early development for CD44 compensation to kick in, and reiterate the importance of CD44 in normal physiology. Indeed, CD44 plays a critical role in the homing and distribution of hematopoietic progenitor cells (Schmits *et al.*, 1997; Lapidot *et al.*, 2005). In normal hematopoietic stem cells (HSCs), the interaction between CD44 and hyaluronic acid is essential for their homing to the bone marrow and spleen (Avigdor *et al.*, 2004). Similarly, CD44 is an important molecule expressed in mesenchymal stem cells (MSCs) involved in cell migration in ECM (Zhu *et al.*, 2006). CD44 has also been reported to mark astrocyte precursor cells (Liu *et al.*, 2004) and multipotent central nervous system (CNS) stem cells (Oishi *et al.*, 2006).

4. CD44 as a Marker for Diverse CSCs

Early studies have implicated CD44H or some of its splice variants (in particular, CD44v6 and CD44v9) in cancer cell adhesion, migration, invasion and metastasis (Gunthert *et al.*, 1991; Lamb *et al.*, 1997; Masellis-Smith *et al.*, 1997; Ponta *et al.*, 2003). The CD44-involved tumor cell invasion and dissemination are generally ascribed to its role as an adhesion and signaling molecule (Ponta *et al.*, 2003), and also to its ability to direct some proteases such as matrix metalloproteinases to the invading lamellipodia (Mori *et al.*, 2002).

4.1. *Leukemic Stem Cells*

In 1992, CD44 was found to be expressed not only on CD34^{+} normal HSCs, but also on CD34^{+} leukemia cells (Reuss-Borst *et al.*, 1992), providing

干细胞之家www.stemcell8.cn ←点击进入

the first hint that CD44 might mark tumorigenic leukemia cells. In 1996, Sutherland *et al.* demonstrated a hierarchical organization of human acute myeloid leukemia (AML) cells; and in 1997, Dick's group showed that only leukemic cells bearing the $CD34^{+}CD38^{-}$ phenotype could transfer the disease to immunodeficient NOD/SCID mice (Bonnet and Dick, 1997). These AML leukemic stem cells (AML-LSCs) were later phenotypically refined as $CD34^{+}CD38^{-}CD71^{-}CD90^{-}$ (Blair *et al.*, 1997; 1998) that also uniquely express IL3Rα (Jordan *et al.*, 2000). Importantly, CD44 is also expressed in LSCs. Although CD44 was not used in the initial identification of LSCs, the variant isoforms are known to be elevated on AML cells and are associated with poor prognosis (Bendall *et al.*, 2000; Legras *et al.*, 1998). In fact, CD44 seems to be crucial in homing LSCs to the right bone marrow microenvironment (i.e., niche) as an activating monoclonal antibody to CD44 (H90) interferes with the transport of AML-LSCs to stem cell-supportive microenvironmental niches, leading to markedly reduced leukemic repopulation (Jin *et al.*, 2006). H90 effectively reduced the tumor burden as well as the engraftment of leukemic cells. The defect in engraftment was specific to AML cells, as human cord blood cells were unaffected by H90. Interestingly, the authors observed that LSCs were somewhat niche-dependent, demonstrating for the first time the link between CSCs and a specialized microenvironment. Furthermore, treatment with the H90 antibody triggered maturation of the LSCs, implicating CD44 as a key regulator of the primitive state of LSCs (Jin *et al.*, 2006).

CD44 expression is also increased in human chronic myeloid leukemia (CML) (Ghaffari *et al.*, 1995) as well as in *BCR-ABL*–expressing progenitors in the bone marrow of mice with CML-like leukemia (Krausse *et al.*, 2006). Using CD44-null mice as donors in a retroviral transplantation model of CML, Krausse *et al.* (2006) found that CD44 was required for bone marrow homing of CML progenitors. As in AML, dependence on CD44 for homing was greatly increased in LSCs compared with normal HSCs (Krausse *et al.*, 2006). Unlike the AML studies, however, direct injection of CML progenitors treated with a CD44 blocking antibody (IM7) into the bone marrow led to a leukemia burden similar to that in control mice, suggesting that CD44 was required for homing but not maintenance of LSCs in the mouse model of CML (Krausse *et al.*, 2006). These studies point to the crucial importance of CD44 in LSC homing, and also highlight the complexity of CD44 signaling and the need to tease apart specific variants that may be involved in different cancers.

干细胞之家www.stemcell8.cn ←点击进入

4.2. *Breast Cancer Stem Cells*

Recent work suggests that CD44 expression may also identify tumor-initiating cells in solid tumors. Studies on breast cancer have generated controversial data over the potential involvement and prognostic value of CD44 (Gotte and Yip, 2006). For instance, hyaluronan is upregulated in breast cancer cells and their surrounding stroma (Auvinen *et al.*, 2000). CD44s is upregulated in invasive breast carcinomas (Auvinen *et al.*, 2005) but, paradoxically, correlated with overall patient survival (Diaz *et al.*, 2005), while some variant forms such as CD44v6 are upregulated in breast cancer but do not correlate with clinical outcome (Auvinen *et al.*, 2005; Diaz *et al.*, 2005). CD44, although promoting the invasion of a variety of cancer cells, somehow seems to attenuate metastatic invasion during breast cancer progression (Lopez *et al.*, 2005). Most of these studies relied on correlative tissue analysis or overexpression experiments in long-term cultured cell lines. The key missing experiment was to purify CD44-expressing breast cancer cells out to ask whether these cells are intrinsically different from the $CD44^-$ tumor cells.

Spurred by the findings that leukemia is driven by LSCs (Bonnet and Dick, 1997), Al-Hajj *et al.* (2003) proceeded to investigate the tumor-initiating capacity of CD44-expressing cells in human primary breast cancer samples. They found that as few as 200 $CD44^+CD24^{-/lo}ESA^+$ cells could generate a tumor in immunodeficient mice; whereas the remaining cancer cells, comprising 95–98% of the population, were much less tumorigenic. Moreover, the $CD44^+CD24^{-/lo}$ population could regenerate the phenotypic heterogeneity of the original tumor. These observations provided the first evidence for a hierarchical organization of cells in a solid tumor. Importantly, they paved the way for the characterization of tumorigenic and non-tumorigenic cells from a single tumor, facilitating the elucidation of the molecular pathways that preferentially mediate resistance to cancer therapies in the tumorigenic population. The $CD44^+CD24^{-/lo}$ breast CSCs can be cultured *in vitro* while maintaining their tumorigenic potential (Ponti *et al.*, 2005). It was recently demonstrated that the Hedgehog signaling pathway, known to regulate self-renewal in mammary stem cells, is aberrantly activated in the $CD44^+CD24^{-/lo}$breast CSCs (Liu *et al.*, 2006).

Many groups are also investigating the correlation between breast CSCs and invasion and metastasis. In a study done with 50 patients in the early stages of breast cancer, an average of 72% of the disseminated tumor cells

干细胞之家www.stemcell8.cn ←点击进入

in the bone marrow were found to be of the $CD44^{+}CD24^{-/lo}$ phenotype (Balic *et al.*, 2006). This finding was striking because cells of the same phenotype were present in <10% of primary tumors, leading to speculation that the breast CSCs mediate metastasis. Another correlative study suggests that the prevalence of $CD44^{+}CD24^{-/lo}$ tumor cells may favor distant metastasis (Abraham, 2005). More recently, Liu *et al.* (2007) compared the gene-expression profiles of breast CSCs with those of normal breast epithelium and generated an "invasive gene signature" (IGS) based on the differential expression of 186 genes. The prognostic power of the criteria used by the National Institutes of Health to determine breast cancer patient survival was significantly strengthened when combined with the IGS. Additionally, the gene signature was associated with prognosis in medulloblastoma, lung cancer, and prostate cancer (Liu *et al.*, 2007). Another independent molecular profiling of $CD24^{+}$ and $CD44^{+}$ breast cancer cells also revealed them to be more differentiated and progenitor-like cells, respectively (Shipitsin *et al.*, 2007). Importantly, the $CD44^{+}$ breast cancer cells were found to preferentially express many known stem-cell markers, and the $CD44^{+}$ cell-specific gene expression correlates with decreased patient survival (Shipitsin *et al.*, 2007). Furthermore, the $CD44^{+}$ breast CSCs are negative for estrogen receptor even in some estrogen receptor-positive tumors (Shipitsin *et al.*, 2007). Finally, $CD44^{+}CD24^{-/lo}$cells have been identified in breast cancer cell lines and have been shown to be highly invasive (Sheridan *et al.*, 2006) and radioresistant, increasing in number after bouts of radiation (Phillips *et al.*, 2006).

The above discussions suggest that, when done properly, the CD44-expressing breast cancer cells in both long-term and short-term cultures as well as in tumors are intrinsically different from the bulk, $CD44^{-}$ breast cancer cells.

4.3. *Prostate Cancer Stem Cells*

Similar to the situation in breast cancer, most early studies on the role of CD44 in prostate cancer (PCa) development and progression have generated conflicting data. One study of 109 cases reported a complete lack of membranous expression of all CD44 isoforms in 93–98% of primary PCa examined (Kallakury *et al.*, 1996). However, another study of 74 PCa lesions reported moderate to high levels of CD44 expression in 60% of primary tumors, with $\sim$14% of metastases expressing low levels of CD44 (Nagabhushan *et al.*, 1996). Yet another study reported significantly reduced CD44 expression in all 94 primary neoplastic foci and 48 metastases (De Marzo *et al.*, 1998). The relationship between CD44 expression and tumor

干细胞之家www.stemcell8.cn ←点击进入

grade is also uncertain — one study showed strong correlation between Gleason grade of the tumor and loss of CD44 expression (De Marzo *et al.*, 1998), whereas another reported no correlation (Paradis *et al.*, 1998). Furthermore, although CD44 expression was reported to be reduced in metastases (Nagabhushan *et al.*, 1996; De Marzo *et al.*, 1998), the CD44$^+$ PCa cells were found to predominate in two visceral metastases (Liu *et al.*, 1999). Similar to expression studies, the potential role of CD44 in PCa development and metastases is controversial — while some studies showed a tumor-suppressive function of CD44 in overexpression experiments (Gao *et al.*, 1997 and 1998), many other studies implicated CD44 in PCa cell proliferation, adhesion, migration and invasion *in vitro* as well as metastatic dissemination *in vivo* (Paradis *et al.*, 1998; Liu *et al.*, 1999; Omara-Opyene *et al.*, 2004; Draffin *et al.*, 2004). Most studies mentioned above utilized either human tissues to carry out correlative immunohistochemistry or bulk-cultured PCa cells to do overexpression experiments, and the key experiment of using purified CD44$^+$ and CD44$^-$ cells from the same culture or tumor to compare their potentially different biological and tumorigenic properties had not been done. Therefore, it was not known whether the minor subset of CD44$^+$ PCa cells in both primary and metastatic lesions were intrinsically different from bulk CD44$^-$ PCa cells.

We addressed this critical issue by isolating human CD44$^+$ PCa cells from multiple cultured PCa cell lines and three xenograft tumors (i.e., Du145, LAPC4, and LAPC9), and compared their *in vitro* biological and *in vivo* tumorigenic properties with the isogenic CD44$^-$ PCa cell populations (Patrawala *et al.*, 2006). We have observed that CD44$^+$ PCa cells are more clonogenic, proliferative, tumorigenic and metastatic than their corresponding negative counterparts (Patrawala *et al.*, 2006). Noticably, CD44$^+$ and CD44$^-$ cells were purified to near homogeneity from freshly dissociated xenograft tumor cells (Fig. 1) and injected into NOD/SCID mice either subcutaneously or orthotopically. In both cases, CD44$^+$ PCa cells were shown to possess higher tumoregenicity than their CD44$^-$ counterparts (Tables 1 and 2).

Moreover, CD44$^+$cells possess many intrinsic traits of stem/progenitor cells, such as preferential activation of genes implicated in self-renewal and maintenance of an undifferentiated state (e.g., *Oct3/4*, *Bmi*, *β-catenin*, *Smoothened*), and intermediate cell-cycle properties. Importantly, CD44$^+$cells are distinct from the fully differentiated androgen receptor (AR)-expressing cells and have the ability to recapitulate the original heterogeneity of the tumor, indicating that they are upstream in the tumor hierarchy (Patrawala

干细胞之家www.stemcell8.cn ←点击进入

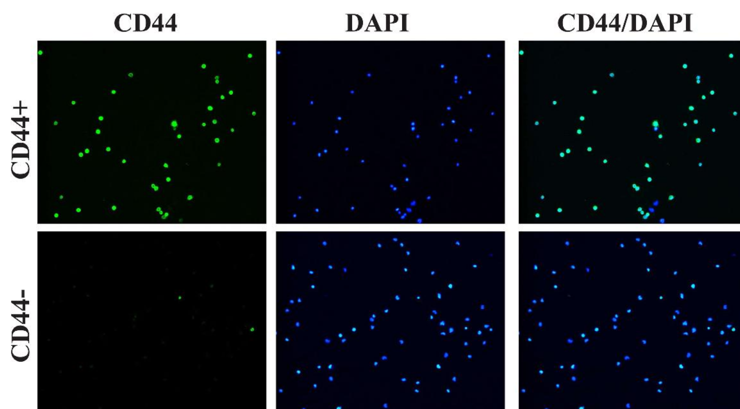


Fig. 1. Purity of the $CD44^+$ and $CD44^-$ PCa cell populations. Tumor cells were acutely purified from xenograft tumors (NOD/SCID mice) and the purity of sorted $CD44^+$ and $CD44^-$ cell populations was analyzed as shown here (LAPC-4 tumor). Cells were stained for CD44 and counterstained by DAPI. Based on supplementary Fig. 2 of: Patrawala *et al.*, *Oncogene*, **25**: 1696–1708 (2006) with permission from Nature Publishing Group.

et al., 2006; Tang *et al.*, 2007). Recently, using a combinatorial sorting strategy, we clearly demonstrated the hierarchical organization of PCa cells in xenograft tumors. Specifically, we found that the $CD44^+\alpha2\beta1^+$ and $CD44^+$ cell populations are very similarly enriched in tumorigenic PCa cells and the $CD44^-\alpha2\beta1^+$ PCa cells show much reduced tumorigenicity whereas the $CD44^-\alpha2\beta1^-$ cells essentially lack tumorigenic potential (Patrawala *et al.*, 2007), suggesting that prostate CSCs reside in the $CD44^+$ cell population and $\alpha2\beta1$ marks prostate tumor progenitors. Since in all of our experiments we routinely carried out lineage selection by removing Lin^+ cells (including blood cells, endothelial cells, fibroblasts and other stromal cells), the prostate CSCs we identified thus possess the phenotype of $CD44^+Lin^-$. Collins *et al.* (2005) utilized *in vitro* approaches to identify putative CSCs in primary human PCa samples. The putative prostate CSCs had the same antigenic profile as normal prostate stem cells, i.e., $CD44^+\alpha2\beta1^{hi}CD133^+$, and were the only cells with the capability to self-renew *in vitro* and generate more differentiated cell types (Collins *et al.*, 2005). Due to technical challenges, there have been no reports to date describing the isolation of tumorigenic prostate CSCs from primary tumor samples. In ~20 PCa patient samples we have worked with, we could detect $CD44^+$ and $CD133^+$ cells, and we are currently studying whether these cells have unique tumor-initiating abilities. Our studies on xenograft tumors clearly indicate that the $CD44^+$ cell population, although enriched in tumorigenic cells, is still heterogeneous. Work is underway to further enrich for more tumorigenic subsets, such as the side population (SP) cells (Patrawala *et al.*, 2005) in the $CD44^+$ PCa cell

干细胞之家www.stemcell8.cn ←点击进入

Table 1 Tumorigenicity of Subcutaneously Injected CD44$^+$ and CD44$^-$ PCa Cells

Cell Type[a]	Cell Nos. Injected	Tumor Incidence[b]	Latency (Days)[c]
Du145 unsorted	100	3/5	61–95 (71)
	1,000	4/6	36–71 (61)
	10,000	5/6	36–95 (36)
	100,000	4/4	36 (36)
Du145-CD44$^+$	100	5/8*	41–67 (41)
	1,000	5/8*	32–50 (40)
Du145-CD44$^-$	100	1/8	108
	1,000	0/6	
	10,000	3/8	96–174 (96)
LAPC4 unsorted	100	10/12	45–85 (63)
	1,000	5/8	42–130 (58)
	10,000	6/6	31–116 (31)
LAPC4-CD44$^+$	100	4/6*	60–102 (67)
	1,000	5/6	41–123(66)
	10,000	3/3	31–78 (47)
LAPC4-CD44$^-$	100	2/14	41–120 (80)
	1,000	13/14	41–137 (90)
	10,000	7/7	35–83 (71)
LAPC9 unsorted	100	3/6	96–124 (96)
	1,000	6/6	60–67 (64)
LAPC9-CD44$^+$	10	1/4	67
	100	10/10*	63–88 (67)
	1,000	10/11	55–82 (64)
	10,000	4/4	53–55 (54)
LAPC9-CD44$^-$	100	0/8	
	1,000	5/6	67–81 (74)
	10,000	4/4	60–67 (64)

[a]Tumor cells were acutely purified from the xenograft tumors maintained in NOD/SCID mice. Cells, either unsorted or sorted as CD44$^+$ or CD44$^-$, were injected in 40 μL of medium/Matrigel (1:1) subcutaneously into the NOD/SCID mice.

[b]The number of tumors/number of injections. $*P < 0.01$ compared with the corresponding CD44$^-$ injections (F-test).

[c]From tumor cell injection to the appearance of a palpable tumor. Values in parentheses are the medians.

Based on Table 1 of: Patrawala *et al.*, *Oncogene*, **25**: 1696–1708 (2006) with permission from Nature Publishing Group.

population. Taken together, studies on breast and prostate CSCs highlight the importance of carrying out prospective *in vivo* tumor experiments using purified populations of cells, and point to CD44 as a CSC marker for both tumor types.

干细胞之家www.stemcell8.cn ←点击进入

Table 2 Tumorigenicity of Orthotopically Implanted LAPC-9 Cells

Cells (Number)[a]	Tumor Incidence[b]	Latency (Days)[c]	Metastasis[d]
LAPC-9/unsorted			
100	0/3		
1,000	0/9		
10,000	4/8 (50%)	46–75 (53)	0/4
100,000	6/9 (67%)	32–69 (44)	0/6
1,000,000	4/4 (100%)	48–69 (56)	0/4
LAPC-9/CD44^{+}			
100	0/5		
1,000	2/8 (25%)	45–61 (53)	1/2
10,000	4/9 (44%)	42–57 (50)	1/4
LAPC-9/CD44^{-}			
100	0/5		
1,000	0/10		
10,000	0/10		
100,000	3/5 (60%)	107 (107)	0/3
500,000	4/5 (80%)	33–107 (60)	0/4

[a]Cells at the indicated numbers were injected in 25 μL of Matrigel into NOD/SCID mouse prostate.
[b]The number of animals that developed tumors/The number of animals injected with tumor cells. Values in parentheses represent the % tumor incidence.
[c]The time (in days) from tumor cell injection to when tumors were observed. The ranges are shown with the median tumor latencies shown in the parentheses.
[d]At necropsy, urogenital organs as well as multiple distant organs were examined for tumor dissemination.
Based on Table 2 of: Patrawala *et al.*, *Oncogene*, **25**: 1696–1708 (2006) with permission from Nature Publishing Group.

4.4. *Pancreatic and Head/Neck Cancer Stem Cells*

CD44 may also mark other CSCs. For example, CD44 has proven useful in isolating CSCs from pancreatic cancer, one of the most lethal cancers. Cells of the phenotype CD44^{+}CD24^{+}ESA^{+}constituted 0.2–0.8% of the pancreatic tumor cells, but were enriched 100-fold in tumorigenic potential compared with the rest of the population and generated tumors that were histologically indistinguishable from the original tumors from which they were derived (Li *et al.*, 2007). The putative pancreatic CSCs showed increased expression of Sonic hedgehog (Shh), presumably leading to dysregulated self-renewal (Li *et al.*, 2007). Similarly, CD44 was found to mark primitive basal-like cells in head and neck squamous cell carcinoma that met all of the criteria to qualify as CSCs. In this case, the *Shh* pathway target *Bmi* was identified as the aberrantly expressed self-renewal gene (Prince *et al.*, 2007).

干细胞之家www.stemcell8.cn ←点击进入

4.5. *Importance of CD44 and Potential Comarkers in CSC Biology*

Considering that CD44 interaction with hyaluronan plays a critical role in colon cancer cell invasion (Kim *et al.*, 2004) and that CD44 expression in a wide variety of cancer cells (including prostate, breast, colon, and bladder cancer cells as well as glioma and melanoma cells) correlates with their malignancy (Patrawala *et al.*, 2006), it stands to reason that CD44 must be involved in the biological activities of a wide variety of CSCs.

It should be noted that in all four solid tumors discussed above, i.e., breast (Al-Hajj *et al.*, 2003), prostate (Collins *et al.*, 2005; Patrawala *et al.*, 2006), pancreatic (Li *et al.*, 2007) and head/neck cancers (Prince *et al.*, 2007), putative CSCs are purified using mainly CD44 as the positive marker. The role of other marker(s) such as CD24 remains unclear. Intriguingly, breast CSCs bear $CD24^{-/low}$ (Al-Hajj *et al.*, 2003), whereas pancreatic CSCs have the phenotype of $CD24^{+}$ (Li *et al.*, 2007). In addition, $CD24^{+}$cells are found to significantly increase in breast cancer metastasis (Shipitsin *et al.*, 2007), and CD24 expression has been associated with tumor growth and metastasis (Baumann *et al.*, 2005). Also, ESA (epithelial specific antigen) is expressed mainly on differentiated cells, so it is unclear why in some purification protocols ESA is used (or needed). At this moment, it is also unclear whether CD44 expression *per se* is required for the manifestation of all CSC properties. The fact that the anti-CD44 antibodies could block the homing of AML (Jin *et al.*, 2006) and CML (Krausse *et al.*, 2006) LSCs suggests that CD44 itself is biologically important. Another point worth mentioning is that CD44 and CD133 — another popular cell surface marker that has been used to identify a variety of normal stem/progenitor cells (Uchida *et al.*, 2000; Kuci *et al.*, 2003) as well as putative CSCs in glioma (Singh *et al.*, 2004), colon cancer (O'Brien *et al.*, 2007; Ricci-Vitiani *et al.*, 2007) and hepatocellular carcinoma (Suetsugu *et al.*, 2006; Yin *et al.*, 2007) — may identify overlapping populations of stem/progenitor cells. For example, putative prostate CSCs reported by Collins *et al.* (2005) are $CD44^{+}CD133^{+}$. Similarly, the $CD133^{+}$ glioma stem cells are also enriched in CD44 (Liu *et al.*, 2006a).

5. CD44-expressing CSCs as Therapeutic Targets

Several types of CSCs have been shown to be more resistant to therapeutics. For example, LSCs are resistant to a wide spectrum of chemodrugs possibly due to overexpression of multi-drug resistance proteins such as ABCG2 (reviewed in Ravandi and Estrov, 2006). The $CD44^{+}CD24^{-/low}$breast cancer cells are radioresistant possibly because they produce lower levels

干细胞之家www.stemcell8.cn ←点击进入

of reactive oxygen species (Phillips *et al.*, 2006). The CD133$^+$ glioma stem cells are also radioresistant due to a preferential activation of DNA damage response in these cells (Bao *et al.*, 2006). If we know the gene expression signatures and/or signal transduction pathways unique to CSCs, we might be able to design specific therapeutics to target them. For example, recent work showed that LSCs seem to have heightened NF-kB prosurvival mechanisms. Parthenolide (PTL), a naturally occurring small molecule, via inhibiting NF-kB activity and inducing generation of reactive oxygen species, causes robust apoptosis in primary human AML cells and blast crisis CML cells while sparing normal hematopoietic cells (Guzman *et al.*, 2005). Similarly, the BMP signaling pathway normally regulates the neural stem cell fate, and BMP4 demonstrates a potent inhibitory effect on CD133$^+$ cell-initiated gliomagenesis (Piccirillo *et al.*, 2006). The CD44$^+$ breast cancer cells seem to have unique activation of the TGFβ signaling pathway due to exclusive expression of TGFβRII, and a TGFβR inhibitor causes their rapid differentiation (Shipitsin *et al.*, 2007). These discussions suggest that CSCs are probably more resistant to therapeutics through many different mechanisms, that CSCs will have to be eradicated if we want to cure the patients of primary tumor burden and prevent recurrence and dissemination, and that it **is** possible to find CSC-unique signaling pathways to specifically target them.

CD44 may represent a good CSC-specific target. It is widely expressed in various CSCs and seems to be required for LSC homing (Jin *et al.*, 2006; Krausse *et al.*, 2006). Since both breast and prostate cancers have a high propensity to metastasize to the bone and CD44$^+$ CSCs might be the cells mediating breast (Abraham, 2005; Balic *et al.*, 2006) and prostate (Patrawala *et al.*, 2006) cancer metastass, it is tempting to speculate that CD44 might also be involved in breast/prostate CSC homing to the bone marrow. Furthermore, disruption of CD44 functions induces apoptosis of many cancer cells (Yu *et al.*, 1997; Ohashi *et al.*, 2007). Therefore, targeting CD44 may help both reduce tumor burden and prevent bone metastasis of these malignancies. Conceivable approaches may include local or imaging-guided administration of anti-CD44 antibodies such as H90 and IM7 (Jin *et al.*, 2006; Krausse *et al.*, 2006), antibody-conjugated cytotoxic agents (or prodrugs), CD44 promoter-driven expression of proapoptotic genes or cytotoxins in retroviral or lentiviral vectors, or hyaluronan polymer-conjugated drugs (such as taxol). Neural stem/progenitor cells as well as mesenchymal stem cells have tremendous migratory ability and high tropism for tumors/ischemic foci (Aboody *et al.*, 2000; Studeny *et al.*, 2002) and have been exploited to carry therapeutic agents to treat tumors, e.g., intracranial brain tumors (e.g., Aboody *et al.*, 2006). Theoretically, CD44$^+$ CSCs (or their

干细胞之家www.stemcell8.cn ←点击进入

normal counterparts) might be utilized in the same way to carry cytotoxic agents to home to the primary tumor and metastatic sites in order to achieve therapeutic efficacy. We envision many exciting developments along these lines in the near future.

Acknowledgments

We thank the other Tang lab members for their assistance in actual experiments. Original work in the lab was supported in part by grants from the NIH (AG023374), American Cancer Society (RSG MGO-105961), Department of Defense, Prostate Cancer Foundation, and Elsa Pardee Foundation. We also acknowledge the support of two Center Grants (CCSG-5 P30 CA166672 and ES07784). L. Patrawala was supported in part by a pre-doctoral fellowship from the Department of Defense (W81XWH-06-1-023). We apologize to the colleagues whose work could not be cited due to space constraints.

References

Aboody KS, Brown A, Rainov NG, *et al*. (2000) Neural stem cells display extensive tropism for pathology in adult brain: evidence from intracranial gliomas. *Proc Natl Acad Sci USA* **97**: 12846–51.

Aboody KS, Bush RA, Garcia E, *et al*. (2006) Development of a tumor-selective approach to treat metastatic cancer. *PLoS ONE* **1**: e23.

Abraham BK, Fritz P, McClellan M, *et al*. (2005) Prevalence of CD44+/CD24−/low cells in breast cancer may not be associated with clinical outcome but may favor distant metastasis. *Clin Cancer Res* **11**: 1154–59.

Al-Hajj M, Wicha MS, Benito-Hernandez A, *et al*. (2003) Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci USA* **100**: 3983–88.

Auvinen P, Tammi R, Parkkinen J, *et al*. (2000) Hyaluronan in peritumoral stroma and malignant cells associates with breast cancer spreading and predicts survival. *Am J Pathol* **156**: 529–36.

Auvinen P, Tammi R, Tammi M, *et al*. (2005) Expression of CD44s, CD44v3 and CD44v6 in benign and malignant breast lesions: correlation and colocalization with hyaluronan. *Histopathology* **47**: 420–28.

Avigdor A, Goichberg P, Shivtiel S, *et al*. (2004) CD44 and hyaluronic acid cooperate with SDF-1 in the trafficking of human CD34+ stem/progenitor cells to bone marrow. *Blood* **103**: 2981–89.

Balic M, Lin H, Young L, *et al*. (2006) Most early disseminated cancer cells detected in bone marrow of breast cancer patients have a putative breast cancer stem cell phenotype. *Clin Cancer Res* **12**: 5615–21.

Bao S, Wu Q, McLendon RE, *et al*. (2006) Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature* **444**: 756–60.

干细胞之家www.stemcell8.cn ←点击进入

Baumann P, Cremers N, Kroese F, *et al*. (2005) CD24 expression causes the acquisition of multiple cellular properties associated with tumor growth and metastasis. *Cancer Res* **65**: 10783–93.

Bendall LJ, Bradstock KF, Gottlieb DJ. (2000) Expression of CD44 variant exons in acute myeloid leukemia is more common and more complex than that observed in normal blood, bone marrow or CD34+ cells. *Leukemia* **14**: 1239–46.

Blair A, Hogge DE, Ailles LE, *et al*. (1997) Lack of expression of Thy-1 (CD90) on acute myeloid leukemia cells with long-term proliferative ability *in vitro* and *in vivo*. *Blood* **89**: 3104–12.

Blair A, Hogge DE, Sutherland HJ. (1998) Most acute myeloid leukemia progenitor cells with long-term proliferative ability *in vitro* and *in vivo* have the phenotype CD34(+)/CD71(−)/HLA-DR-. *Blood* **92**: 4325–35.

Bonnet D, Dick JE. (1997) Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med* **3**: 730–37.

Clarke MF, Dick JE, Dirks PB, *et al*. (2006) Cancer stem cells — perspectives on current status and future directions: AACR Workshop on Cancer Stem Cells. *Cancer Res* **66**: 9339–44.

Collins AT, Berry PA, Hyde C, *et al*. (2005) Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Res* **65**: 10946–51.

Dalerba P, Cho RW, Clarke MF. (2007) Cancer stem cells: models and concepts. *Ann Rev Med* **58**: 267–84.

De Marzo AM, Bradshaw C, Sauvageot J, *et al*. (1998) CD44 and CD44v6 downregulation in clinical prostatic carcinoma: relation to Gleason grade and cytoarchitecture. *Prostate* **34**: 162–68.

Diaz LK, Zhou X, Wright ET, *et al*. (2005) CD44 expression is associated with increased survival in node-negative invasive breast carcinoma. *Clin Cancer Res* **11**: 3309–14.

Draffin JE, McFarlane S, Hill A, *et al*. (2004) CD44 potentiates the adherence of metastatic prostate and breast cancer cells to bone marrow endothelial cells. *Cancer Res* **64**: 5702–11.

Gao AC, Lou W, Sleeman JP, Isaacs JT. (1998) Metastasis suppression by the standard CD44 isoform does not require the binding of prostate cancer cells to hyaluronate. *Cancer Res* **58**: 2350–52.

Gao AC, Lou W, Dong JT, Isaacs JT. (1997) CD44 is a metastasis suppressor gene for prostatic cancer located on human chromosome 11p13. *Cancer Res* **57**: 846–49.

Ghaffari S, Dougherty GJ, Lansdorp PM, *et al*. (1995) Differentiation-associated changes in CD44 isoform expression during normal hematopoiesis and their alteration in chronic myeloid leukemia. *Blood* **86**: 2976–85.

Gotte M, Yip GW. (2006) Heparanase, hyaluronan, and CD44 in cancers: a breast carcinoma perspective. *Cancer Res* **66**: 10233–37.

Gunthert U, Hofmann M, Rudy W, *et al*. (1991) A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. *Cell* **65**: 13–24.

干细胞之家www.stemcell8.cn ←点击进入

Guzman ML, Rossi RM, Karnischky L, *et al*. (2005) The sesquiterpene lactone parthenolide induces apoptosis of human acute myelogenous leukemia stem and progenitor cells. *Blood* **105**: 4163–69.

Jin L, Hope KJ, Zhai Q, *et al*. (2006) Targeting of CD44 eradicates human acute myeloid leukemic stem cells. *Nat Med* **12**: 1167–74.

Jordan CT, Upchurch D, Szilvassy SJ, *et al*. The interleukin-3 receptor alpha chain is a unique marker for human acute myelogenous leukemia stem cells. *Leukemia* **14**: 1777–84.

Kallakury BV, Yang F, Figge J, *et al*. (1996) Decreased levels of CD44 protein and mRNA in prostate carcinoma. Correlation with tumor grade and ploidy. *Cancer* **78**: 1461–69.

Kaya G, Rodriguez I, Jorcano JL, *et al*. (1997) Selective suppression of CD44 in keratinocytes of mice bearing an antisense CD44 transgene driven by a tissue-specific promoter disrupts hyaluronate metabolism in the skin and impairs keratinocyte proliferation. *Genes Dev* **11**: 1996–2007.

Kim HR, Wheeler MA, Wilson CM, *et al*. (2004) Hyaluronan facilitates invasion of colon carcinoma cells *in vitro* via interaction with CD44. *Cancer Res* **64**: 4569–76.

Krause DS, Lazarides K, von Andrian UH, Van Etten RA. (2006) Requirement for CD44 in homing and engraftment of BCR-ABL-expressing leukemic stem cells. *Nat Med* **12**: 1175–80.

Kuci S, Wessels JT, Buhring HJ, *et al*. (2003) Identification of a novel class of human adherent CD34- stem cells that give rise to SCID-repopulating cells. *Blood* **101**: 869–76.

Lamb RF, Hennigan RF, Turnbull K, *et al*. (1997) AP-1-mediated invasion requires increased expression of the hyaluronan receptor CD44. *Mol Cell Biol* **17**: 963–76.

Lapidot T, Dar A, Kollet O. (2005) How do stem cells find their way home? *Blood* **106**: 1901–10.

Legras S, Gunthert U, Stauder R, *et al*. (1998) A strong expression of CD44-6v correlates with shorter survival of patients with acute myeloid leukemia. *Blood* **91**: 3401–13.

Li C, Heidt DG, Dalerba P, *et al*. (2007) Identification of pancreatic cancer stem cells. *Cancer Res* **67**: 1030–37.

Liu AY, True LD, LaTray L, *et al*. (1999) Analysis and sorting of prostate cancer cell types by flow cytometry. *Prostate* **40**: 192–99.

Liu G, Yuan X, Zeng Z, *et al*. (2006a) Analysis of gene expression and chemoresistance of CD133+ cancer stem cells in glioblastoma. *Mol Cancer* **5**: 67.

Liu S, Dontu G, Mantle ID, *et al*. (2006b) Hedgehog signaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells. *Cancer Res* **66**: 6063–71.

Liu R, Wang X, Chen GY, *et al*. (2007) The prognostic role of a gene signature from tumorigenic breast-cancer cells. *N Engl J Med* **356**: 217–26.

Liu Y, Han SS, Wu Y, *et al*. (2004) CD44 expression identifies astrocyte-restricted precursor cells. *Dev Biol* **276**: 31–46.

干细胞之家www.stemcell8.cn ←点击进入

Lopez JI, Camenisch TD, Stevens MV, *et al*. (2005) CD44 attenuates metastatic invasion during breast cancer progression. *Cancer Res* **65**: 6755–63.

Masellis-Smith A, Belch AR, Mant MJ, Pilarski LM. (1997) Adhesion of multiple myeloma peripheral blood B cells to bone marrow fibroblasts: a requirement for CD44 and alpha4beta7. *Cancer Res* **57**: 930–36.

Mori H, Tomari T, Koshikawa N, *et al*. (2002) CD44 directs membrane-type 1 matrix metalloproteinase to lamellipodia by associating with its hemopexin-like domain. *EMBO J* **21**: 3949–59.

Murakami D, Okamoto I, Nagano O, *et al*. (2003) Presenilin-dependent gamma-secretase activity mediates the intramembranous cleavage of CD44. *Oncogene* **22**: 1511–16.

Nagabhushan M, Pretlow TG, Guo YJ, *et al*. (1996) Altered expression of CD44 in human prostate cancer during progression. *Am J Clin Pathol* **106**: 647–51.

Nagano O, Murakami D, Hartmann D, *et al*. (2004) Cell-matrix interaction via CD44 is independently regulated by different metalloproteinases activated in response to extracellular Ca(2+) influx and PKC activation. *J Cell Biol* **165**: 893–902.

O'Brien CA, Pollett A, Gallinger S, Dick JE. (2007) A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature* **445**: 106–10.

Ohashi R, Takahashi F, Cui R, *et al*. (2007) Interaction between CD44 and hyaluronate induces chemoresistance in non-small cell lung cancer cell. *Cancer Lett*. Epub ahead of print.

Oishi K, Ito-Dufros Y. (2006) Angiogenic potential of CD44+ CD90+ multipotent CNS stem cells *in vitro*. *Biochem Biophys Res Commun* **349**: 1065–72.

Omara-Opyene AL, Qiu J, Shah GV, Iczkowski KA. (2004) Prostate cancer invasion is influenced more by expression of a CD44 isoform including variant 9 than by Muc18. *Lab Invest* **84**: 894–907.

Paradis V, Eschwege P, Loric S, *et al*. (1998) *De novo* expression of CD44 in prostate carcinoma is correlated with systemic dissemination of prostate cancer. *J Clin Pathol* **51**: 798–802.

Patrawala L, Calhoun T, Schneider-Broussard R, *et al*. (2005) Side population (SP) is enriched in tumorigenic, stem-like cancer cells whereas $ABCG2^+$ and $ABCG2^-$ cancer cells are similarly tumorigenic. *Cancer Res* **65**: 6207–19.

Patrawala L, Calhoun T, Schneider-Broussard R, *et al*. (2006) Highly purified CD44+ prostate cancer cells from xenograft human tumors are enriched in tumorigenic and metastatic progenitor cells. *Oncogene* **25**: 1696–708.

Patrawala L, Calhoun-Davis T, Schneider-Broussard R, Tang DG. (2007) Hierarchical organization of prostate cancer cells in xenograft tumors: the $CD44^+\alpha2\beta1^+$ cell population is enriched in tumor-initiating cells. *Cancer Res*. In press.

Phillips TM, McBride WH, Pajonk F. (2006) The response of CD24(−/low)/CD44+ breast cancer-initiating cells to radiation. *J Natl Cancer Inst* **98**: 1777–85.

Piccirillo SG, Reynolds BA, Zanetti N, *et al*. (2006) Bone morphogenetic proteins inhibit the tumorigenic potential of human brain tumour-initiating cells. *Nature* **444**: 761–65.

干细胞之家www.stemcell8.cn ←点击进入

Ponta H, Sherman L, Herrlich PA. (2003) CD44: from adhesion molecules to signalling regulators. *Nat Rev Mol Cell Biol* **4**: 33–45.

Ponti D, Costa A, Zaffaroni N, *et al*. (2005) Isolation and *in vitro* propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. *Cancer Res* **65**: 5506–11.

Prince ME, Sivanandan R, Kaczorowski A, *et al*. (2007) Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. *Proc Natl Acad Sci USA* **104**: 973–78.

Protin U, Schweighoffer T, Jochum W, Hilberg F. (1999) CD44-deficient mice develop normally with changes in subpopulations and recirculation of lymphocyte subsets. *J Immunol* **163**: 4917–23.

Ravandi F, Estrov Z. (2006) Eradication of leukemia stem cells as a new goal of therapy in leukemia. *Clin Cancer Res* **12**: 340–44.

Reya T, Morrison SJ, Clarke MF, Weissman IL. (2001) Stem cells, cancer, and cancer stem cells. *Nature* **414**: 105–11.

Reuss-Borst MA, Buhring HJ, Klein G, Muller CA. (1992) Adhesion molecules on CD34+ hematopoietic cells in normal human bone marrow and leukemia. *Ann Hematol* **65**:169–74.

Ricci-Vitiani L, Lombardi DG, Pilozzi E, *et al*. (2007) Identification and expansion of human colon-cancer-initiating cells. *Nature* **445**: 111–15.

Schmits R, Filmus J, Gerwin N, *et al*. (1997) CD44 regulates hematopoietic progenitor distribution, granuloma formation, and tumorigenicity. *Blood* **90**: 2217–33.

Screaton GR, Bell MV, Jackson DG, *et al*. (1992) Genomic structure of DNA encoding the lymphocyte homing receptor CD44 reveals at least 12 alternatively spliced exons. *Proc Natl Acad Sci USA* **89**: 12160–64.

Sheridan C, Kishimoto H, Fuchs RK, *et al*. (2006) CD44+/CD24− breast cancer cells exhibit enhanced invasive properties: an early step necessary for metastasis. *Breast Cancer Res* **8**: R59.

Shipitsin M, Campbell LL, Argani P, *et al*. (2007) Molecular definition of breast tumor heterogeneity. *Cancer Cell* **11**: 259–73.

Singh SK, Hawkins C, Clarke ID, *et al*. (2004) Identification of human brain tumour initiating cells. *Nature* **432**: 396–401.

Stamenkovic I, Aruffo A, Amiot M, Seed B. (1991) The hematopoietic and epithelial forms of CD44 are distinct polypeptides with different adhesion potentials for hyaluronate-bearing cells. *EMBO J* **10**: 343–48.

Studeny M, Marini FC, Champlin RE, *et al*. (2002) Bone marrow-derived mesenchymal stem cells as vehicles for interferon beta delivery into tumors. *Cancer Res* **62**: 3603–608.

Suetsugu A, Nagaki M, Aoki H, *et al*. (2006) Characterization of CD133+ hepatocellular carcinoma cells as cancer stem/progenitor cells. *Biochem Biophys Res Commun* **351**: 820–24.

Sutherland HJ, Blair A, Zapf RW. (1996) Characterization of a hierarchy in human acute myeloid leukemia progenitor cells. *Blood* **87**: 4754–61.

干细胞之家www.stemcell8.cn ←点击进入

Tang DG, Patrawala L, Calhoun T, *et al*. (2007) Prostate cancer stem/progenitor cells: identification, characterization, and implications. *Mol Carcinogenesis* **46**: 1–14.

Uchida N, Buck DW, He D, *et al*. (2000) Direct isolation of human central nervous system stem cells. *Proc Natl Acad Sci USA* **97**: 14720–25.

Weber GF, Ashkar S, Glimcher MJ, Cantor H. (1996) Receptor-ligand interaction between CD44 and osteopontin (Eta-1). *Science* **271**: 509–12.

Yin S, Li J, Hu C, *et al*. (2007) CD133 positive hepatocellular carcinoma cells possess high capacity for tumorigenicity. *Int J Cancer* **120**: 1436–42.

Yu Q, Toole BP, Stamenkovic I. (1997) Induction of apoptosis of metastatic mammary carcinoma cells *in vivo* by disruption of tumor cell surface CD44 function. *J Exp Med* **186**: 1985–96.

Zhu H, Mitsuhashi N, Klein A, *et al*. (2006) The role of the hyaluronan receptor CD44 in mesenchymal stem cell migration in the extracellular matrix. *Stem Cells* **24**: 928–35.

干细胞之家www.stemcell8.cn ←点击进入

Chapter 13

Neural Stem Cell-mediated Therapy of Primary and Metastatic Solid Tumors

Joseph Najbauer*,†, Mary K. Danks§, Nils-Ole Schmidt¶, Seung U. Kim‖ and Karen S. Aboody*,†,‡

The poor prognosis for patients with metastatic cancer and the toxic side effects of currently available treatments necessitate the development of more effective tumor-selective therapies. Dosage of systemically administered chemotherapeutic agents is limited by the toxicity to normal tissues, often precluding achievement of therapeutic indices of sufficient levels to affect complete cures. Malignant brain tumors pose further limitations to available therapies due to the infiltrative nature of the tumor cells throughout the brain and the presence of the blood–brain barrier. Novel anti-cancer treatment approaches must be more tumor-localized and tumor cell-selective to improve effectiveness and clinical outcome.

Neural stem (and/or progenitor) cells (NSCs) display inherent tumor-tropic properties that can be exploited for targeted delivery of anti-cancer agents to invasive and metastatic tumors. We and others have previously demonstrated that NSCs can deliver bioactive therapeutic agents to elicit a significant anti-tumor response in animal models of intracranial glioma, medulloblastoma and melanoma brain metastases. Recent studies demonstrate retention of tumor-tropic properties when NSCs are injected into the peripheral vasculature, localizing to multiple tumor sites in animal models of disseminated neuroblastoma and orthotopic breast carcinoma, with little accumulation in normal tissues. We postulate that this NSC-mediated, tumor-selective approach can maximize local concentrations of anti-cancer agents

*Corresponding authors. JNajbauer@coh.org, KAboody@coh.org
†Division of Hematology/Hematopoietic Cell Transplantation and
‡Division of Neurosciences, City of Hope National Medical Center and Beckman Research Institute, Duarte, CA, USA
§Department of Molecular Pharmacology, St. Jude Children's Research Hospital, Memphis, TN, USA
¶Department of Neurosurgery, University Medical Center Hamburg-Eppendorf, Hamburg, Germany
‖Division of Neurology, UBC Hospital, University of British Columbia, Vancouver, Canada; and Institute for Regenerative Medicine, Gachon University, Inchon, Korea

干细胞之家www.stemcell8.cn ←点击进入

to tumor foci, while minimizing toxicity to normal tissues. This would potentially achieve therapeutic indices sufficient to eradicate invasive tumors that are otherwise lethal.

Several well-characterized immortalized murine and human NSC lines have been extensively studied for the determination of their therapeutic potential in animal tumor models of glioblastoma, melanoma brain metastases, medulloblastoma, and disseminated neuroblastoma. In all invasive and metastatic solid tumor models, 70–90% therapeutic efficacy was achieved as measured by increased long-term survival or decreased tumor burden. Other studies in the literature demonstrated the achievement of similar results with nonimmortalized NSC pools modified with various therapeutic genes, further verifying the NSC tumor tropism phenomenon. However, these pools are more difficult to keep consistent over time and passage, adding more variability to pre-clinical trials and more difficulty in creating uniform master cell banks for clinical trials.

We suggest that using a stable, sustainable and easily expandable clonal NSC line will circumvent the problems associated with characterization, senescence, and replenishment sources of primary stem cell pools. It would also allow for cost-effective and accessible patient trials. At the very least, such NSC lines can serve in proof-of-concept pre-clinical studies, where tumor types and therapeutic regimens are being tested. Preliminary biodistribution studies indicate that one such immortalized human NSC line (HB1.F3) is safe, non-immunogenic and non-tumorigenic. Furthermore, these cells retain their tumor-tropic property when modified to express therapeutic transgenes. Genomic stability and retention of tumor-tropic properties over time and passage has also been confirmed, making it a very promising line for clinical trials.

Regardless of which NSCs move towards therapeutic cancer trials, safety and feasibility would be best assessed first in patients with no alternative treatments, such as recurrent high-grade gliomas. These aggressive tumor cells infiltrate normal brain, and are not readily treatable by resection, irradiation, chemotherapy or gene therapy. In rodent models of orthotopic human brain tumors, it has been demonstrated that HB1.F3 NSCs can selectively target invasive tumor cells and distant micro tumor foci, achieving therapeutic efficacy with an enzyme/prodrug strategy. This serves as one example of many different anti-cancer agents that could be similarly delivered to tumor sites throughout the body.

Keywords: Neural stem cells; cancer therapy; gene therapy; targeted therapy; glioma; medulloblastoma, melanoma, neuroblastoma, breast cancer, cytosine deaminase, carboxylesterase, 5-fluorocytosine, 5-FC, 5-fluorouracil, 5-FU, irinotecan, CPT-11, SN-38; prodrug-activating systems.

1. Introduction

1.1. *The Challenges of Effective Cancer Treatment*

The poor prognosis for patients with metastatic cancer and the toxic side effects of currently available treatments necessitate the development of more effective tumor-selective therapies. The molecular basis for treatment failure is undoubtedly multi-factorial, but depends partly on the intrinsic sensitivity of each tumor type to specific therapeutic approaches. Additionally, dosage

干细胞之家www.stemcell8.cn ←点击进入

of systemically administered treatments is limited by the toxicity to normal tissues, often precluding achievement of therapeutic indices of sufficient levels to affect complete cures. Novel anti-cancer treatment approaches require improvement in tumor cell selectivity to improve effectiveness and clinical outcome. Limiting toxicity to normal tissues would also provide improved quality of life during treatment regimens.

Malignant brain tumors present additional treatment difficulties due to their location and invasive nature. Treatment failure of gliomas is largely attributable to the inability of current strategies to effectively address the diffuse and highly infiltrative nature of these tumors. Extensive surgical removal of the tumor can lead to permanent damage to nervous tissue and does not eliminate cancer cells that have migrated throughout the normal brain parenchyma. Drug delivery has been ineffective due to the inability of many chemotherapeutic drugs to efficiently cross the blood–brain barrier and/or poor diffusion of drugs within the brain and within the tumor (Minchinton and Tannock, 2006). In addition, systemic toxicity of traditional chemotherapeutic agents limits their usefulness. The inability to deliver therapeutic agents to where they are needed similarly decreases the effectiveness of gene therapy. Cell-mediated vector delivery is limited by the ability of the carrier cell line to migrate with sufficient distribution throughout primary and distant tumor sites. Immunotherapeutic strategies have also failed to show significant impact at the clinical level, largely due to their inability to eradicate disseminated tumor foci throughout the brain (Ehtesham *et al.*, 2005). Despite aggressive treatments, the recurrence rate for glioma is 98%, with 80% occurring within 2 cm of the resection cavity, and with a median survival of 3–6 months (Hochberg and Pruitt, 1980; Stupp *et al.*, 2005; Price *et al.*, 2007).

1.2. *Stem Cell-mediated Gene Therapy: A Tumor-selective Therapeutic Approach*

Stem cells hold the promise of improved cancer treatment without the current limitations of traditional surgical therapies, radio therapies and chemotherapies. Utilized as a delivery vehicle to target therapeutic gene products to tumor sites, stem/progenitor cells may overcome the major challenges facing current gene therapy strategies, by allowing effective delivery and distribution of a therapeutic agent throughout the tumor mass and selectively to metastatic tumor foci. Neural stem cells (NSCs), neural progenitor cells (NPCs) and mesenchymal stem cells (MSCs) from bone marrow or adipose tissue migrate *in vivo* to sites of pathology, including tumors

干细胞之家www.stemcell8.cn ←点击进入

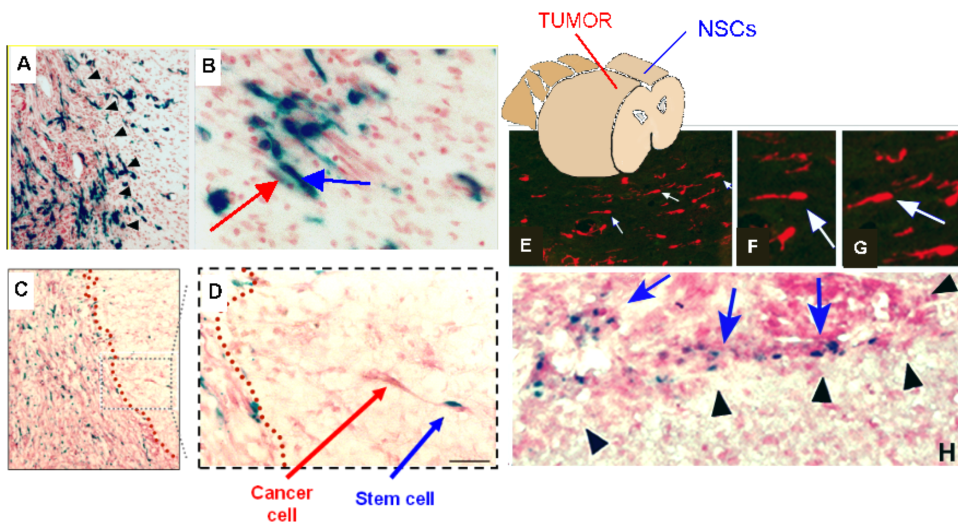


Fig. 1. NSCs target invading tumor cells and distant tumor sites. **(A,B)** Administered directly into the tumor in a preclinical orthotopic glioma model, NSCs (blue, blue arrows) distribute throughout the main tumor mass and co-localize with islands of brain tumor cells (red, red arrows). **(C,D)** NSC (blue) "tracking" single invasive tumor cell (red) infiltrating into normal tissue away from primary tumor mass. **(E–H)** When injected into the contralateral hemisphere, NSCs (blue) migrate across white matter tracks [(E–G), red] to enter tumor [(H), red] on opposite side. Based on Figs. 2 and 3 from: Aboody *et al.*, *Proc Natl Acad Sci USA* **97**: 12846–12851 (2000) with permission from the National Academy of Sciences (USA).

(Aboody *et al.*, 2000; Brown *et al.*, 2003; Kabos *et al.*, 2003; Studeny *et al.*, 2004; Aboody *et al.*, 2006a and 2006b). NSCs in particular have demonstrated inherently robust migratory and tumor-tropic properties that can be exploited for targeted delivery and distribution of anti-cancer agents to invasive and metastatic tumors. An example in orthotopic glioma models is shown with the well-characterized C17.2 clonal, murine NSC line (Fig. 1).

The tumor tropism of these types of cells appears to be independent of immune response, as evident in studies using syngeneic and immunodeficient animal tumor models. The molecular mechanism responsible for the tumor tropism of stem and progenitor cells is poorly understood, but it is unlikely that species specificity plays a dominant role since cells of murine origin migrate to tumors derived from humans, rats or mice (Aboody *et al.*, 2000; Brown *et al.*, 2003; Aboody *et al.*, 2006a). Further, NSCs of human or murine origin migrate to numerous invasive tumor types including melanoma, glioblastoma, medulloblastoma, prostate and breast carcinoma, and neuroblastoma in pre-clinical tumor models (Aboody *et al.*, 2000; Brown *et al.*, 2003; Kabos *et al.*, 2003; Studeny *et al.*, 2004; Aboody *et al.*, 2006a and 2006b; Kim *et al.*, 2006a). These studies demonstrate NSC localization to

干细胞之家www.stemcell8.cn ←点击进入

metastases throughout the body, including the liver, ovaries, and bone marrow, with little accumulation in nontumor tissues. Our laboratories have been investigating the possibility that the tumor tropism of NSCs might be exploited to deliver therapeutic cDNA products selectively to tumor foci, to maximize therapeutic efficacy and minimize toxic side effects in metastatic solid tumor models both inside and outside the brain. Specifically, we have evaluated the potential use of v-*myc*–immortalized murine and human clonal NSC lines (C17.2 and HB1.F3, respectively) as delivery vehicles for therapeutic cDNAs including interferon-β (Dickson *et al.*, 2007), cytosine deaminase (Aboody *et al.*, 2000 and 2006a; Kim *et al.*, 2006a) and carboxylesterase (Aboody *et al.*, 2006b; Danks *et al.*, 2007). We most recently reported the long-term efficacy for the latter approach, documenting unchanged plasma carboxylesterase activities and active drug (SN-38) levels within ranges tolerated by pediatric patients (Furman *et al.*, 1999; Ma *et al.*, 2000; Furman *et al.*, 2006) at doses of prodrug (CPT-11) that produced 1-year disease-free survival in 90% of NSC-treated mice. Other laboratories have achieved significant results with NSCs modified to express alternative therapeutic transgenes in various animal tumor models (summarized in Table 1). These results are promising for extending NSC applications to other types of invasive solid tumors and eventual clinical use.

1.3. *Use of Clonal, Immortalized NSC Lines for Tumor-selective Therapy*

1.3.1. *Advantages of clonal NSC Lines*

Immortalized NSC lines, generated by the introduction of oncogenes (e.g., v-*myc*), have demonstrated advantageous characteristics for pre-clinical cell therapy and gene therapy studies (Snyder *et al.*, 1992; Hoshimaru *et al.*, 1996; Martinez-Serrano and Bjorklund, 1997; Martinez-Serrano *et al.*, 2001; Yip *et al.*, 2003; Muller *et al.*, 2006; Kim, 2007). Advantages of well-characterized, clonal NSC lines over primary and autologous stem cell pools include: (1) stable NSC lines can be readily expanded to large numbers in culture, negating the need for a constant replenishment source of cells/tissue; (2) NSC lines are generated from a single clone and comprise an expandable line that can be specifically characterized for genomic stability and retention of tumor tropism over time and passage, providing a constant variable across pre-clinical studies; (3) stable transgene expression can be readily achieved by retroviral (Aboody *et al.*, 2000; Brown *et al.*, 2003) or lentiviral transduction (Aboody, unpublished data), and high transient transgene expression

Table 1 Genetically Modified NSCs Demonstrate Tumor Localization and/or Therapeutic Efficacy, as Measured by a Significant Decrease in Tumor Burden and/or Increased Long-term Survival, in Pre-clinical Tumor Models.

NSC Type (Source)	Route of Administration	Tumor Type (Source) Location	Tumor Host	Therapeutic Transgene	Therapeutic Efficacy	References
C17.2 (mouse)	Intratumoral	CNS-1 (rat glioma) Intracranial	*Nu/nu* mouse	HSV-1 vector	Not tested	Herrlinger *et al.* (2000)
C57.npr.IL-4 (mouse)	Intratumoral	GL261 (mouse glioma) Intracranial	C57BL/6J mouse	IL-4	Extended survival	Benedetti *et al.* (2000)
ST14A.IL-4.3 (rat)	Intratumoral	C6 (rat glioma) Intracranial	Sprague — Dawley rat	IL-4	Extended survival	Benedetti *et al.* (2000)
C17.CD2 (mouse)	Intracranial — C17.2 + CNS-1	CNS-1 (rat glioma) Intracranial	*Nu/nu* mouse	CD (5-FC i.p.)	~ 80% reduction in tumor volume	Aboody *et al.* (2000)
NSC-IL-12 (mouse)	Intratumoral	GL26 (mouse glioma) Intracranial	Sprague — Dawley rat	IL-12	Extended survival	Ehtesham *et al.* (2002a)
NSC-TRAIL (mouse)	Intracranial/ Intratumoral	U343MG (human glioma) Intracranial	*Nu/nu* mouse	TRAIL	Tumor size reduction	Ehtesham *et al.* (2002b)
ST14A (rat)	Intracranial — ST14A mixed with C6 cells	C6 (rat glioma) Intracranial	Sprague–Dawley rat	CD (5-FC i.p.)	~ 50% reduction in tumor growth	Barresi *et al.* (2003)

(*Continued*)

Table 1 (*Continued*)

NSC Type (Source)	Route of Administration	Tumor Type (Source) Location	Tumor Host	Therapeutic Transgene	Therapeutic Efficacy	References
C17.2, C17.CD2 (mouse)	Intravascular	CNS-1 (rat glioma) U-251 (human glioma) SMHM3 (human melanoma) PC3 (human prostate cancer) SH-SY5Y (human neuroblastoma) Intracranial, Subcutaneous	*Nu/nu* mouse	N/A	Distribution study	Brown *et al.* (2003)
NPC-FLsTRAIL C17.2 (mouse)	Intracranial	Gli36-RL (human glioma) Intracranial	*Nu/nu* mouse	S-TRAIL	>80% reduction in tumor growth	Shah *et al.* (2005b)
HB1.F3-PEX (human)	Intracranial	U87MG (human glioma) Intracranial	Swiss nude mouse	PEX	90% reduction in tumor volume	Kim *et al.* (2005)
BM-NSC-IL23 (mouse)	Intracranial	GL261 (mouse glioma)	C57BL/6, *Nu/nu*, CD4 T-cell KO	IL-23	Extended survival	Yuan *et al.* (2006)
C17.CD2 (mouse)	Intracranial	B16/F10 (mouse melanoma) Intracranial	C57BL/6J mouse	CD (5-FC i.p.)	∼ 70% reduction in tumor volume	Aboody *et al.* (2006a)

(*Continued*)

Table 1 (*Continued*)

NSC Type (Source)	Route of Administration	Tumor Type (Source) Location	Tumor Host	Therapeutic Transgene	Therapeutic Efficacy	References
HB1.F3.CD (human)	Intratumoral	Daoy (human medulloblastoma) Intracranial	Nu/nu mouse	CD (5-FC i.p.)	76% reduction in tumor volume	Kim *et al.* (2006a)
HB1.F3.rCE (human)	Intravascular (tail vein)	NB-1691, NB-1643, SK-N-AS (human neuroblastoma) Disseminated	Es1^{e} SCID mice	CE (CPT-11 i.v.)	100% tumor-free survival for >6 months	Aboody *et al.* (2006b)
HB1.F3.rCE (human)	Intravascular (tail vein)	NB-1691, NB-1643, SK-N-AS (human neuroblastoma) Disseminated	Es1^{e} SCID mice	CE (CPT-11 i.v.)	90% tumor-free survival for 1 year	Danks *et al.* (2007)
HB1.F3. IFN-β (human)	Intravascular (tail vein)	NB-1691 (human neuroblastoma) Disseminated	Es1^{e} SCID mice	IFN-β (i.v.)	Significant tumor reduction/ Slowed disease progression	Dickson *et al.* (2007)
HB1.F3 (human)	Intracranial	U251, D566 (human glioma) Intracranial	Nu/nu mouse	N/A	Distribution study	Lin *et al.* (2007)

Abbreviations: 5-FC, 5-fluorocytosine; 5-FU, 5-fluorouracil; BM-NSC, bone marrow-derived neural stem-like cells; CD, cytosine deaminase; i.p., intraperitoneal; i.v., intravenous; IFN-β, interferon-β; PEX, a naturally-occurring fragment of human metalloproteinase-2; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; S-TRAIL, secreted TRAIL; CPT-11, irinotecan; rCE, rabbit carboxylesterase (CPT-11–activating enzyme).

干细胞之家www.stemcell8.cn ←点击进入

by adenoviral transduction (Aboody *et al.*, 2006b; Danks *et al.*, 2007); (4) a well-characterized, tested parental line can be engineered to express a variety of therapeutic gene products that can then be used sequentially or in combination to hit multiple tumor targets for enhanced treatment strategies; and (5) clonal expandability allows for the generation of a Master Cell Bank and the development of an off-the-shelf treatment, which would significantly decrease costs and increase availability for patient trials.

In recent studies, the c-*myc* gene (cellular homolog of v-*myc*) has been shown to contribute to long-term maintenance of the stem cell phenotype (Cartwright *et al.*, 2005; Takahashi and Yamanaka, 2006), suggesting that it serves as a stemness gene and that v-*myc* introduced into primary neural stem/progenitor cells may similarly act as a stemness gene, leading to the long-term maintenance of stem cell characteristics of immortalized NSCs such as the human HB1.F3 cells (see below).

Of significance, immortalized NSCs have emerged as a highly effective source for *ex vivo* manipulation, and for transplantation into various focal and global CNS disease models for delivery of therapeutic gene products and/or integration into damaged or developing host brain (Renfranz *et al.*, 1991; Snyder *et al.*, 1992; Martinez-Serrano and Bjorklund, 1997; Flax *et al.*, 1998). The current literature demonstrates the use of v-*myc*–immortalized NSCs both as a vehicle to target therapeutic agents to tumors (Aboody *et al.*, 2000; Brown *et al.*, 2003; Kim *et al.*, 2005; Aboody *et al.*, 2006b) and for regenerative purposes in pre-clinical models of Huntington's disease (Ryu *et al.*, 2004; Goldman, 2005; Lee *et al.*, 2005 and 2006; Kim, 2007), Parkinson's disease (Ourednik *et al.*, 2002; Kim, 2004; Goldman, 2005; Ryu *et al.*, 2005; Kim *et al.*, 2006b), cerebral ischemia (Jeong *et al.*, 2003; Chu *et al.*, 2004; Imitola *et al.*, 2004; Kim, 2004; Muller *et al.*, 2006) and spinal cord injury (Kim *et al.*, 2002; Goldman, 2005; Sykova and Jendelova, 2005; Kim, 2007).

1.3.2. *Disadvantages of primary (non-immortalized/clonal) NSC pools*

Our own culture studies of NSCs/neurospheres derived from human fetal brain tissue indicate that continuous passage over time results in decreased proliferative capacity and increased terminal differentiation into neurons or glial cells (S. Kim, unpublished data). In addition, primary pools are problematic for clinical applications because: (1) they show drift of cell population, proliferation rate and differentiation ability over time and across conditions, making them difficult to characterize and necessitating a constant replenishment source of cells/tissue and retesting; (2) individual cell characteristics are unstable over passage and time, with cell population drift

干细胞之家www.stemcell8.cn ←点击进入

adding undesired variability to pre-clinical studies; and (3) instability of cell pools and limited expansion make them impractical for generating a Master Cell Bank.

1.3.3. *Disadvantages of autologous stem cells*

Like primary NSCs, the use of autologous stem cells has a number of disadvantages, including: (1) they have questionable availability and time course to expand autologous cultures; (2) identification and testing of autologous stem cell sources that will efficiently target tumors has not been done; (3) these cells have limited expansion capabilities in cultures; (4) autologous cells may contain the genetic defect that originally facilitated tumor development; and (5) this type of therapy encompasses considerably higher costs per treatment, and would be less widely available.

1.3.4. *HB1.F3 NSCs as potential off-the-shelf human tumor-homing cell line*

We suggest that using a stable, sustainable and easily expandable clonal NSC line will circumvent the problems associated with characterization, senescence and replenishment sources of primary stem cell pools. It would also allow for cost-effective and accessible patient trials. At the very least, they may serve as standard NSC lines in proof-of-concept pre-clinical studies, where tumor types and therapeutic regimens are being tested.

One such immortalized human NSC line (HB1.F3), derived by v-*myc* immortalization of human fetal telencephalon cells, has been extensively studied and characterized. With various genetic modifications, these NSCs have demonstrated selective tumor localization (to invasive tumor cells and distant tumor microfoci) and/or therapeutic efficacy in pre-clinical models of glioma (Kim *et al.*, 2005), medulloblastoma (Kim *et al.*, 2006a), disseminated neuroblastoma (Aboody *et al.*, 2006b; Danks *et al.*, 2007), and breast carcinoma (Aboody *et al.*, unpublished data). In all invasive and metastatic solid tumor models, 70–90% therapeutic efficacy was achieved as measured by increased long-term survival or decreased tumor burden. HB1.F3 NSCs retain their tumor-tropic property when modified to express various transgenes. Because of their highly specific tumor-targeting ability, HB1.F3 and other clonal NSC lines hold great promise for successful localized therapy for glioma and other malignant cancer patients. Within the framework of carefully designed and monitored clinical trials, the likely benefits appear much greater than the potential risk factors. This has yet to be shown.

干细胞之家www.stemcell8.cn ←点击进入

2. Literature Summary

We and others have used NSCs that have been modified to express a variety of therapeutic transgenes to target tumors in numerous pre-clinical models. These experimental trials are summarized in Table 1.

3. Generation of Immortalized Human HB1.F3 Neural Stem Cells

Human fetal telencephalon (15-week gestation) brain cells were dissociated into single cells through trypsin treatment and grown in serum-free medium consisting of Dulbecco's modified Eagle medium (DMEM) supplemented with insulin, transferrin, selenium, hydrocortisone, triiodothyotrophin, antioxidants and basic fibroblast growth factor (Ryu *et al.*, 2003; Kim *et al.*, 2004). Neurospheres formed within three days in culture, comprised of small aggregates of cells including neurons, astrocytes and neural stem/progenitor cells. Within seven days in culture, many neurospheres adhered to the surface of the plastic culture dish, displaying extensive outgrowth of neuronal or glial processes. Neurons and neural stem cells migrated from the neurosphere core and grew in a monolayer on top of cellular sheets of astrocytes. An amphotropic replication-incompetent retroviral vector encoding v-*myc*, pLSN.v-*myc*, was used to infect human telencephalon cells [Fig. 2(A)]. This method was previously used to generate v-*myc*-immortalized, clonal murine NSC lines (Snyder *et al.*, 1997 and 1992; Ryder *et al.*, 1990). Following G418 selection, large clusters of cells were individually isolated and grown in poly-L-lysine coated dishes [Fig. 2(B)]. Further clonal selection by limited dilution yielded several propagated NSC lines, including HB1.F3 and HB1.F5, used in our studies. The HB1.F3 clone expressed v-*myc*, as detected by RT-PCR and Western blotting [Fig. 2(C)], and displayed a normal 46XX karyotype [Fig. 2(D)]. Gene expression analysis of HB1.F3 human NSCs, carried out by RT-PCR, showed mRNAs for ABCG2 and nestin, type-specific markers of NSCs. In addition, HB1.F3 cells expressed specific receptors involved in cellular migration including CXCR4, VEGFR-1, VEGFR-2, c-Kit and EGFR (Lee *et al.*, 2005).

HB1.F3 cells appear to replicate for a maximum of ~48 h following *in vivo* administration, as assessed by immunostaining for Ki-67, PCNA and BrdU. Downregulation of the NSC propagating gene product v-*myc* occurs constitutively and spontaneously, becoming undetectable 48 h following *in vivo* engraftment (Flax *et al.*, 1998). These data suggest that v-*myc* is regulated by the normal developmental mechanisms that downregulate endogenous

干细胞之家www.stemcell8.cn ←点击进入

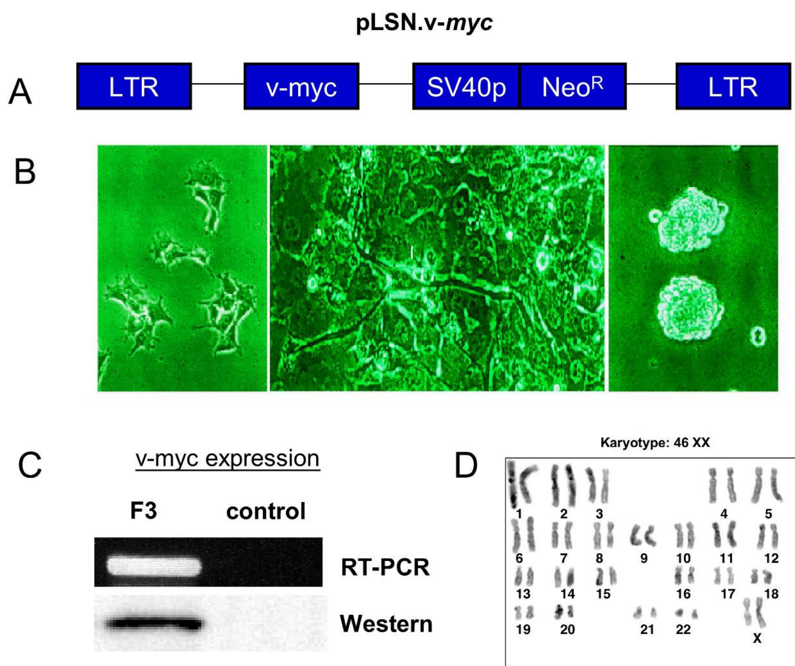


Fig. 2. Isolation of human NSC line HB1.F3. **(A)** HB1.F3 cells were immortalized using a retrovirus vector (pLSN.v-*myc*, modified from pLCNX, which was purchased from Clonetech) carrying the v-*myc* gene. Cells were selected for neomycin resistance. The producer line was PA317. **(B)** HB1.F3 cells shown under phase contrast microscopy. **(C)** v-*myc* expression was confirmed by RT-PCR and Western blotting. **(D)** The karyotype displayed normal 46 XX chromosomes. Reproduced from: Kim, *Neuropathology*, **24**: 159–171 (2004) with permission of the Japanese Society of Neuropathology and Blackwell Publishing.

cellular *myc* in CNS precursors during mitotic arrest and/or differentiation. The loss of v-*myc* expression from murine and human NSC lines following intraparenchymal or intravascular administration is consistent with the absence of tumors in short- and long-term studies in rodents (Danks *et al.*, 2007; Aboody *et al.*, 2006a and 2006; Kim *et al.*, 2006a and 2005; Lee *et al.*, 2005; Jeong *et al.*, 2004; Chu *et al.*, 2004; Snyder *et al.*, 1992) and primates (Redmond *et al.*, 2007).

4. Introduction of Therapeutic Genes into Neural Stem Cells

NSCs can be engineered to express various anti-tumor gene products, including prodrug-activating enzymes, apoptosis-inducing or cell differentiating agents, cell cycle modulators, anti-angiogenesis factors, immune-enhancing agents and oncolytic factors. Additionally, these engraftable

干细胞之家www.stemcell8.cn ←点击进入

migratory NSCs may also be engineered to serve as viral vector producer cells, allowing extended delivery of lethal virus-mediated genes to tumor cells. When administered into the tumor-bearing animal with any of these strategies, NSCs can target their therapeutic "payload" selectively to invasive tumor sites, thereby increasing localization of therapy and minimizing exposure to normal tissues.

4.1. *Cytosine Deaminase*

The principal advantage of a directed enzyme/prodrug approach compared to conventional chemotherapy is its potential to achieve tumor cell-selective cytotoxicity. One example of this therapeutic strategy uses NSCs modified to express the prodrug-activating enzyme *E. coli* cytosine deaminase (HB1.F3.CD). This CD-expressing NSC line was generated from the v-*myc*-immortalized HB1.F3 parental line, using an amphotropic replication-incompetent retroviral vector encoding *E. coli* cytosine deaminase (CD; EC 3.5.4.1), transcribed from the retrovirus LTR plus *puromycin*^{R} transcribed from an internal SV40 early promoter. Following puromycin selection and cloning by limited dilution, selected CD-expressing NSC lines were established and expanded for further characterization. The HB1.F3.CD21 line has been used in most of our studies. This line stably expresses the functional CD enzyme, which converts 5-fluorocytosine (5-FC) into 5-fluorouracil (5-FU), a pyrimidine analog that acts as an anti-neoplastic anti-metabolite by interfering with DNA synthesis, blocking the thymidylate synthetase conversion of deoxyuridylic acid to thymidylic acid (Fig. 3). Expression of CD in HB1.F3.CD cells was confirmed by FACS and immunofluorescence microscopy, and the karyotype was also tested for chromosomal stability (Aboody *et al.*, unpublished data). The HB1.F3.CD NSCs retain their inherent tumor-tropic properties, thereby localizing the prodrug-activating CD enzyme to primary and metastatic tumor sites (Fig. 4). Systemically administered 5- FC crosses the blood–brain barrier, is converted to the active cytotoxic 5-FU by CD expressed in the NSCs, and readily diffuses out to selectively kill surrounding, dividing tumor cells (Fig. 4). This CD/5-FC prodrug strategy is known to have a large bystander effect (Huber *et al.*, 1994). In effect, the NSCs serve as a platform for the production of tumor-localized chemotherapy. This allows the achievement of concentrated drug levels selectively at tumor sites, minimizing toxicity associated with systemic admistration. In regards to brain tumors, this strategy circumvents the inability of 5-FU and other therapeutic agents to cross the blood–brain barrier (see Sec. 6 for *in vivo* results).

干细胞之家www.stemcell8.cn ←点击进入

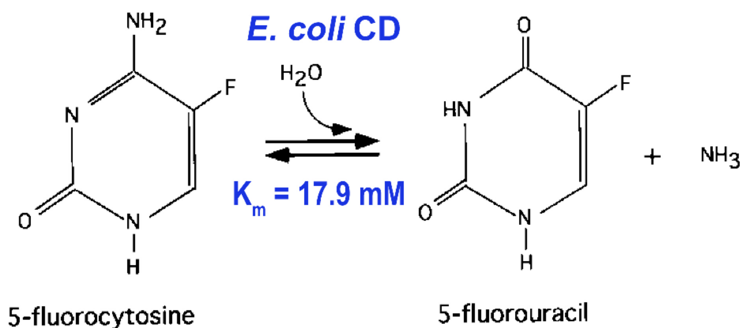


Fig. 3. Conversion of 5-fluorocytosine prodrug to 5-fluorouracil anti-cancer drug by cytosine deaminase. The K_m value of the *E. coli* CD enzyme for 5-FC is 17.9 mM (Springer and Niculescu-Duvaz, 2000; Ireton *et al.*, 2002).

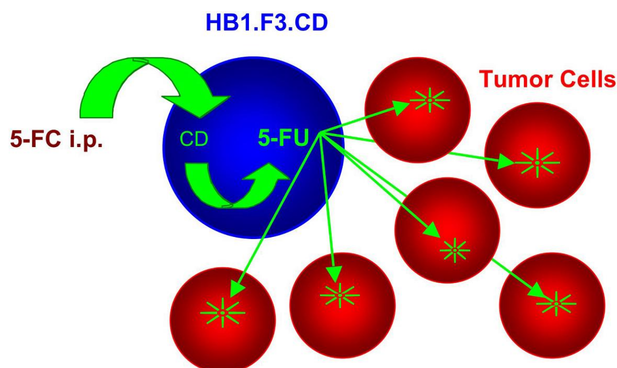


Fig. 4. Production of 5-FU chemotherapeutic agent localized to immediate vicinity of stem cells. NSCs expressing the enzyme *E. coli* cytosine deaminase (HB1.F3.CD) migrate to tumor sites. Systemically administered prodrug 5-FC crosses the blood–brain barrier and is converted to 5-FU by the CD in the NSCs. 5-FU and its toxic metabolites readily diffuse out of the cell to selectively kill dividing tumor cells. This CD enzyme/5-FC prodrug strategy is known to have a large bystander effect, predicting that one NSC can kill off many surrounding tumor cells.

4.2. *Carboxylesterase*

Another enzyme/prodrug strategy, useful in neuroblastoma and perhaps other metastatic solid tumor models, uses intravascularly administered NSCs engineered to express the activating enzyme rabbit carboxylesterase (rCE), which efficiently converts the prodrug CPT-11 (irinotecan) to the active metabolite SN-38, a potent topoisomerase I inhibitor (Fig. 5). Using a disseminated neuroblastoma mouse model, we postulated that rCE-secreting NSCs (HB1.F3.AdCMVrCE) would localize selectively to disseminated tumor foci throughout the body. The secreted rCE enzyme at tumor

干细胞之家www.stemcell8.cn ←点击进入

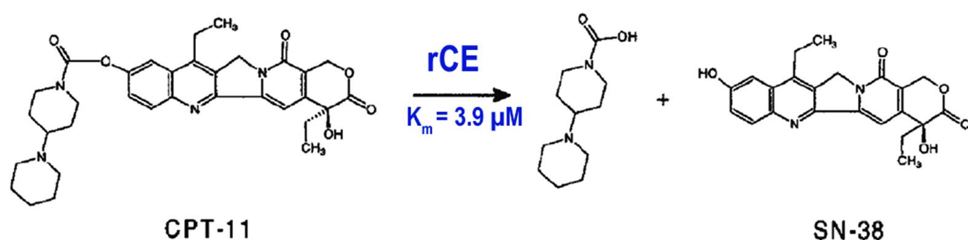


Fig. 5. Metabolic activation of CPT-11 to SN-38 (a potent topoisomerase inhibitor) by esterases (Guichard *et al.*, 1998; Pommier, 2006). Rabbit carboxylesterase (rCE) and its K_m for CPT-11 are shown.

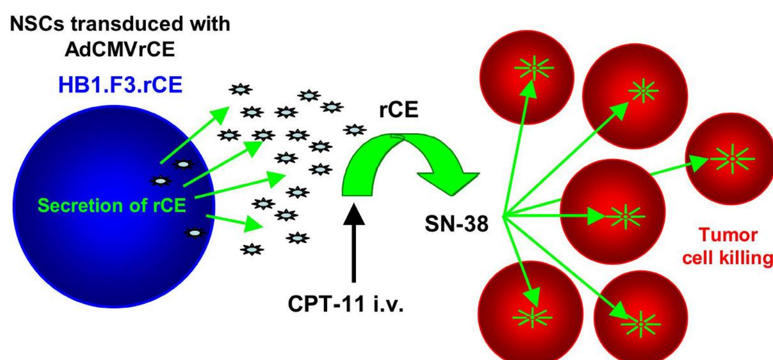


Fig. 6. HB1.F3.AdCMVrCE neural stem cells migrate to tumor sites and secrete rabbit carboxylesterase (rCE). The secreted rCE is a prodrug-activating enzyme produced locally in the tumor by stem cells which have accumulated in the tumor. The CPT-11 prodrug is then administered systemically, and becomes converted to the active cytotoxic drug SN-38 by rCE locally in the tumor tissue. SN-38 is a potent topoisomerase inhibitor that selectively kills surrounding dividing tumor cells.

sites activates systemically administered CPT-11 to the highly chemotoxic agent SN-38, leading to selective killing of dividing cancer cells (Fig. 6) (see Sec. 6b for *in vivo* results).

Replication-deficient adenovirus expressing a secreted form of rabbit carboxylesterase (rCE; EC 3.1.1.1) under the control of the cytomegalovirus (CMV) promoter (AdCMVrCE) was constructed using standard methods, as described previously (Danks *et al.*, 1998; Potter *et al.*, 1998; Danks *et al.*, 1999). HB1.F3 cells were co-cultured with AdCMVrCE for 24 h prior to intravenous injection. The enzyme activity assay used to quantitate the level of expression of rCE by HB1.F3 cells transduced with adenovirus, and the enzymatic mechanisms of CPT-11 prodrug activation (Fig. 5) have been reported previously (Guichard *et al.*, 1998; Danks *et al.*, 1999; Pommier, 2006).

干细胞之家www.stemcell8.cn ←点击进入

4.3. *Other Therapeutic Agents*

4.3.1. *Interleukins*

The genes of various cytokines have been introduced into NSCs to treat experimental brain tumors in mice and rats. SV40 large T antigen-immortalized primary mouse NSCs were transduced with the gene for interleukin 4 (*IL-4*) (Benedetti *et al.*, 2000), a cytokine that promotes T-cell–mediated immune response against tumor cells (Faber *et al.*, 2000). In another study, C57BL6J mouse primary NSCs were retrovirally transduced to express the *IL-4* gene and these NSCs were used to treat mice with established syngeneic GL261 brain glioblastomas (Benedetti *et al.*, 2000). This treatment led to a 90-day survival of $>80\%$ of mice when tumor cells were co-injected with IL-4–secreting NSCs, and 70% of mice survived when IL-4–secreting NSCs were injected into mice with already established tumors (all tumor-bearing, untreated mice died by day 30). Similar results were obtained by implanting immortalized NSCs derived from Sprague–Dawley rats into C6 glioblastomas (Benedetti *et al.*, 2000). IL-12–secreting NSCs that had been derived from the fronto-parietal region of embryonic day 15 mice and transduced with adenovirus vector carrying an IL-12 expression cassette were used for treatment of glioblastoma-bearing mice. The treatment with NSC–IL-12 cells led to significantly prolonged survival and resulted in long-term anti-tumor immunity, as compared to animals treated with nonmodified NSCs (Jean *et al.*, 1998; Ehtesham *et al.*, 2002a). Bone marrow-derived neural stem-like cells (BM-NSCs) were used to express and deliver interleukin-23 (IL-23), which showed protective effects on intracranial tumor-bearing C57BL/6 mice (Yuan *et al.*, 2006). Depletion of $CD8^+$ T cells showed that this subpopulation of lymphocytes was critical for the anti-tumor immunity elicited by IL-23–expressing BM-NSCs, and that $CD4^+$ T cells and natural killer cells also participated in the anti-tumor activity. Importantly, the IL-23–expressing BM-NSC–treated survivors were resistant to tumor re-challenge associated with enhanced IFN-gamma, but not IL-17, expression in the brain tissue. These data suggest that IL-23–expressing BM-NSCs can induce anti-tumor immunity against intracranial gliomas.

4.3.2. *TRAIL*

TRAIL (TNF-α-related apoptosis-inducing ligand, or APO-2L) is a member of the TNF ligand family (Pitti *et al.*, 1996). TRAIL is trimeric and binds to

干细胞之家www.stemcell8.cn ←点击进入

DR4 or DR5 death receptors, and induces their trimerization which leads to recruitment of the death-inducing signaling complex (DISC). Formation of DISC induces activation of caspases and downstream intrinsic death signaling pathways (Walczak and Krammer, 2000). TRAIL can induce apoptosis in numerous tumor cells, including gliomas (Pollack *et al.*, 2001; Shah *et al.*, 2003), while being relatively nontoxic to most normal cells (Ashkenazi *et al.*, 1999); thus, it has attracted great interest as a cancer therapeutic agent. NSCs derived from embryonic day 15 mice were transduced with an adenoviral expression vector for human TRAIL, and were used in experiments on intracranial glioma (Ehtesham *et al.*, 2002b). Inoculation of human glioblastoma xenografts with TRAIL-secreting NSCs resulted in dramatic induction of apoptosis in treated tumors and tumor satellites, and was associated with significant inhibition of tumor growth (Ehtesham *et al.*, 2002b). For more efficient protein delivery, a secretable version of TRAIL (S-TRAIL) was designed and introduced into NSCs to deliver S-TRAIL directly to invasive tumor cells (Shah *et al.*, 2004). For monitoring of highly malignant gliomas and migration of NSCs, cell lines were also engineered with luminescent and fluorescent transgenes for dual bioluminescence imaging (Shah *et al.*, 2005a and 2005b). A highly malignant human glioma model expressing *Renilla* luciferase was used. Intracranially implanted NSCs expressing both firefly luciferase and S-TRAIL were shown to migrate into the gliomas and have significant anti-tumor effects (>80% reduction in tumor growth) (Shah *et al.*, 2005a). More recent results show that simultaneous triggering of the TRAIL-mediated death receptor pathway and downregulation of *Bcl-2* by shRNA leads to highly enhanced eradication of gliomas in experimental animals (Kock *et al.*, 2007).

4.3.3. *PEX*

The human metalloproteinase-2 fragment, PEX, acts as an inhibitor of glioma and endothelial cell proliferation, migration and angiogenesis (Brooks *et al.*, 1998; Bello *et al.*, 2001). The anti-tumor activity of PEX-producing human NSCs against malignant glioma was investigated using HB1.F3 cells in mouse models of human glioma. HB1.F3-PEX cells labeled with CM-DiI migrated to the tumor boundary and caused 90% reduction of tumor volume (Kim *et al.*, 2005). The reduction in tumor volume in HB1.F3-PEX–treated animals was associated with a decrease in angiogenesis and cell proliferation, supporting the use of NSCs as a delivery vehicle for targeting therapeutic genes to human gliomas.

干细胞之家www.stemcell8.cn ←点击进入

4.3.4. *Interferon-β*

NSC-mediated interferon-β therapy has been investigated in aminal models of human neuroblastoma (Dickson *et al.*, 2007). Disseminated neuroblastoma was established in SCID mice by tail vein injection of tumor cells. Mice were treated by intravenous injection of human HB1.F3 NSCs which had been transduced with a replication-deficient adenoviral vector to express hIFN-β (HB1.F3–IFN-β). Control mice received NSCs transduced with empty vector adenovirus. Progression of disease was monitored using bioluminescence imaging, which demonstrated a significant decrease in disease progression in mice receiving HB1.F3–IFN-β cells. Necropsy of control mice revealed bulky tumor in multiple organs, while the organs of mice receiving HB1.F3–IFN-β therapy appeared normal with the exception of small residual tumor nodules within the kidneys. Importantly, systemic levels of hIFN-β in the treated mice remained below detectable levels, suggesting that the therapeutic effect was due to hIFN-β secreted by NSCs at tumor sites. This NSC-mediated IFN-β therapy may circumvent limitations associated with systemic toxicity of IFN-β.

5. Tropism of Neural Stem Cells to Tumors *In vitro*

5.1. *HB1.F3 NSCs Migrate to Tumor-conditioned Medium*

The migratory capabilities of the immortalized human HB1.F3 line was examined in response to conditioned medium from human glioma (U251, U251.eGFP, U251.ffluc), breast carcinoma (MCF7, MDA) and neuroblastoma (SK-N-AS) cell lines. Modified Boyden chamber assays were used to assess the *in vitro* tropism of HB1.F3 cell lines to tumor cell-conditioned media or to control media, using standard methods. Briefly, HB1.F3 cells were detached by trypsinization, washed and resuspended in DMEM containing 5% BSA. NSCs (3×10^4 cells/100 μL) were placed in upper chambers and tumor cell-conditioned medium in lower chambers (10% FCS was used as positive control for chemoattraction). Tumor cell-conditioned medium in both the upper and lower chambers comprised the chemokinesis control without a chemoattractant gradient. Cells were allowed to migrate for 4 h in a cell culture incubator at 37°C and 6% CO_2. The number of migrated HB1.F3 cells in the lower chamber of each well was quantitated using CyQuant GR fluorescent dye (Chemicon) and a fluorescence microplate reader (Molecular Devices, Sunnyvale, CA). Assays were performed in triplicate.

Tumor-conditioned media from all tumor cell lines investigated (U251, SK-N-AS, MCF7, MDA) significantly stimulated the directional migration of

干细胞之家www.stemcell8.cn ←点击进入

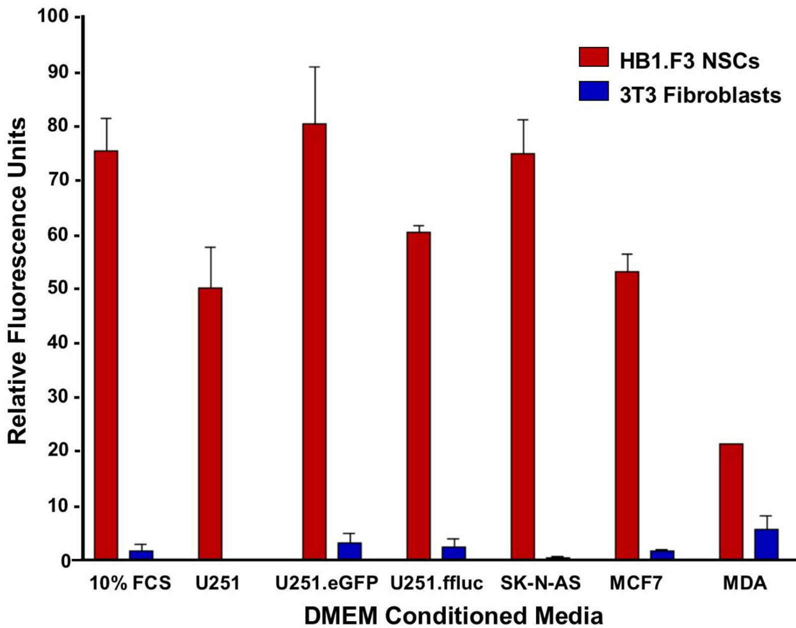


Fig. 7. NSC migration to tumor-conditioned media. HB1.F3 cell migration in response to conditioned media from human tumor lines, including U251 glioma, U251.eGFP, U251.ffluc, SK-N-AS neuroblastoma, MCF7 and MDA breast carcinoma. Note the lack of migration of control 3T3 fibroblasts (Aboody *et al.*, unpublished data).

the NSCs compared to 3T3 cells (Fig. 7). In control chemokinesis assay, minimal cell migration was detected, confirming NSC migration due to chemotaxis (data not shown).

5.2. *Neural Stem Cells Transduced with Therapeutic or Reporter Genes Retain Their Tumor-tropic Properties*

Insertion of the *E. coli* cytosine deaminase gene into the HB1.F3 parental line was successfully achieved by retroviral transduction. Directed migration in response to conditioned media from human tumor cell lines (UPN029, U251, U87 and U87ffLuc) was assessed in 96-well Boyden chamber assays (Fig. 8). The results demonstrated that insertion of the CD transgene into NSCs has no significant effect on migratory and tumor-tropic properties, supporting their use for delivery of CD directly to tumor sites.

Furthermore, HB1.F3 cells transduced with adenovirus carrying the rCE prodrug activating enzyme gene (MOI of 0, 5, 10 or 20 AdCMVrCE), or lentivirus carrying the eGFP reporter gene, also showed retention of tumor

干细胞之家www.stemcell8.cn ←点击进入

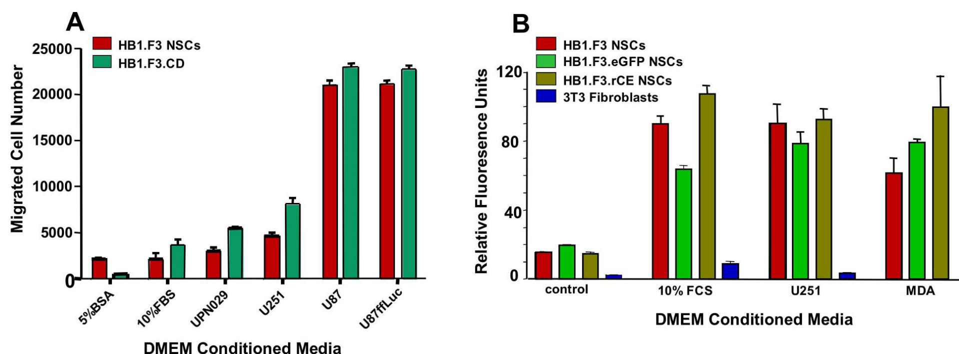


Fig. 8. NSCs retain their tropism for tumor-conditioned media (Boyden chamber assays). **(A)** HB1.F3 at passage 12 (dark red) and HB1.F3.CD cells (green; retroviral transduction) at p20 retained their migratory properties to media conditioned by a variety of glioma cell lines (UPN029, U251 and U87$^{+/-}$ ffLuc). **(B)** Migratory properties of HB1.F3 cells (red) were retained following lentiviral transduction with a reporter gene (HB2.F3.eGFP; green) or adenoviral transduction with a therapeutic gene encoding the prodrug activating enzyme rCE (HB1.F3.AdCMVrCE; olive). Control 3T3 fibroblasts (blue) showed little or no migration.

tropism in Boyden chamber migration assays to U251 glioma and MDA breast carcinoma cell-conditioned media [Fig. 8(B)].

6. NSC-Mediated Cancer Therapy: Pre-Clinical Tumor Models

6.1. *Brain Tumor Models*

6.1.1. *Glioma*

Treatment failure of gliomas is largely attributable to the inability of current strategies to address the diffuse and highly infiltrative nature of these tumors. Due to extensive dissemination of malignant glial cells away from the primary site, there is no clear border between normal brain and tumor edge, with invasive cells and distant microfoci proving exceptionally difficult to treat with surgical and radiotherapies. Drug delivery has been ineffective due to the inability of many drugs to cross the blood–brain barrier and/or poor diffusion of drugs within the brain and within the tumor. In addition, systemic toxicity of traditional chemotherapeutic agents limits their usefulness. An inability to deliver therapeutic agents with sufficient distribution throughout primary and distant tumor sites limits the effectiveness of gene therapy. Immunotherapeutic strategies have also failed to show significant impact at the clinical level, largely due to their inability to eradicate disseminated tumor foci throughout the brain (Ehtesham *et al.*, 2005). Despite aggressive treatments, the recurrence rate for glioma is 98%, with

干细胞之家www.stemcell8.cn ←点击进入

80% occurring within 2 cm of the resection cavity and with a median survival of 3–6 months. To improve the clinical outcome, novel therapies must address the invasive tumor cells that escape currently available treatments, while limiting toxicity to normal tissues.

In vitro tumor cell killing by NSCs expressing the prodrug activating enzyme CD. We determined the sensitivity of the U87 human glioma cell line to the 5-FU anti-cancer drug and 5-FC prodrug *in vitro*. The half-maximal killing effect was detected at $\sim 5\,\mu g/mL$ 5-FU whereas $500\,\mu g/mL$ of 5-FC was necessary to achieve the same effect, showing the high potency of 5-FU compared to its precursor prodrug 5-FC (MTT assay). When U87 glioma cells were co-cultured *in vitro* at increasing ratios of murine NSCs expressing the active CD enzyme (C17.2.lacZ.CD), increased tumor cell killing was observed in the presence of 5-FC prodrug, but not in its absence. However, only 5% of C17.2.lacZ.CD stem cells were necessary to induce a tumor cell growth inhibition of ~50%, indicating a strong bystander effect of CD-expressing NSCs (Schmidt *et al.*, unpublished data). We observed a similar tumor cell killing effect by HB1.F3.CD human NSCs when co-cultured with U251 human glioma cells in the presence of 5-FC prodrug (Aboody *et al.*, unpublished data).

Therapeutic effectiveness of tumor-homing NSCs expressing the CD transgene. We then investigated the *in vivo* therapeutic effectiveness of glioma-targeting NSCs expressing CD to convert systemically administered prodrug 5-FC to 5-FU. 5-FU and its toxic metabolites diffused out of the NSCs to selectively kill dividing tumor cells in their vicinity (Fig. 4). Murine C17.2.lacZ.CD NSCs (or C17.2.lacZ as a control) were stereotactically implanted distant to established orthotopic U87 glioma xenografts in adult nude mice. Systemic treatment with 5-FC (500 mg/kg/day i.p. ×2 weeks) was initiated three days after implantation of NSCs (Fig. 9).

Intracerebral implantation of C17.2.lacZ.CD cells caudal-lateral to the tumor site, followed by systemic administration of 5-FC, significantly inhibited tumor growth (>65%) relative to controls (CD-expressing NSCs only, control NSCs +5-FC, tumor only) as assessed by magnetic resource imaging (MRI) 14 days after the beginning of treatment (Fig. 10). The anti-tumor effect of NSC-mediated, tumor-localized CD enzyme/5-FC prodrug chemotherapy resulted in a significant increase of survival of treated glioma-bearing mice (Schmidt *et al.*, unpublished data). These results support the use of NSCs as a novel tumor-selective delivery vehicle to target therapeutic gene products to invasive glioma foci.

干细胞之家www.stemcell8.cn ←点击进入

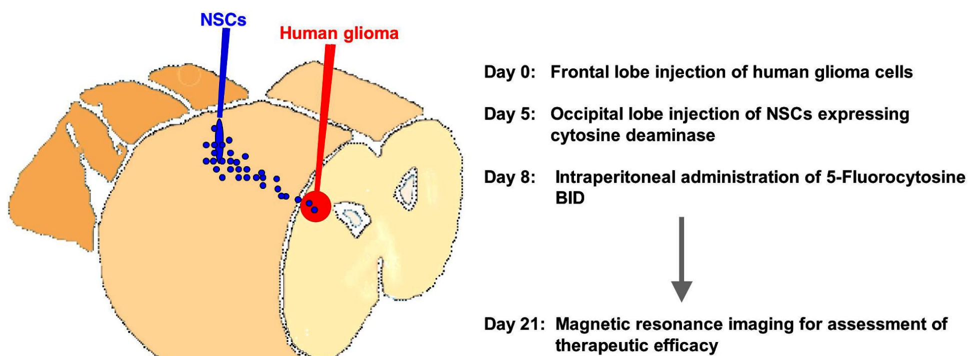


Fig. 9. Therapeutic homing and anti-glioma activity of CD-NSCs. Murine C17.2.lacZ.CD (expressing CD) or control C17.2.lacZ (no CD) NSCs were stereotactically implanted distant to established orthotopic U87 human glioma xenografts in adult nude mice. Systemic treatment with 5-FC at 500 mg/kg/day started three days after NSC injection, and continued for 14 days. (Schmidt *et al.*, unpublished data).

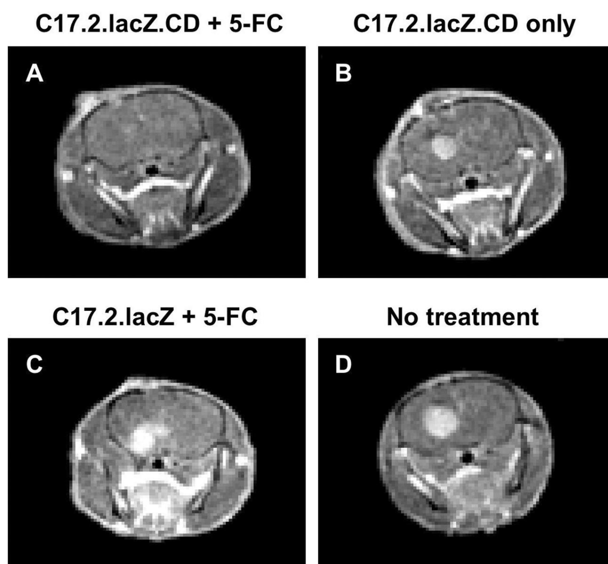


Fig. 10. Tumor growth assessed by T1-weighted, gadolinium-enhanced magnetic resonance imaging. Note the significantly reduced hypodense signal (indicating reduced tumor size) in representative mouse treated with C17.2.lacZ.CD NSCs and 5-FC prodrug **(A)**, when compared to brains of control mice **(B–D)** (Schmidt *et al.*, unpublished data).

Visualization and modeling of spatial distribution of neural stem cells within intracranial glioma. We used an orthotopic mouse model of human glioma, high-resolution fluorescent confocal microscopy, and three-dimensional (3D) modeling to develop an algorithm that allows the following

干细胞之家www.stemcell8.cn ←点击进入

analyses: (1) estimation of primary tumor boundary and centroid; (2) identification of cancer cells and NSCs in the tumor; (3) determination of coordinates and 3D distribution of NSCs and (4) estimation of tumor volume that would be eradicated by NSCs (using a cell-killing radius) with a defined intratumoral and surrounding microfoci distribution. U251.eGFP human glioma was established in the brain of athymic mice (for 1 or 2 weeks), followed by sterotactic injection of CM-DiI-labeled HB1.F3 NSCs posterior-lateral to the tumor site. This novel algorithm allowed 3D visualization of tumor cells and NSCs in and around the tumor site (Fig. 11). Furthermore, this model predicted that a single administration of NSCs would affect the

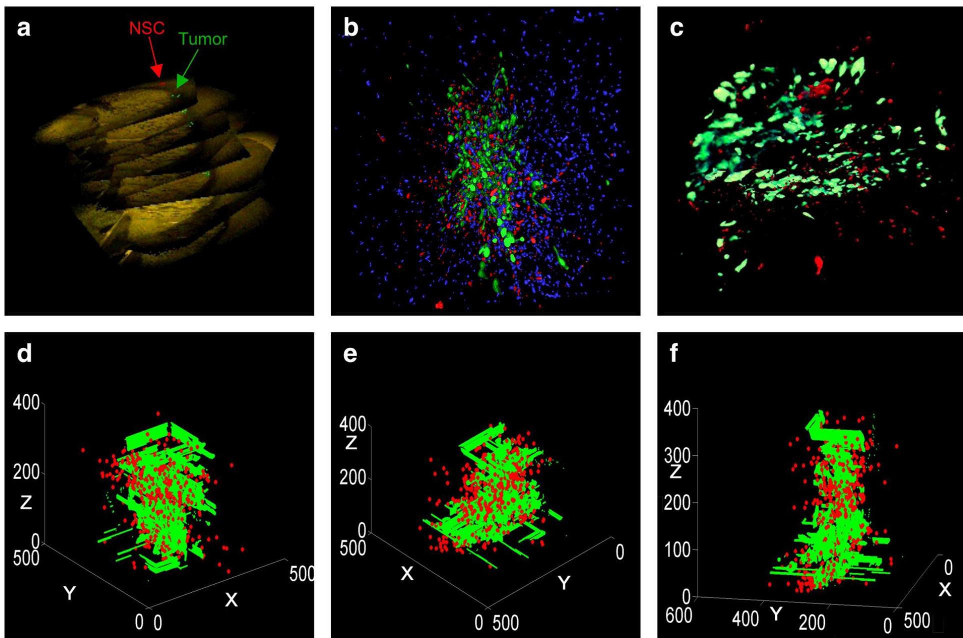


Fig. 11. 3D reconstructions show extent of NSC distribution in primary glioma mass. **(a)** 3D AMIRA model of aligned 2.5× serial images at 100 μm intervals. The NSC injection site (red) and tumor site (green) (*inset*) can be traced downward through the image series. Tumors were established via orthotopic injection of U251.eGFP glioma cells, followed by posterior-lateral stereotactic injection of CM-DiI-labeled NSCs 7 days [Mouse A; (**b–f**)] or 14 days (Mouse B) after implantation of U251.eGFP cells. **(b)** AMIRA surface reconstruction of tumor bulk in Mouse A showing NSCs (red), tumor cells (green) and nuclei (blue). **(c)** AMIRA voxel projection of tumor in Mouse A showing NSCs (red) and tumor (green). **(d–f)** MATLAB plots of tumor boundary and NSC locations in Animal A shown in various orientations; NSCs (red) and tumor mass (green). Based on Fig. 4 from: Lin *et al.*, *Neuroimage*, Epub ahead of print (2007) with permission of Elsevier.

干细胞之家www.stemcell8.cn ←点击进入

vast majority (70–90%) of tumor cells within the primary mass and surrounding infiltrative tumor foci. This method may provide a quantitative framework for projecting the therapeutic potential of a given NSC-expressed transgene in glioma or other solid tumor treatment.

6.1.2. *Medulloblastoma*

Medulloblastoma is a malignant pediatric brain tumor that is incurable in about 30% of patients despite multimodal therapies. The main cause of death is recurrence associated with tumor dissemination, at which point current therapeutic options have little efficacy (Patrice *et al.*, 1995; Graham *et al.*, 1997). Consequently, there is substantial need for novel, effective, low-toxicity therapies for children with medulloblastoma. Based on the inherent tropism of NSCs toward malignant gliomas (Aboody *et al.*, 2000; Yip *et al.*, 2003) and the secretion of growth factors and chemokines common to glioma and medulloblastoma, we hypothesized that NSCs could also target therapeutic gene products to medulloblastoma.

We investigated the migratory ability of HB1.F3.CD NSCs to medulloblastoma both *in vitro* and *in vivo*. The migratory capacity of HB1.F3 cells was confirmed by an *in vitro* cell migration assay, and then corroborated *in vivo* by injecting CM-DiI–labeled HB1.F3 cells into the hemisphere contralateral to established medulloblastoma tumors in nude mice (Kim *et al.*, 2006a). *In vitro* studies showed a tumor cell-killing effect of HB1.F3.CD NSCs on Daoy medulloblastoma cells in co-culture experiments. For *in vivo* therapeutic studies, animals bearing intracranial medulloblastoma were injected ipsilaterally with HB1.F3.CD cells followed by systemic 5-FC treatment. Histologic analysis demonstrated HB1.F3.CD NSC migration to the tumor bed and its boundary and, following systemic 5-FC treatment, resulted in a 76% reduction of tumor volume compared to the control groups ($P < 0.01$) (Kim *et al.*, 2006a). These studies showed for the first time the potential of human NSCs as an effective delivery system to target and disseminate therapeutic agents to medulloblastoma.

6.1.3. *Solid tumor metastases to brain: melanoma*

The most common type of brain tumors are those that develop as distant metastases from solid tumors in other organs, such as lung, prostate, breast or melanoma. Metastatic tumors to the brain occur 10 times more frequently than primary brain tumors (Hutter *et al.*, 2003; Patchell, 2003). Malignant melanoma is the third most common cause of intracranial metastases (Saha *et al.*, 1994). Each year, more than 100,000 patients in the United States are

干细胞之家www.stemcell8.cn ←点击进入

diagnosed with brain metastases; and although treatment for systemic disease has been improving, drug delivery to brain metastases remains ineffective. Although solitary metastasis can be surgically removed or treated with stereotactic radiosurgery, patients with multiple brain metastases have very limited therapeutic options (Westphal *et al.*, 2003). NSC-mediated therapy shows promise to overcome these current treatment limitations, due to their inherent ability to migrate throughout the brain parenchyma and target tumor sites, including intracerebral solid tumor metastases.

Therapeutic paradigm for NSC-mediated CD enzyme/5-FC prodrug strategy in a syngeneic model of melanoma brain metastases. For our *in vivo* therapeutic paradigm, we established melanoma brain metastases by intracarotid injection of B16/F10 melanoma cells in a syngeneic mouse model (Fig. 12). One week later, animals received rostral and caudal intracranial injections of murine C17.2.CD NSCs (Aboody *et al.*, 2006a). On day 4 following NSC injections, systemic 5-FC treatment was given for eight days. Results demonstrated a significant reduction in resultant tumor burden (> 70%) in CD-expressing NSC treated animals, as compared to controls (non-CD expressing NSCs +5-FC, tumor only) (Fig. 13). These data provide proof of principle for the use of NSCs for targeted delivery of therapeutic gene products to melanoma brain metastases.

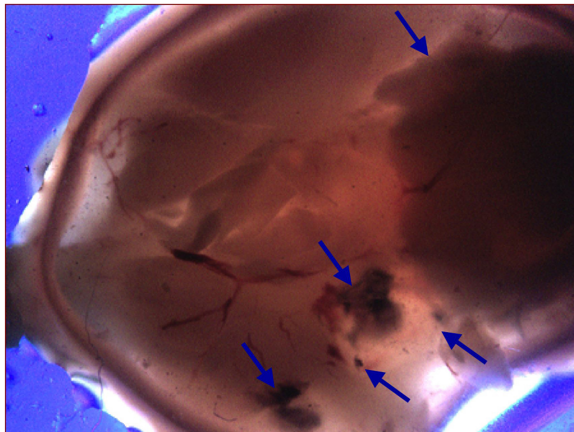


Fig. 12. *In vivo* syngeneic experimental model of melanoma metastases to the brain. The trans-illuminated whole brain shows a wide range of sizes of pigmented melanoma metastases 19 days after injection of melanoma cells (arrows) into the carotid artery. Scale bar, 2 mm. Based on Fig. 2 from: Aboody *et al.*, *Neuro-Oncol* **8**: 119–126 (2006) with permission of the Society for Neuro-Oncology.

干细胞之家www.stemcell8.cn ←点击进入

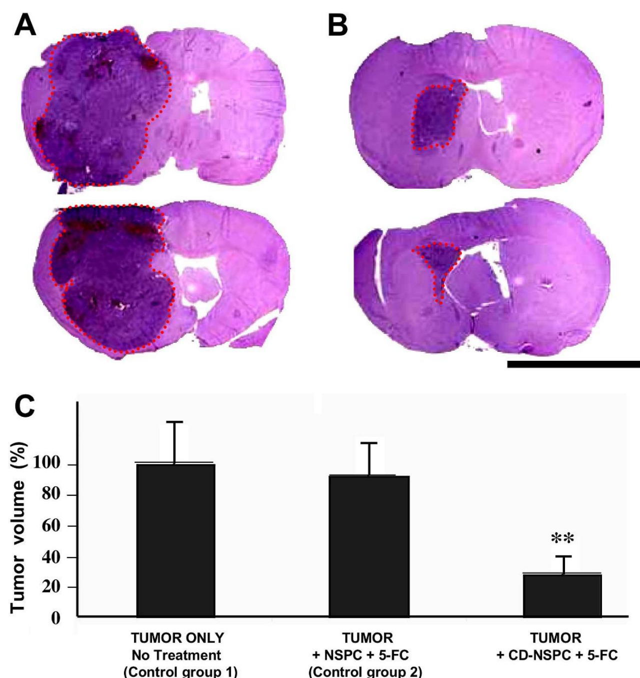


Fig. 13. *In vivo* schematic for therapeutic paradigm of C17.2.CD cells +5-FC for treatment of brain metastases of melanoma. Tumor areas (outlined in red) in representative brains of mice that **(A)** had received injections of melanoma cells only, and **(B)** had received injections of melanoma cells and were subsequently treated with CD-NSCs and 5-FC. Scale bar, 5 mm. **(C)** Quantitative analysis of tumor volume showing 71% and 69% less tumor burden in mice that had been treated with CD-NSCs and 5-FC when compared to control group 1 (tumor only, no treatment) or control group 2 (tumor + NSCs + 5-FC), respectively (mean ± SD; Student's t-test; $n = 4$ in each group). Tumor volumes were expressed as a percentage of control group 1 tumor (no treatment was taken as 100%). All mice were sacrificed 19 days following tumor injection. Based on Fig. 4 from: Aboody *et al.*, *Neuro-Oncol* **8**: 119–126 (2006) with permission of the Society for Neuro-Oncology.

6.2. *Disseminated Solid Tumor Models: Neuroblastoma*

Neuroblastoma is the most common extracranial solid tumor in children. Typically, patients diagnosed with high-risk disease demonstrate a good initial response to therapy, but as many as 80% of these patients relapse with metastatic disease that is refractory to therapy. Like other solid tumors, when neuroblastomas metastasize, they are very difficult to treat, and a majority of children with high-risk metastatic neuroblastoma die of their disease (Evans *et al.*, 1994; Matthay *et al.*, 1994; Brodeur, 2003). Current treatments have anti-tumor efficacy but also produce undesirable side effects to normal tissue, limiting the treatment dose that can be administered. Novel effective

干细胞之家www.stemcell8.cn ←点击进入

approaches for the treatment of neuroblastoma are needed. In the following studies, we exploited the tumor-tropic property of NSCs to selectively localize to multiple solid tumor foci outside the brain, following intravascular administration. The specific goal of these studies was to determine if intravenous administration of HB1.F3 cells expressing a secreted form of the CPT-11 (irinotecan)-activating enzyme rCE (HB1.F3.AdCMVrCE; see Fig. 6) would significantly increase the anti-tumor effect of tolerated doses of CPT-11 in mice bearing disseminated neuroblastoma tumors. Since no effective treatments are available for most metastatic tumors, demonstrating that stem or progenitor cells of fetal or adult origin can be used to improve the prognosis of patients with fatal metastatic disease would be highly significant. The approach described might also be adapted to developing a treatment for patients with other types of metastatic solid tumors and/or residual disease.

6.2.1. *In vitro killing of neuroblastoma cells by NSCs secreting rabbit carboxylesterase*

In order to determine whether the HB1.F3.AdCMVrCE cells are capable of killing neuroblastoma cells, we carried out *in vitro* cytotoxicity assays. HB1.F3.AdCMVrCE stem cells efficiently killed SK-N-AS.ffLuc neuroblastoma cells in the presence of CPT-11 prodrug, with half-maximal cytototoxicity achieved at $\sim 0.1\ \mu$M CPT-11 (Fig. 14).

6.2.2. *HB1.F3 NSCs target disseminated human neuroblastoma foci in liver, ovary and bone of SCID mice*

To investigate whether HB1.F3 stem cells can target human neuroblastoma tumors *in vivo*, we administered CM-DiI–labeled HB.F3 cells via tail vein injection to mice with established human NB1643 neuroblastoma tumors. Immunohistochemical analysis revealed significant homing of HB1.F3 cells to multiple metastatic tumor sites, including micrometastases in the liver (Fig. 15), ovary and bone marrow. Note NSCs appearing to extravasate out of the blood vessels to infiltrate the surrounding tumor parenchyma [Fig. 15(E)]. No NSCs were found in non–tumor-bearing brain, kidney, heart, intestine, or skin, and were rarely found in non–tumor-bearing tissues (lung, liver and spleen) at 2–4 days post-injection (Aboody *et al.*, 2006b).

6.2.3. *Neural stem/progenitor cell-directed enzyme pro-drug therapy (NDEPT)*

We postulated that HB1.F3.AdCMVrCE cells would preferentially localize to neuroblastoma tumor foci following intravascular injection. The

干细胞之家www.stemcell8.cn ←点击进入

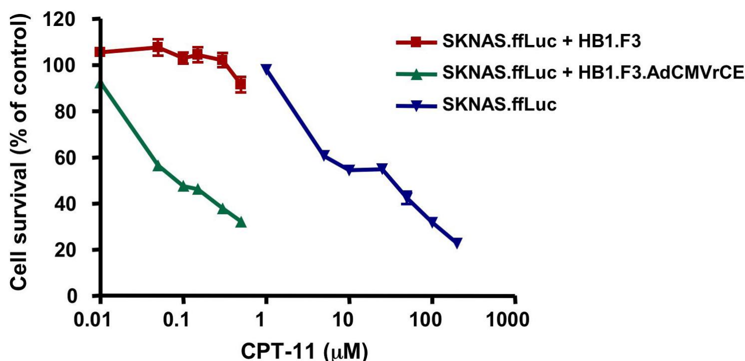


Fig. 14. *In vitro* killing of neuroblastoma cells by NSCs secreting rabbit carboxylesterase. Neuroblastoma cells (SK-N-AS) were co-cultured with neural stem cells transduced with adenovirus to express the secreted form of rCE (HB1.F3.AdCMVrCE) or HB1.F3 parental stem cell line at a 2:1 ratio of NSC-to-tumor cells in 96-well plated (100 μL/well). SKNAS cultured without NSCs were included as controls. Following 24 h of culture, CPT-11 was added in 2× concentration (100 μL/well) to give a final of 1× concentration indicated above in 200 μL/well total volume. Cells were incubated at 37°C, 6% CO_2 for an additional 48 to 72 h. Sulforhodamine B (SRB) assay was performed to detect the total remaining cell mass. Optical density (OD) was measured at 570 mm.

rCE secreted into the extracellular milieu would then convert systemically administered CPT-11 to its active form, producing high local extracellular concentrations of SN-38 (up to 1000 times more toxic to tumor cells than CPT-11 alone) (Fig. 16).

Therapeutic efficacy trials involved tail vein administration of rCE expressing NSCs into neuroblastoma-bearing animals, followed by 5 days of CPT-11 treatment and immediately followed by another round of NSCs and CPT-11. Following two weeks of treatment, the NSC/CPT-11 paradigm was repeated and animals followed for long-term survival. Of great significance, animals receiving rCE-secreting NSCs +15 mg/kg/day CPT-11 prodrug demonstrated 90% one-year survival compared to < 75 day survival in animals with no treatment, and only 30% one-year survival of animals treated with the CPT-11 alone [Fig. 17(B)]. Importantly, in our long-term NSC-treated animals, we found no evidence of residual tumor or NSCs, suggesting a complete cure (Danks *et al.*, 2007). Furthermore, plasma carboxylesterase activity and SN-38 levels in mice receiving both rCE-expressing HB1.F3.AdCMVrCE cells and CPT-11 were comparable to those in mice receiving CPT-11 only (Danks *et al.*, 2007). These data support the hypothesis that the anti-tumor effect of the described neural stem cell–directed enzyme prodrug therapy (NDEPT) is mediated by the production of high concentrations of active drug selectively at tumor sites, thereby

干细胞之家www.stemcell8.cn ←点击进入

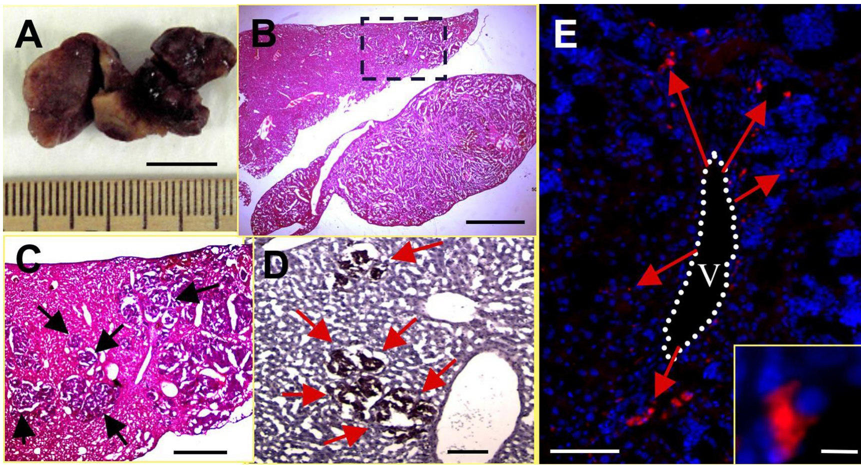


Fig. 15. Intravascularly administered NSCs (HB1.F3) target human neuroblastoma liver metastases in SCID mice. **(A)** Dissected liver from a representative animal with hepatic metastases. **(B)** Low- and **(C)** high-power magnification of a section of tumor-involved liver, stained with hematoxylin and eosin. Tumor cells appear dark purple (black arrows). **(D)** Liver section stained with an anti-human mitochondrial protein antibody and counterstained with hematoxylin. Tumor micrometastases stain dark brown (red arrows). **(E)** Immunofluorescence microscopy of liver tumor section. Tumor foci are identified by areas of densely packed DAPI (blue)-stained tumor cell nuclei. The red arrows show extravasated CM-DiI (red)–labeled HB1.F3.cells proximal to a hepatic vein (v). Inset is high magnification of a CM-DiI–labeled HB1.F3 cell within the tumor. Scale bars: (**A**) 1 cm, (**B**) 2 mm, (**C**) 500 μm, (**D**,**E**) 100 μm and **(E)** inset 10 μm. Based on Fig. 3 from: Aboody *et al.*, *PLoS ONE* **1**: e23. (2006) with permission.

maximizing the anti-tumor an effect of CPT-11. NDEPT approaches merit further investigation as an effective, targeted therapy for metastatic tumors. We propose that the described approach may have the greatest use for eradicating minimum residual disease.

6.2.4. *NSC-mediated therapeutic efficacy in disseminated neuroblastoma model*

With 1-year survival as the end point, the Kaplan–Meier plot shows a clear dose–response relationship for mice treated with 7.5 mg/kg CPT-11 [Fig. 17(A)] compared with 15 mg/kg CPT-11 [Fig. 17(B)], both for mice treated with CPT-11 alone (10% survival versus 30%, respectively) and with CPT-11 as a component of NDEPT (50% versus 90%, respectively).

7. Factors Involved in NSC Tumor Tropism

Numerous cytokines and growth factors and their receptors have been identified that affect NSC migration under normal and pathological conditions,

干细胞之家www.stemcell8.cn ←点击进入

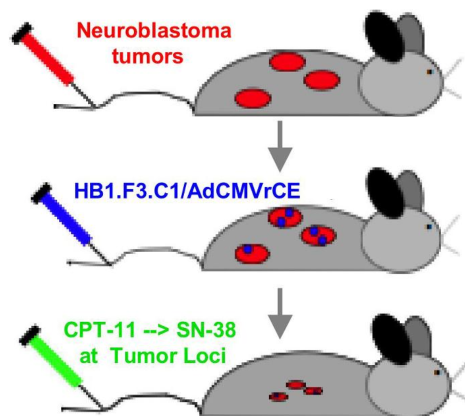


Fig. 16. Schematic representation of neural stem/progenitor cell-directed enzyme prodrug therapy (NDEPT). Mice bearing disseminated neuroblastoma receive i.v. injections of HB1.F3 cells transduced to express a secreted form of rCE. These cells have been shown to migrate selectively to tumors *in vivo*. The rCE at tumor sites systemically converts the given CPT-11 to its active form, SN-38. High concentrations of SN-38 at tumor loci are cytotoxic to tumors, without additional toxic side effects. Of note, no detectable levels of SN-38 were found in the blood. Based on Fig. 1 from: Danks *et al.*, *Cancer Res* **67**: 22–25 (2007) with permission of the American Association for Cancer Research.

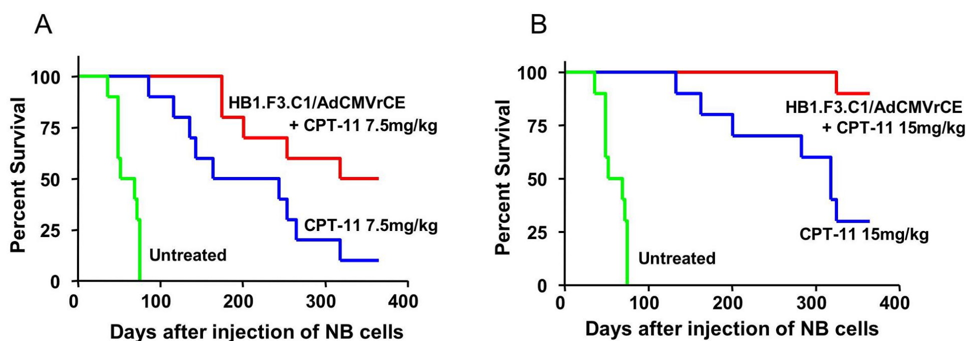


Fig. 17. Kaplan–Meier plots of tumor-bearing mice treated with HB1. F3.C1/AdCMVrCE and CPT-11. Mice (10 per group) were injected i.v. with 500,000 SK-N-AS cells to produce disseminated neuroblastoma (NB) tumors in 100% of mice. Two weeks later, mice were treated with rCE/CPT-11 NDEPT by the following treatment protocol: week 1, 2 million HB1.F3.C1 cells transduced with a MOI of 20 of AdCMVrCE followed by CPT-11 daily × five i.v. at the indicated doses; week 2, repeat of week 1; weeks 3 and 4, no treatment; and weeks 5 and 6, same as weeks 1 and 2. The doses of CPT-11 given were (**A**) 7.5 mg/kg or (**B**) 15 mg/kg. Based on Fig. 3 from: Danks *et al.*, *Cancer Res* **67**: 22–25 (2007) with permission of the American Association for Cancer Research.

干细胞之家www.stemcell8.cn ←点击进入

including cancer. Such agents include SDF-1/CXCR4 (Imitola *et al.*, 2004; Kucia *et al.*, 2004; Peng *et al.*, 2004; Kucia *et al.*, 2005), SCF/c-Kit (Erlandsson *et al.*, 2004; Sun *et al.*, 2004), HGF/c-Met (Heese *et al.*, 2005), VEGF/VEGFR (Schmidt *et al.*, 2005), MCP-1/CCR2 (Widera *et al.*, 2004) and HMGB1/RAGE (Palumbo and Bianchi, 2004). Extracellular matrix proteins, especially laminin and tenascin have been shown to serve as strong modulators of NSC migration (Ziu *et al.*, 2006). Among transcription factors, hypoxia-inducible factor-1 (HIF-1) plays a crucial role in the regulation/expression of many cytokines and their receptors in tumors (Semenza, 2003). Gliomas have been extensively characterized with regard to the cytokines and growth factors they release, and stimulate the directed migration of exogenous and endogenous NSCs into the tumor microenvironment. There is evidence that cancer cells and stem cells may share common molecular mechanisms used for cell trafficking (Kucia *et al.*, 2005). It should be noted that in addition to gliomas, many cancers release cytokines that can serve as potent chemoattractants for stem cells, which suggests the feasibility of stem cell-mediated delivery of therapeutic genes to tumors of various types. Clearly, the identification and characterization of cancer-associated cytokines will be important for the optimization of stem cell-mediated therapies.

8. NSC-mediated Therapeutic Applications to Invasive and Metastatic Tumors: Future Directions

The poor prognosis for patients with metastatic cancer and the toxic side effects of currently available treatments necessitates the development of more effective tumor-selective therapies. We postulate that the inherent tumor-tropic properties of NSCs can be harnessed to develop safe and effective targeted therapy for invasive and metastastic cancers. This tumor-selective NSC-mediated delivery approach has the potential to maximize local concentrations of anti-cancer agents while minimizing toxicity to normal tissues, making them a very attractive platform for localized chemotherapy. Specifically, NSCs can be engineered to deliver a wide range of cDNAs encoding therapeutic transgenes specifically to tumor foci. Indeed, the prodrug-activating enzyme genetic strategy represents one of many potential approaches to treating malignant solid tumors. Other candidates include genes encoding proteins that induce differentiation of neoplastic cells and/or their signal-transduction mediators, cell cycle modulators, apoptosis-promoting agents, anti-angiogenesis factors, immune-enhancing agents, fusion agents, and oncolytic factors. Furthermore, NSC-mediated targeted tumor therapy is likely not limited to tumor types discussed.

干细胞之家www.stemcell8.cn ←点击进入

Preliminary data suggest that NSCs also display a tropism for malignant breast carcinoma and lung cancer (Aboody *et al.*, unpublished data). Current studies are in progress to elucidate the chemotactic factors and signaling pathways involved in this stem cell tumor tropism.

In order to move this novel therapeutic approach towards clinical trials, further long-term biodistribution data must be generated to evaluate the safety and potential toxicity, tumorgenicity, immunogenecity, and cell fate. Optimization of tumor-specific therapeutic regimens must also be investigated. Additionally, allogeneic, autologous and clonal lines as well as primary NSC pools must be compared for strength of tumor tropism, stability of expansion potential as well as feasibility and practicality.

Acknowledgments

This work was supported by the National Institutes of Health/National Cancer Institute (CA113446, CA79763, CA76202 and CA21765), Stop Cancer Foundation, The Rosalinde and Arthur Gilbert Foundation, Neidorf Family Foundation, H.L. Snyder Foundation, and American Lebanese Syrian Associated Charities (ALSAC).

References

Aboody KS, Brown A, Rainov NG, *et al.* (2000) Neural stem cells display extensive tropism for pathology in adult brain: evidence from intracranial gliomas. *Proc Natl Acad Sci USA* **97**: 12846–51.

Aboody KS, Najbauer J, Schmidt NO, *et al.* (2006a) Targeting of melanoma brain metastases using engineered neural stem/progenitor cells. *Neuro Oncol* **8**: 119–26.

Aboody KS, Bush RA, Garcia E, *et al.* (2006b) Development of a tumor-selective approach to treat metastatic cancer. *PLoS ONE* **1**: e23.

Ashkenazi A, Pai RC, Fong S, *et al.* (1999) Safety and antitumor activity of recombinant soluble Apo2 ligand. *J Clin Invest* **104**: 155–62.

Barresi V, Belluardo N, Sipione S, *et al.* (2003) Transplantation of prodrug-converting neural progenitor cells for brain tumor therapy. *Cancer Gene Ther* **10**: 396–402.

Bello L, Lucini V, Carrabba G, *et al.* (2001) Simultaneous inhibition of glioma angiogenesis, cell proliferation, and invasion by a naturally occurring fragment of human metalloproteinase-2. *Cancer Res* **61**: 8730–36.

Benedetti S, Pirola B, Pollo B, *et al.* (2000) Gene therapy of experimental brain tumors using neural progenitor cells. *Nat Med* **6**: 447–50.

Brodeur GM. (2003) Neuroblastoma: biological insights into a clinical enigma. *Nat Rev Cancer* **3**: 203–16.

干细胞之家www.stemcell8.cn ←点击进入

Brooks PC, Silletti S, von Schalscha TL, *et al.* (1998) Disruption of angiogenesis by PEX, a noncatalytic metalloproteinase fragment with integrin binding activity. *Cell* **92**: 391–400.

Brown AB, Yang W, Schmidt NO, *et al.* (2003) Intravascular delivery of neural stem cell lines to target intracranial and extracranial tumors of neural and non-neural origin. *Hum Gene Ther* **14**: 1777–85.

Cartwright P, McLean C, Sheppard A, *et al.* (2005) LIF/STAT3 controls ES cell self-renewal and pluripotency by a Myc-dependent mechanism. *Development* **132**: 885–96.

Chu K, Kim M, Park KI, *et al.* (2004) Human neural stem cells improve sensorimotor deficits in the adult rat brain with experimental focal ischemia. *Brain Res* **1016**: 145–53.

Conti L, Cattaneo E. (2002) Gene therapy using neural stem cells. *Meth Mol Biol* **198**: 233–44.

Danks MK, Morton CL, Pawlik CA, Potter PM (1998) Overexpression of a rabbit liver carboxylesterase sensitizes human tumor cells to CPT-11. *Cancer Res* **58**: 20–22.

Danks MK, Morton CL, Krull EJ, *et al.* (1999) Comparison of activation of CPT-11 by rabbit and human carboxylesterases for use in enzyme/prodrug therapy. *Clin Cancer Res* **5**: 917–24.

Danks MK, Yoon KJ, Bush RA, *et al.* (2007) Tumor-targeted enzyme/prodrug therapy mediates long-term disease-free survival of mice bearing disseminated neuroblastoma. *Cancer Res* **67**: 22–25.

Dickson PV, Hamner JB, Burger RA, *et al.* (2007) Intravascular administration of tumor tropic neural progenitor cells permits targeted delivery of interferon-beta and restricts tumor growth in a murine model of disseminated neuroblastoma. *J Pediatr Surg* **42**: 48–53.

Ehtesham M, Stevenson CB, Thompson RC. (2005) Stem cell therapies for malignant glioma. *Neurosurg Focus* **19**: E5.

Ehtesham M, Kabos P, Kabosova A, *et al.* (2002a) The use of interleukin 12-secreting neural stem cells for the treatment of intracranial glioma. *Cancer Res* **62**: 5657–63.

Ehtesham M, Kabos P, Gutierrez MA, *et al.* (2002b) Induction of glioblastoma apoptosis using neural stem cell-mediated delivery of tumor necrosis factor-related apoptosis-inducing ligand. *Cancer Res* **62**: 7170–74.

Erlandsson A, Larsson J, Forsberg-Nilsson K. (2004) Stem cell factor is a chemoattractant and a survival factor for CNS stem cells. *Exp Cell Res* **301**: 201–10.

Evans AE, August CS, Kamani N, *et al.* (1994) Bone marrow transplantation for high risk neuroblastoma at the Children's Hospital of Philadelphia: an update. *Med Pediatr Oncol* **23**: 323–27.

Faber C, Terao E, Morga E, Heuschling P. (2000) Interleukin-4 enhances the *in vitro* precursor cell recruitment for tumor-specific T lymphocytes in patients with glioblastoma. *J Immunother* (1997) **23**: 11–16.

干细胞之家www.stemcell8.cn ←点击进入

Flax JD, Aurora S, Yang C, *et al.* (1998) Engraftable human neural stem cells respond to developmental cues, replace neurons, and express foreign genes. *Nat Biotechnol* **16**: 1033–39.

Furman WL, Stewart CF, Poquette CA, *et al.* (1999) Direct translation of a protracted irinotecan schedule from a xenograft model to a phase I trial in children. *J Clin Oncol* **17**: 1815–24.

Furman WL, Crews KR, Billups C, *et al.* (2006) Cefixime allows greater dose escalation of oral irinotecan: a phase I study in pediatric patients with refractory solid tumors. *J Clin Oncol* **24**: 563–70.

Goldman S. (2005) Stem and progenitor cell-based therapy of the human central nervous system. *Nat Biotechnol* **23**: 862–71.

Graham ML, Herndon JE 2nd, Casey JR, *et al.* (1997) High-dose chemotherapy with autologous stem-cell rescue in patients with recurrent and high-risk pediatric brain tumors. *J Clin Oncol* **15**: 1814–23.

Guichard SM, Morton CL, Krull EJ, *et al.* (1998) Conversion of the CPT-11 metabolite APC to SN-38 by rabbit liver carboxylesterase. *Clin Cancer Res* **4**: 3089–94.

Heese O, Disko A, Zirkel D, *et al.* (2005) Neural stem cell migration toward gliomas *in vitro*. *Neuro Oncol* **7**: 476–84.

Herrlinger U, Woiciechowski C, Sena-Esteves M, *et al.* (2000) Neural precursor cells for delivery of replication-conditional HSV-1 vectors to intracerebral gliomas. *Mol Ther* **1**: 347–57.

Hochberg FH, Pruitt A. (1980) Assumptions in the radiotherapy of glioblastoma. *Neurology* **30**: 907–11.

Hoshimaru M, Ray J, Sah DW, Gage FH. (1996) Differentiation of the immortalized adult neuronal progenitor cell line HC2S2 into neurons by regulatable suppression of the v-myc oncogene. *Proc Natl Acad Sci USA* **93**: 1518–23.

Huber BE, Austin EA, Richards CA, *et al.* (1994) Metabolism of 5-fluorocytosine to 5-fluorouracil in human colorectal tumor cells transduced with the cytosine deaminase gene: significant antitumor effects when only a small percentage of tumor cells express cytosine deaminase. *Proc Natl Acad Sci USA* **91**: 8302–306.

Hutter A, Schwetye KE, Bierhals AJ, McKinstry RC. (2003) Brain neoplasms: epidemiology, diagnosis, and prospects for cost-effective imaging. *Neuroimaging Clin N Am* **13**: 237–50, x–xi.

Imitola J, Raddassi K, Park KI, *et al.* (2004) Directed migration of neural stem cells to sites of CNS injury by the stromal cell-derived factor 1alpha/CXC chemokine receptor 4 pathway. *Proc Natl Acad Sci USA* **101**: 18117–22.

Ireton GC, McDermott G, Black ME, Stoddard BL. (2002) The structure of *Escherichia coli* cytosine deaminase. *J Mol Biol* **315**: 687–97.

Jean WC, Spellman SR, Wallenfriedman MA, *et al.* (1998) Interleukin-12-based immunotherapy against rat 9L glioma. *Neurosurgery* **42**: 850–56; discussion 856–57.

干细胞之家www.stemcell8.cn ←点击进入

Jeong SW, Chu K, Jung KH, *et al.* (2003) Human neural stem cell transplantation promotes functional recovery in rats with experimental intracerebral hemorrhage. *Stroke* **34**: 2258–63.

Kabos P, Ehtesham M, Black KL, Yu JS. (2003) Neural stem cells as delivery vehicles. *Expert Opin Biol Ther* **3**: 759–70.

Kim SK, Cargioli TG, Machluf M, *et al.* (2005) PEX-producing human neural stem cells inhibit tumor growth in a mouse glioma model. *Clin Cancer Res* **11**: 5965–70.

Kim SK, Kim SU, Park IH, *et al.* (2006a) Human neural stem cells target experimental intracranial medulloblastoma and deliver a therapeutic gene leading to tumor regression. *Clin Cancer Res* **12**: 5550–56.

Kim SU. (2004) Human neural stem cells genetically modified for brain repair in neurological disorders. *Neuropathology* **24**: 159–71.

Kim SU. (2007) Genetically engineered human neural stem cells for brain repair in neurological diseases. *Brain Dev* **29**: 193–201.

Kim SU, Nakagawa E, Hatori K, *et al.* (2002) Production of immortalized human neural crest stem cells. *Meth Mol Biol* **198**: 55–65.

Kim SU, Park IH, Kim TH, *et al.* (2006b) Brain transplantation of human neural stem cells transduced with tyrosine hydroxylase and GTP cyclohydrolase 1 provides functional improvement in animal models of Parkinson disease. *Neuropathology* **26**: 129–40.

Kock N, Kasmieh R, Weissleder R, Shah K. (2007) Tumor therapy mediated by lentiviral expression of shBcl-2 and S-TRAIL. *Neoplasia* **9**: 435–42.

Kucia M, Reca R, Miekus K, *et al.* (2005) Trafficking of normal stem cells and metastasis of cancer stem cells involve similar mechanisms: pivotal role of the SDF-1-CXCR4 Axis. *Stem Cells*.

Kucia M, Jankowski K, Reca R, *et al.* (2004) CXCR4-SDF-1 signalling, locomotion, chemotaxis and adhesion. *J Mol Histol* **35**: 233–45.

Lee ST, Chu K, Park JE, *et al.* (2005) Intravenous administration of human neural stem cells induces functional recovery in Huntington's disease rat model. *Neurosci Res* **52**: 243–49.

Lee ST, Park JE, Lee K, *et al.* (2006) Noninvasive method of immortalized neural stem-like cell transplantation in an experimental model of Huntington's disease. *J Neurosci Meth* **152**: 250–54.

Lin D, Najbauer J, Salvaterra PM, *et al.* (2007) Novel method for visualizing and modeling the spatial distribution of neural stem cells within intracranial glioma. *Neuroimage*.

Ma MK, Zamboni WC, Radomski KM, *et al.* (2000) Pharmacokinetics of irinotecan and its metabolites SN-38 and APC in children with recurrent solid tumors after protracted low-dose irinotecan. *Clin Cancer Res* **6**: 813–19.

Martinez-Serrano A, Bjorklund A. (1997) Immortalized neural progenitor cells for CNS gene transfer and repair. *Trends Neurosci* **20**: 530–38.

干细胞之家www.stemcell8.cn ←点击进入

Martinez-Serrano A, Rubio FJ, Navarro B, *et al.* (2001) Human neural stem and progenitor cells: *in vitro* and *in vivo* properties, and potential for gene therapy and cell replacement in the CNS. *Curr Gene Ther* **1**: 279–99.

Matthay KK, Seeger RC, Reynolds CP, *et al.* (1994) Allogeneic versus autologous purged bone marrow transplantation for neuroblastoma: a report from the Childrens Cancer Group. *J Clin Oncol* **12**: 2382–89.

Minchinton AI, Tannock IF. (2006) Drug penetration in solid tumours. *Nat Rev Cancer* **6**: 583–92.

Muller FJ, Snyder EY, Loring JF. (2006) Gene therapy: can neural stem cells deliver? *Nat Rev Neurosci* **7**: 75–84.

Nakamizo A, Marini F, Amano T, *et al.* (2005) Human bone marrow-derived mesenchymal stem cells in the treatment of gliomas. *Cancer Res* **65**: 3307–18.

Ourednik J, Ourednik V, Lynch WP, *et al.* (2002) Neural stem cells display an inherent mechanism for rescuing dysfunctional neurons. *Nat Biotechnol* **20**: 1103–10.

Ourednik V, Ourednik J, Park KI, *et al.* (2000) Neural stem cells are uniquely suited for cell replacement and gene therapy in the CNS. *Novartis Found Symp* **231**: 242–62; discussion 262–49, 302–246.

Palumbo R, Bianchi ME. (2004) High mobility group box 1 protein, a cue for stem cell recruitment. *Biochem Pharmacol* **68**: 1165–70.

Patchell RA. (2003) The management of brain metastases. *Cancer Treat Rev* **29**: 533–40.

Patrice SJ, Tarbell NJ, Goumnerova LC, *et al.* (1995) Results of radiosurgery in the management of recurrent and residual medulloblastoma. *Pediatr Neurosurg* **22**: 197–203.

Peng H, Huang Y, Rose J, *et al.* (2004) Stromal cell-derived factor 1-mediated CXCR4 signaling in rat and human cortical neural progenitor cells. *J Neurosci Res* **76**: 35–50.

Pitti RM, Marsters SA, Ruppert S, *et al.* (1996) Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. *J Biol Chem* **271**: 12687–90.

Pollack IF, Erff M, Ashkenazi A. (2001) Direct stimulation of apoptotic signaling by soluble Apo2l/tumor necrosis factor-related apoptosis-inducing ligand leads to selective killing of glioma cells. *Clin Cancer Res* **7**: 1362–69.

Pommier Y. (2006) Topoisomerase I inhibitors: camptothecins and beyond. *Nat Rev Cancer* **6**: 789–802.

Potter PM, Pawlik CA, Morton CL, *et al.* (1998) Isolation and partial characterization of a cDNA encoding a rabbit liver carboxylesterase that activates the prodrug irinotecan (CPT-11). *Cancer Res* **58**: 2646–51.

Price SJ, Jena R, Burnet NG, *et al.* (2007) Predicting patterns of glioma recurrence using diffusion tensor imaging. *Eur Radiol* **17**: 1675–84.

Redmond DE Jr, Bjugstad KB, Teng YD, *et al.* (2007) Behavioral improvement in a primate Parkinson's model is associated with multiple homeostatic effects of human neural stem cells. *Proc Natl Acad Sci USA* **104**: 12175–80.

干细胞之家www.stemcell8.cn ←点击进入

Renfranz PJ, Cunningham MG, McKay RD. (1991) Region-specific differentiation of the hippocampal stem cell line HiB5 upon implantation into the developing mammalian brain. *Cell* **66**: 713–29.

Ryder EF, Snyder EY, Cepko CL. (1990) Establishment and characterization of multipotent neural cell lines using retrovirus vector-mediated oncogene transfer. *J Neurobiol* **21**: 356–75.

Ryu JK, Kim J, Cho SJ, *et al.* (2004) Proactive transplantation of human neural stem cells prevents degeneration of striatal neurons in a rat model of Huntington disease. *Neurobiol Dis* **16**: 68–77.

Ryu MY, Lee MA, Ahn YH, *et al.* (2005) Brain transplantation of neural stem cells cotransduced with tyrosine hydroxylase and GTP cyclohydrolase 1 in Parkinsonian rats. *Cell Transplant* **14**: 193–202.

Saha S, Meyer M, Krementz ET, *et al.* (1994) Prognostic evaluation of intracranial metastasis in malignant melanoma. *Ann Surg Oncol* **1**: 38–44.

Schmidt NO, Przylecki W, Yang W, *et al.* (2005) Brain tumor tropism of transplanted human neural stem cells is induced by vascular endothelial growth factor. *Neoplasia* **7**: 623–29.

Semenza GL. (2003) Targeting HIF-1 for cancer therapy. *Nat Rev Cancer* **3**: 721–32.

Shah K, Tang Y, Breakefield X, Weissleder R. (2003) Real-time imaging of TRAIL-induced apoptosis of glioma tumors *in vivo*. *Oncogene* **22**: 6865–72.

Shah K, Jacobs A, Breakefield XO, Weissleder R. (2004) Molecular imaging of gene therapy for cancer. *Gene Ther* **11**: 1175–87.

Shah K, Tung CH, Breakefield XO, Weissleder R. (2005a) *In vivo* imaging of S-TRAIL-mediated tumor regression and apoptosis. *Mol Ther* **11**: 926–31.

Shah K, Bureau E, Kim DE, *et al.* (2005b) Glioma therapy and real-time imaging of neural precursor cell migration and tumor regression. *Ann Neurol* **57**: 34–41.

Snyder EY, Deitcher DL, Walsh C, *et al.* (1992) Multipotent neural cell lines can engraft and participate in development of mouse cerebellum. *Cell* **68**: 33–51.

Snyder EY, Park KI, Flax JD, *et al.* (1997) Potential of neural "stem-like" cells for gene therapy and repair of the degenerating central nervous system. *Adv Neurol* **72**: 121–32.

Springer CJ, Niculescu-Duvaz I. (2000) Prodrug-activating systems in suicide gene therapy. *J Clin Invest* **105**: 1161–67.

Studeny M, Marini FC, Dembinski JL, *et al.* (2004) Mesenchymal stem cells: potential precursors for tumor stroma and targeted-delivery vehicles for anticancer agents.*J Natl Cancer Ins*t **96**: 1593–1603.

Stupp R, Mason WP, van den Bent MJ, *et al.* (2005) Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med* **352**: 987–96.

Sun L, Lee J, Fine HA. (2004) Neuronally expressed stem cell factor induces neural stem cell migration to areas of brain injury. *J Clin Invest* **113**: 1364–74.

Sykova E, Jendelova P. (2005) Magnetic resonance tracking of implanted adult and embryonic stem cells in injured brain and spinal cord. *Ann N Y Acad Sci* **1049**: 146–60.

干细胞之家www.stemcell8.cn ←点击进入

Takahashi K, Yamanaka S. (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**: 663–76.

Uchida N, Buck DW, He D, *et al.* (2000) Direct isolation of human central nervous system stem cells. *Proc Natl Acad Sci USA* **97**: 14720–25.

Walczak H, Krammer PH. (2000) The CD95 (APO-1/Fas) and the TRAIL (APO-2L) apoptosis systems. *Exp Cell Res* **256**: 58–66.

Westphal M, Heese O, de Wit M. (2003) Intracranial metastases: therapeutic options. *Ann Oncol* **14** (Suppl 3): iii4–10.

Widera D, Holtkamp W, Entschladen F, *et al.* (2004) MCP-1 induces migration of adult neural stem cells. *Eur J Cell Biol* **83**: 381–87.

Yip S, Aboody KS, Burns M, *et al.* (2003) Neural stem cell biology may be well suited for improving brain tumor therapies. *Cancer J* **9**: 189–204.

Yuan X, Hu J, Belladonna ML, *et al.* (2006) Interleukin-23-expressing bone marrow-derived neural stem-like cells exhibit antitumor activity against intracranial glioma. *Cancer Res* **66**: 2630–38.

Zhou C, Wen ZX, Wang ZP, *et al.* (2003) Green fluorescent protein-labeled mapping of neural stem cells migrating towards damaged areas in the adult central nervous system. *Cell Biol Int* **27**: 943–45.

Ziu M, Schmidt NO, Cargioli TG, *et al.* (2006) Glioma-produced extracellular matrix influences brain tumor tropism of human neural stem cells. *J Neurooncol* **79**: 125–33.

干细胞之家www.stemcell8.cn ←点击进入

Chapter 14

Antitumor Activity of Adenovirally Transduced CD34$^+$ Cells Expressing Membrane-bound TRAIL

Carmelo Carlo-Stella*,†,§, Cristiana Lavazza†,§,
Antonino Carbone‡ and Alessandro M. Gianni*,†,§

Based on preclinical studies demonstrating that tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) exerts a potent and cancer cell-specific pro-apoptotic activity, recombinant TRAIL as well as agonistic anti–TRAIL-R1 and anti–TRAIL-R2 antibodies recently entered clinical trials. Additionally, gene therapy approaches using TRAIL-encoding adenovirus (Ad-TRAIL) are currently being developed to overcome the limitations inherent to TRAIL receptor targeting, i.e., pharmacokinetics of soluble TRAIL, pattern of receptor expression and tumor cell resistance. To optimize gene therapy approaches, CD34$^+$ cells transduced with Ad-TRAIL (CD34-TRAIL$^+$) have been investigated as tumor-homing cellular vehicles for TRAIL delivery. Transduced cells exhibit a potent tumor-killing activity on a variety of lympho-hematopoietic tumor cell types both *in vitro* and *in vivo*, and are also cytotoxic against tumor cells resistant to soluble TRAIL. Studies in tumor-bearing nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice suggest that the antitumor effect of CD34-TRAIL$^+$ cells is mediated by both direct tumor cell killing due to apoptosis and indirect tumor cell killing due to vascular-disrupting mechanisms. The clinical translation of cell and gene therapy approaches represents a challenging strategy that might achieve systemic tumor targeting and efficient intratumor delivery of the therapeutic agent.

Keywords: TRAIL; TRAIL receptors; Ad-TRAIL; CD34+ cells; gene therapy; tumor homing.

*Corresponding authors. carmelo.carlostella@unimi.it; Alessandro.Gianni@unimi.it
†"Cristina Gandini" Medical Oncology Unit, Fondazione IRCCS Istituto Nazionale Tumori, Milano, Italy
‡Department of Pathology, Fondazione IRCCS Istituto Nazionale Tumori, Milano, Italy
§Medical Oncology, University of Milano, Milano, Italy

干细胞之家www.stemcell8.cn ←点击进入

1. Introduction

Dysregulated apoptosis plays a key role in the pathogenesis and progression of neoplastic disorders, allowing tumor cells to survive beyond their normal life span and to eventually acquire chemo-radioresistance (Laconi *et al.*, 2000; Pommier *et al.*, 2004). Thus, apoptotic pathways represent attractive therapeutic targets for restoring apoptosis sensitivity of malignant cells or for activating agonists of apoptosis. Several strategies which target either the mitochondria-dependent (intrinsic) or the death receptor-dependent (extrinsic) pathways of apoptosis can be envisaged to modulate apoptotic genes and proteins (Waxman and Schwartz, 2003). Death receptor ligands of the tumor necrosis factor α (TNFα) superfamily are attractive anti-cancer agents due to their ability to induce cell death. These cytokines are type II transmembrane proteins capable of cell targeting upon cell-cell contact or after protease-mediated release to the extracellular space (Ashkenazi, 2002). Four members of this family — including Fas ligand (FasL), TNFα, TL1A, and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) — stand out because of their ability to induce cell death (Wiley *et al.*, 1995; Wajant, 2003).

TRAIL, in its soluble form, is emerging as a relevant anti-cancer agent due to its cancer cell specificity and potent antitumor activity. *In vitro*, several sets of evidence demonstrated that TRAIL selectively induces apoptosis in a variety of transformed cell lines (Pitti *et al.*, 1996; Mariani *et al.*, 1997; Almasan and Ashkenazi, 2003); *in vivo* administration of TRAIL to mice exerts a remarkable activity against tumor xenografts of various cancer types (Ashkenazi *et al.*, 1999; Walczak *et al.*, 1999; Mitsiades *et al.*, 2001; Pollack *et al.*, 2001; Fulda *et al.*, 2002; LeBlanc *et al.*, 2002). Unlike other apoptosis-inducing TNF family members, soluble TRAIL appears to be inactive against normal healthy tissue (Ashkenazi *et al.*, 1999), and reports in which TRAIL induced apoptosis in normal cells could be attributed to the specific preparations of TRAIL used in the experiments (Lawrence *et al.*, 2001). The physiological functions of TRAIL are not yet fully understood, but mouse gene knockout studies indicated that this agent has an important role in antitumor surveillance by immune cells, mediates thymocyte apoptosis, and is involved in the induction of autoimmune diseases (Cretney *et al.*, 2002; Lamhamedi-Cherradi *et al.*, 2003; Smyth *et al.*, 2003). TRAIL signals by interacting with its receptors. So far, five receptors have been identified, including the two agonistic receptors TRAIL-R1 (Pan *et al.*, 1997b) and TRAIL-R2 (Walczak *et al.*, 1997) as well as the three antagonistic receptors (Sheridan *et al.*, 1997)

干细胞之家www.stemcell8.cn ←点击进入

TRAIL-R3 (Pan *et al.*, 1997a), TRAIL-R4 (Degli-Esposti *et al.*, 1997) and osteoprotegerin (OPG) (Emery *et al.*, 1998). Both TRAIL-R1 and TRAIL-R2 are type I transmembrane proteins containing a cytoplasmic death domain (DD) motif that engage apoptotic machinery upon ligand binding (Almasan and Ashkenazi, 2003), whereas the other three receptors either act as decoys or transduce antiapoptotic signals (Wang and El-Deiry, 2003). TRAIL-R3 and TRAIL-R4 have close homology to the extracellular domains of agonistic receptors. TRAIL-R4 has a truncated, nonfunctional cytoplasmic DD, while TRAIL-R3 exists on the plasma membrane as a glycophospholipid-anchored protein lacking the cytosolic tail. The physiological relevance of OPG as a soluble receptor for TRAIL is unclear, but a recent study suggests that cancer-derived OPG may be an important survival factor in hormone-resistant prostate cancer cells (Holen *et al.*, 2002).

While apoptosis induction in response to most DNA-damaging drugs usually requires the function of the tumor suppressor p53, which engages primarily the mitochondria-dependent apoptotic pathway, activation of the agonistic TRAIL receptors represents a unique opportunity to exploit the extrinsic apoptotic pathway to destroy cancer cells, regardless of p53 status. Therefore, it might be a useful therapeutic strategy, particularly in cells in which the p53-response pathway has been inactivated, thus helping to circumvent resistance to chemotherapy and radiotherapy.

2. TRAIL-induced Apoptosis as a Therapeutic Window

2.1. *TRAIL-induced Apoptosis Signaling*

TRAIL forms homotrimers that bind three receptor molecules, each at the interface between two of its subunits. A Zn atom bound to cysteine residues in the trimeric ligand is essential for trimer stability and optimal biologic activity. Binding of TRAIL to the extracellular domain of agonistic receptors results in trimerization of the receptors and clustering of the intracellular DDs, which leads to the recruitment of the adaptor molecule Fas-associated protein with death domain (FADD). Subsequently, FADD recruits initiator caspase-8 and -10, leading to the formation of the death-inducing signaling complex (DISC), where initiator caspases autoactivate by proteolysis. Once they become enzymatically active, caspase-8 and/or -10 are released from the DISC and signal through two different proteolytic pathways that converge on caspase-3 and lead to cellular disassembly (Kaufmann and Steensma, 2005). In type I cells, activation of initiator caspases upon death receptor ligation is sufficient to directly activate downstream effector

干细胞之家www.stemcell8.cn ←点击进入

caspases, such as caspase-3 and/or -7 (Scaffidi *et al.*, 1998). This extrinsic pathway is independent of the mitochondria and is not blocked by overexpression of Bcl-2. In type II cells, the commitment of death receptor ligation to apoptosis is less direct (Scaffidi *et al.*, 1998). The amount of initially cleaved caspase-8 and/or -10 is not enough to directly trigger effector caspase activation. Consequently, apoptotic signaling requires an amplification loop by mitochondrial pathway engagement through caspase 8-mediated cleavage of Bid (BH3 interacting death domain agonist), which in turn induces the cytosolic Bcl-2 family member Bax (Bcl-2–associated X protein) and/or the loosely bound mitochondrial homolog Bak (Bcl-2 antagonist/killer) to insert into the mitochondrial membrane, where they contribute to the mitochondrial release of cytochrome *c* (Lucken-Ardjomande and Martinou, 2005). In the cytosol, cytochrome *c* binds the adaptor protein Apaf-1 (apoptotic protease activating factor 1) to form an apoptosome with recruitment and activation of the apoptosis-initiating caspase-9, which proteolytically activates additional caspase-3. These events are further amplified by apoptogenic factors released from the mitochondrial space, including Smac/DIABLO (second mitochondrial activator of caspases/direct IAP-binding protein with low p*I*), which displaces the X-chromosome-linked inhibitor of apoptosis protein (XIAP) from caspase-3, -7 and -9 (Verhagen and Vaux, 2002).

2.2. *TRAIL Resistance*

Tumor cells may have an impaired apoptotic response to TRAIL due to resistance mechanism(s) occurring at different points along the TRAIL signaling pathway (Zhang and Fang, 2005). Mutations and defects in either the death receptors TRAIL-R1 or TRAIL-R2, as well as the adaptor protein FADD and caspase-8, can lead to TRAIL resistance, due to their essential role in the DISC complex assembly (Degli-Esposti *et al.*, 1997; Pan *et al.*, 1997a; Pan *et al.*, 1997b; Sheridan *et al.*, 1997). Overexpression of cellular FADD-like interleukin-1beta–converting enzyme-inhibitory protein (cFLIP) correlates with TRAIL resistance in several types of cancer. Overexpression of Bcl-2 or Bcl-XL, loss of Bax or Bak function, high expression of inhibitor of apoptosis proteins and reduced release of Smac/DIABLO from the mitochondria to the cytosol have all been reported to result in TRAIL resistance in mitochondria-dependent type II cancer cells (Fulda *et al.*, 2002; LeBlanc *et al.*, 2002). Finally, activation of different subunits of mitogen-activated protein kinases (MAPKs) or nuclear factor-kappa B (NF-kB) can lead to development of TRAIL resistance in certain types of cancer cells (Sheridan *et al.*, 1997).

干细胞之家www.stemcell8.cn ←点击进入

The mechanism(s) to overcome TRAIL resistance remain largely unclear. A prolonged exposure to the drug or very high doses of TRAIL might allow overcoming resistance (Johnston *et al.*, 2003; Mouzakiti and Packham, 2003; Mathas *et al.*, 2004; Hasegawa *et al.*, 2005). However, because of the short half-life of TRAIL in plasma (Ashkenazi *et al.*, 1999) and its rapid elimination (Walczak *et al.*, 1999), achieving prolonged exposure at high concentrations is difficult. Although *in vivo* studies using a trimerized (Walczak *et al.*, 1999) or a non-tagged (Ashkenazi *et al.*, 1999; Hao *et al.*, 2004) form of TRAIL have demonstrated a good toxicity profile of the molecule in mice and nonhuman primates, organ toxicity might occur when using high doses of soluble TRAIL. As shown in several experimental anticancer models, co-administration of TRAIL and DNA-damaging chemotherapeutic drugs might increase TRAIL-induced apoptosis and overcome TRAIL resistance due to up-regulation of TRAIL-R1 and/or TRAIL-R2 (Wen *et al.*, 2000; LeBlanc and Ashkenazi, 2003). Additionally, irradiation appears to specifically upregulate TRAIL-R2 receptor expression, and combining irradiation with TRAIL treatment has an additive or synergistic effect (Chinnaiyan *et al.*, 2000). Alternatively, upregulation of TRAIL-R1 or TRAIL-R2 using small molecules, such as the proteasome inhibitor bortezomib (Johnson *et al.*, 2003) or inhibitors of histone deacetylase (Nakata *et al.*, 2004); might overcome TRAIL resistance.

2.3. *Pharmacological Approaches for Targeting TRAIL Agonistic Receptors*

Based on promising preclinical observations, recombinant TRAIL as well as agonistic anti–TRAIL-R1 and anti–TRAIL-R2 antibodies recently entered clinical trials (Gajewski, 2007). Recombinant TRAIL is currently being co-developed by Genentech (San Francisco, CA, USA) and Amgen (Thousand Oaks, CA, USA). Phase I studies using recombinant TRAIL as a targeted therapy for solid tumors and hematological malignancies have been initiated, but results are not yet available (Buchsbaum *et al.*, 2006). HGS-ETR1 (Mapatumumab; Human Genome Sciences, Rockville, MD, USA) is a fully human agonistic monoclonal antibody that targets TRAIL-R1 (Tolcher *et al.*, 2007). This antibody is currently in phase II clinical development as a single agent in patients with non–small-cell lung cancer and colorectal cancer (reviewed in Rowinsky, 2005). Clinical activity of HGS-ETR1 is suggested by three partial responses observed in a recently reported multicenter phase II trial in relapsed non-Hodgkin lymphoma patients receiving either 3 mg/kg

干细胞之家www.stemcell8.cn ←点击进入

or 10 mg/kg HGS-ETR1 every 3 weeks for six cycles or until disease progression (Rowinsky, 2005). Additional phase Ib trials with HGS-ETR1 in combination with carboplatin/paclitaxel and cisplatin/gemcitabine have been initiated in patients with advanced solid malignancies (Rowinsky, 2005). Fully human antibodies to TRAIL-R2 (HGS-ETR2 and HGS-TR2J; Human Genome Sciences) have also entered the clinic and are currently in phase I clinical development (Rowinsky, 2005; Marini, 2006).

While recombinant TRAIL interacts with both TRAIL-R1 and TRAIL-R2 as well as the decoy receptors, agonist monoclonal antibodies selectively bind and activate TRAIL-R1 (HGS-ETR1) or TRAIL-R2 (HGS-ETR2 and HGS-TR2J). Thus, monoclonal antibodies restrict the therapeutic target to tumors with a distinct receptor expression profile. Soluble TRAIL may either have a wider therapeutic spectrum or a narrower and more unpredictable therapeutic window compared with the highly specific antibodies. The biologic relevance of the decoy receptors, their ability to inhibit TRAIL signaling, and the expression profile of the decoy receptors have not yet been fully investigated.

2.4. *Gene Therapy Approaches for Targeting TRAIL Agonistic Receptors*

Several gene therapy approaches are currently being developed to enhance tumor cell targeting and overcome the limitations inherent to death receptor targeting, i.e., pharmacokinetics, pattern of receptor expression and tumor cell resistance. Recently, a TRAIL-expressing adenoviral vector (Ad-TRAIL) has been shown to cause direct tumor cell killing as well as a potent bystander effect through the presentation of TRAIL by transduced normal cells (Lee *et al.*, 2002). Thus, using Ad-TRAIL might be an alternative to systemic delivery of soluble TRAIL, possibly resulting in better tumor cell targeting and increased tumoricidal activity (Griffith *et al.*, 2000; Griffith and Broghammer, 2001; Kagawa *et al.*, 2001; Lee *et al.*, 2002; Armeanu *et al.*, 2003). However, systemic Ad-TRAIL–based gene therapy requires efficient infection of target tumor cells as well as avoidance of immune clearance, and is limited by several safety and toxicity issues related to intravenous adenovector administration (Harrington *et al.*, 2002). Intratumoral injection of TRAIL-encoding adenovectors has been successfully explored in a number of experimental models; however, this approach results in local antitumor activity and has little, if any, value in the treatment of disseminated tumors.

干细胞之家www.stemcell8.cn ←点击进入

3. CD34$^+$ Cells as Tumor-homing Vectors for Membrane-bound TRAIL Gene Therapy

In order to optimize the use of TRAIL-encoding adenovectors for the treatment of systemic tumors, we have recently investigated a cell-based approach using CD34$^+$ cells transduced with a replication-deficient Ad-TRAIL (CD34-TRAIL$^+$) (Carlo-Stella *et al.*, 2006b). Several lines of evidence support the use of gene-modified CD34$^+$ cells as optimal vehicles of anti-tumor molecules. CD34$^+$ cells are already widely used in the clinical setting. Additionally, they can migrate from the bloodstream into tumor tissues due to the expression of adhesion receptors that specifically interact with counter-receptors on endothelial cells in the tumor microenvironment (Verfaillie, 1998; Kaplan *et al.*, 2005; Burger and Kipps, 2006). Moreover, upregulation of inflammatory chemo-attractants in the tumor microenvironment provides a permissive milieu that potentially allows for homing of systemically delivered CD34-TRAIL$^+$cells and efficient tumor targeting (Jin *et al.*, 2006). Finally, upon adenovector transduction, CD34$^+$ cells express high levels of the transgene for only a few days (Bregni *et al.*, 1998). This transient expression, partly related to dilution of the intracellular adenoviral episome through multiple cell divisions, enhances the safety of cell-based gene therapy.

3.1. *Ad-TRAIL Transduction of CD34$^+$ Cells*

We used a replication-deficient Ad-TRAIL encoding a human, full-length membrane-bound TRAIL (mTRAIL) under the control of the CMV promoter (University of Iowa Gene Transfer Vector Core, Iowa City, Iowa, USA) (Griffith *et al.*, 2000). To optimize transduction efficiency, CD34$^+$ cells were exposed to Ad-TRAIL for 6 h, and then the BoosterExpress™(Gene Therapy System, San Diego, CA, USA) chemical cocktail was added. Using a multiplicity of infection (MOI) of 500, this transduction protocol consistently resulted in a transduction efficiency higher than 80% (range 70–96%), a high level expression of mTRAIL, and a cell viability ≥85%. Under these experimental conditions, flow cytometry analysis of CD34-TRAIL$^+$ cells showed significant levels of transgene expression for at least 96 h after transduction, and Western blot analysis revealed the presence of 32- and 55-kDa proteins, which are the expected products for full-length monomer and dimer TRAIL, respectively (Carlo-Stella *et al.*, 2006b).

干细胞之家www.stemcell8.cn ←点击进入

3.2. *In Vitro Antitumor Activity of CD34-TRAIL$^+$ Cells*

The *in vitro* antitumor activity of mTRAIL-expressing cells was investigated using two human cell lines, including the multiple myeloma (MM) cell line KMS-11 (Ronchetti *et al.*, 2001) and the prolymphocytic leukemia (PLL) cell line JVM-2 (Carlo-Stella *et al.*, 2006a), which were selected as being sensitive (KMS-11) or resistant (JVM-2) to apoptosis induced by soluble TRAIL. Transduced cells were co-cultured with tumor cells at a 1:1 effector:target cell ratio for 24–48 h, and then cell death was evaluated by annexin-V/propidium iodide (PI) double staining (Carlo-Stella *et al.*, 2006b).

Co-culturing of CD34-TRAIL$^+$ and KMS-11 cells resulted in a highly significant and time-dependent induction of apoptosis and necrosis, with an average 70% and 80% tumor cell death being detected following 24 and 48 h of co-culture [Fig. 1(A)]. Thus, the level of cell death induced by mTRAIL-armed cells was comparable to that induced by 100 ng/mL soluble TRAIL. In contrast, no tumor cell death could be detected by either co-culturing mock-transduced CD34$^+$ cells and tumor cells or preventing cell-cell contact between effector cells and tumor cells using transwell culture dishes.

Exposure of JVM-2 cells to CD34-TRAIL$^+$ cells resulted in significant levels (up to 50%) of tumor cell death detected after a 48-hour co-culture; in striking contrast, no cell death could be detected by incubating JVM-2 cells with 100 ng/mL soluble TRAIL or co-culturing mock-transduced CD34$^+$ cells and tumor cells [Fig. 1(B)]. This cell death induction reflected a significantly higher biological activity of membrane-bound TRAIL compared to soluble TRAIL. The capacity of CD34-TRAIL$^+$ cells to overcome resistance to soluble TRAIL was further confirmed by analyzing mTRAIL-induced cell death in a variety of TRAIL-resistant tumor cell lines, including lymphoma, multiple myeloma, as well as epithelial cancers (Carlo-Stella *et al.*, 2006b). In all instances, the membrane-bound form of the ligand was capable of triggering apoptosis and overcoming tumor resistance to the soluble ligand. This peculiar functional property of mTRAIL might be due to a differential activation of TRAIL-R1 and TRAIL-R2 by soluble and membrane TRAIL, as suggested by studies demonstrating that TRAIL-R1 signals apoptosis upon triggering by both the soluble and membrane-bound form of the ligand, whereas TRAIL-R2 becomes only activated by mTRAIL or soluble TRAIL cross-linked by antibodies (Muhlenbeck *et al.*, 2000; Wajant *et al.*, 2001).

Co-culture of the TRAIL-sensitive KMS-11 cell line with CD34-TRAIL$^+$ cells resulted in caspase-3, -8 and -9 activation and in PARP cleavage, i.e., the same pattern of biochemical events achieved by incubating KMS-11

干细胞之家www.stemcell8.cn ←点击进入

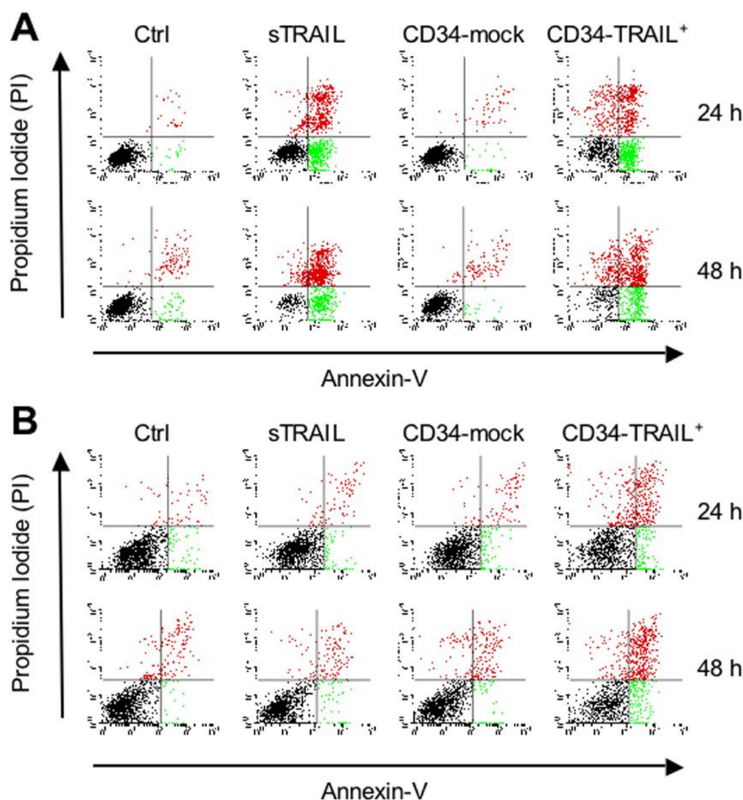


Fig. 1. CD34-TRAIL^{+} cells induce significant levels of cell death in both TRAIL-sensitive and TRAIL-resistant cell lines. Annexin-V/PI double staining (A&B) was used to distinguish between apoptotic (annexin-V^{+}/PI^{-}) and nonapoptotic, that is dead cells (annexin-V^{+}/PI^{+} + annexin V^{-}/PI^{+}). **(A)** KMS-11 cell line exposed to soluble TRAIL (100 ng/mL) or co-cultured (effector:target ratio = 1:1) with CD34-mock or CD34-TRAIL^{+} cells for 24 h and 48 h. To selectively detect KMS-11 cell death and rule out any contribution of CD34-TRAIL^{+} cells to apoptosis and necrosis, co-cultures were additionally stained with an anti-CD45 monoclonal antibody that binds CD34^{+} cells but not tumor cells. Co-culturing CD34-TRAIL^{+} cells and KMS-11 cells resulted in a marked, time-dependent cell death which was comparable to that induced by 100 ng/mL soluble TRAIL. Negligible levels of cell death could be detected by co-culturing mock-transduced CD34^{+} cells and tumor cells. **(B)** JVM-2 cell line exposed to soluble TRAIL (100 ng/mL) or co-cultured (effector:target ratio = 1:1) with CD34-mock or CD34-TRAIL^{+} cells for 24 h and 48 h. To selectively detect JVM-2 cell death and rule out any contribution of CD34-TRAIL^{+} cells to apoptosis and necrosis, co-cultures were additionally stained with an anti-CD19 monoclonal antibody that binds tumor cells but not CD34^{+} cells. Co-culturing CD34-TRAIL^{+} and JVM-2 cells resulted in significant levels of tumor cell death, whereas no cell death could be detected by incubating JVM-2 cells with soluble TRAIL or co-culturing mock-transduced CD34^{+} cells and tumor cells. Based on Figs. 3(a) and 3(c) of: Carlo-Stella *et al.*, *Hum Gene Ther* **17**: 1225–1240, with permission from MA Liebert.

干细胞之家www.stemcell8.cn ←点击进入

cells with soluble TRAIL (Debatin and Krammer, 2004). Even more interestingly, caspase activation and PARP cleavage were also observed by exposing the TRAIL-resistant JVM-2 cells to mTRAIL-armed cells but not to soluble TRAIL. The specificity of tumor cell cytotoxicity induced by mTRAIL was demonstrated by the lack of caspase activation or PARP cleavage upon co-culture of KMS-11 or JVM-2 cells with mock-transduced $CD34^+$ cells. Overall, these data demonstrate that the antitumor activity of CD34-$TRAIL^+$ cells effectively depends on TRAIL receptor ligation by mTRAIL-expressing $CD34^+$ cells (Carlo-Stella *et al.*, 2006b).

3.3. *In Vivo Antitumor Activity of CD34-TRAIL$^+$ Cells*

In addition to *in vitro* cell-killing effects, CD34-$TRAIL^+$ cells showed potent *in vivo* tumoricidal activity in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice xenografted with either the TRAIL-sensitive KMS-11 or the TRAIL-resistant JVM-2 and SU-DHL-4V cell lines. Treatment of NOD/SCID mice bearing an advanced-stage KMS-11 xenograft resulted in a significant increase of median survival over controls (83 vs 55 days, $P \leq 0.0001$), with 28% of NOD/SCID mice being alive and disease-free at the end of the 150-day observation period [Fig. 2(A)]. Additionally, mTRAIL-armed cells induced a significantly better survival as compared to soluble TRAIL (83 vs 66 days, $P \leq 0.0001$). CD34-$TRAIL^+$ cells could also overcome TRAIL resistance *in vivo*. In fact, four weekly injections of CD34-$TRAIL^+$ cells resulted in a significant prolongation of the median survival times of mice xenografted with JVM-2 (40 vs 31 days, $P \leq 0.0001$) cells [Fig. 2(B)] and SU-DHL-4V (38 vs 30 days, $P \leq 0.0001$) cells [Fig. 2(C)].

3.4. *In Vivo Toxicity of CD34-TRAIL$^+$ Cells*

The use of soluble TRAIL in the treatment of tumors *in vivo* may result in potential undesirable liver and brain toxicities (Jo *et al.*, 2000; Leverkus *et al.*, 2000; Nitsch *et al.*, 2000). To investigate the potential liver toxicity of CD34-$TRAIL^+$ cells, we monitored on a weekly basis liver enzyme activity in mice receiving a single injection of Ad-TRAIL–transduced cells (5×10^6 cells/mouse). Indeed, these mice failed to show any liver toxicity, as compared to controls (Carlo-Stella *et al.*, 2006b). In contrast, injection of NOD/SCID mice with the Ad-TRAIL vector (1×10^9 pfu/mouse) resulted in an early appearing and long-lasting liver toxicity. The lack of liver toxicity by CD34-$TRAIL^+$ cells reflects tolerability differences when TRAIL is administered as a membrane-bound ligand on the surface of $CD34^+$ cells.

干细胞之家www.stemcell8.cn ←点击进入

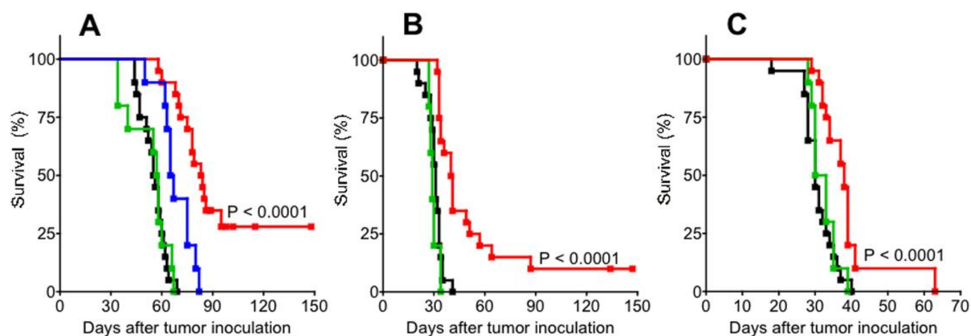


Fig. 2. *In vivo* antitumor activity of CD34-TRAIL$^+$ cells. **(A)** Survival curves of NOD/SCID mice xenografted with the TRAIL-sensitive KMS-11 cell line (0.5×10^6 cells/mouse) and treated for an advanced-stage disease. Mice received four weekly intravenous injections of PBS ($n = 20$, black line), CD34-mock cells ($n = 20$, green line), CD34-TRAIL$^+$cells ($n = 20$, red line), and soluble TRAIL ($n = 10$, blue line). Weekly administrations of mock- or Ad-TRAIL–transduced CD34$^+$ cells (1×10^6 cells/mouse) were started 14 days after tumor injection. Mice treated with recombinant soluble TRAIL received four weekly subcutaneous injections (50 μg/mouse/injection) starting 14 days after tumor inoculation. **(B)** Survival curves of NOD/SCID mice xenografted with the TRAIL-resistant JVM-2 (1×10^6 cells/mouse) cell line. **(C)** Survival curves of NOD/SCID mice xenografted with the TRAIL-resistant SU-DHL-4V (1×10^6 cells/mouse) cell line. For experiments shown in panels **(B)** and **(C)**, mice received four weekly intravenous injections of PBS ($n = 20$, black line), CD34-mock cells ($n = 10$, green line) and CD34-TRAIL$^+$ cells ($n = 20$, red line). Weekly administrations of mock- or Ad-TRAIL–transduced CD34$^+$ cells (1×10^6 cells/mouse) were started on day 1 after tumor inoculation. Based on Figs. 5(b)–5(d) of: Carlo-Stella *et al.*, *Hum Gene Ther* **17**: 1225–1240 with permission from MA Liebert.

Additional studies were carried out using an adenovector encoding murine TRAIL. The intravenous injection of 1×10^9 pfu/mouse induced an average AST and ALT increase of 20- and 65-fold, respectively (Carlo-Stella *et al.*, 2006b). Conversely, mice receiving 5×10^6 CD34$^+$ cells transduced with the same vector, and expressing high levels of the murine TRAIL, failed to show evidence of liver toxicity. These experiments, which closely mimic a clinical scenario of somatic cell therapy, suggest that cell-mediated mTRAIL administration does not result in liver toxicity, the greatest toxicity concern for systemically administered TNF family members.

3.5. *Mechanism(s) of Action of CD34-TRAIL$^+$ Cells*

To gain insight into the antitumor mechanism(s) of mTRAIL-armed cells, we initially addressed the issue of tumor homing of transduced cells by injecting CD34-TRAIL$^+$ cells in NOD/SCID mice bearing subcutaneous tumor

干细胞之家www.stemcell8.cn ←点击进入

nodules. Following a single intravenous injection of CD34-TRAIL$^+$ cells, nodules were excised and immunostained with the anti-human CD45 antibody. Transduced cells were detected in the tumors as early as 30 min following injection, peaked after 24 h and persisted up to 48 h post-treatment. At peak, the percentage of CD45$^+$ cells per tumor section ranged from 0.2% to 0.4%, as evaluated using the NIH imaging software ImageJ (Carlo-Stella *et al.*, 2006b).

We then determined histological changes associated with administration of CD34-TRAIL$^+$ cells to mice bearing subcutaneous tumors. Consistent with the early tumor homing of transduced cells, hematoxylin and eosin staining revealed that injection of CD34-TRAIL$^+$ cells, but not mock-transduced CD34$^+$ cells, induced tumor hemorrhage that could be detected as early as 0.5 h following treatment and necrosis which was detectable 5 h following infusion (Fig. 3). Hemorrhagic and necrotic findings initially detected in the peripheral area of the tumor extended over time to the inner

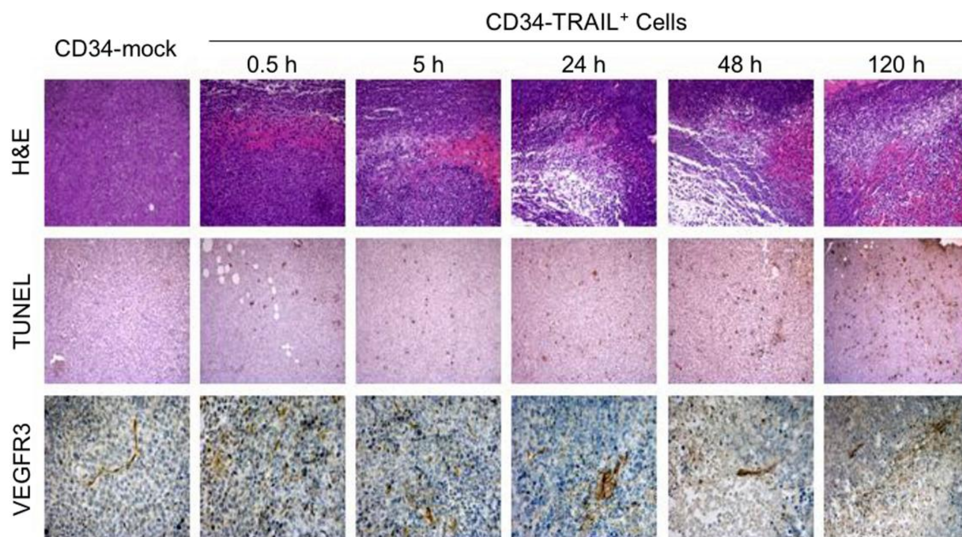


Fig. 3. Injection of CD34-TRAIL$^+$ cells induces tumor apoptosis, necrosis and endothelial cell damage. Hematoxylin and eosin (H&E) (magnification, ×20), TUNEL (magnification, ×20) and anti-mouse VEGFR-3 (magnification, ×40) staining of sections from tumor nodules growing in mice injected subcutaneously with the KMS-11 cell line. Tumor nodules (approximately 10 mm in diameter) were excised and stained at different time points following injections of mock-transduced CD34$^+$ cells or CD34-TRAIL$^+$ cells (5×10^6/mouse). Injection of mTRAIL-expressing cells was associated with intratumor hemorrhage, apoptosis, necrosis and vascular damage detected as early as 0.5 h following treatment. Based on Fig. 7(c) of: Carlo-Stella *et al.*, *Hum Gene Ther* **17**: 1225–1240, with permission from MA Liebert.

干细胞之家www.stemcell8.cn ←点击进入

portion of the nodule, thus becoming a prominent finding in nodules analyzed 120 h following treatment. TUNEL staining revealed the presence of apoptotic cells, detectable as early as 0.5 h post-treatment and increasing in a time-dependent manner up to 120 h post-injection (Fig. 3). Interestingly, injection of CD34-TRAIL$^+$ cells also induced significant levels of tumor apoptosis in mice bearing subcutaneous nodules of the TRAIL-resistant JVM-2 cell line (Carlo-Stella *et al.*, 2006b).

Since the early appearing apoptotic and necrotic areas detected in mice receiving CD34-TRAIL$^+$ cells were distributed along the tumor vasculature, we analyzed tumor blood vessels by staining endothelial cells with a monoclonal antibody directed against the vascular endothelial growth factor receptor-3 (VEGFR3). As early as 0.5 h following injection of CD34-TRAIL$^+$ cells, we observed signs of vascular damage leading to a progressive disintegration of the vascular bed, which co-localized with apoptotic and necrotic phenomena (Fig. 3). Staining with an anti–TRAIL-R2 antibody showed that this receptor is widely expressed on tumor endothelial cells, although with variable intensity, suggesting that tumor endothelial cells represent an early target of CD34-TRAIL$^+$ cells, and that binding of these cells to endothelial TRAIL-R2 might represent the first step of a complex cascade of events leading to tumor cell death (Carlo-Stella *et al.*, 2006b).

4. Conclusions

4.1. *CD34$^+$ Cells Target Disseminated Lympho-hematopoietic Tumors*

Genetically modified neural or mesenchymal stem cells have been demonstrated to exert an efficient antitumor activity following intratumor or systemic injection in xenograft models of glioma (Ehtesham *et al.*, 2002a and 2002b; Lee *et al.*, 2003; Nakamizo *et al.*, 2005), melanoma (Studeny *et al.* 2002) and breast cancer (Studeny *et al.*, 2004). Our data demonstrated that targeting of disseminated lympho-hematopoietic tumors can also be achieved by intravenous injection of CD34$^+$ cells. These cells are already widely used in the clinic (Carlo-Stella *et al.*, 2006b) and might indeed easily become a therapeutically applicable approach.

CD34$^+$ cells were selected as vehicles for mTRAIL delivery due to their migratory properties as well as then capacity of specifically interacting with endothelial cells through receptor-mediated mechanisms (Verfaillie, 1998; Lapidot *et al.*, 2005). Due to inflammatory characteristics of tumor microenvironment, systemically delivered CD34$^+$ cells can efficiently home in the

干细胞之家www.stemcell8.cn ←点击进入

tumor by rolling on tumor vasculature, adhering to it and finally extravasating into the tumor (Jin *et al.*, 2006). In our study, genetically modified CD34$^+$ cells exhibited a tumor homing capacity similar to the bone marrow homing capacity of CD34$^+$ cells injected in non-irradiated recipients (De Palma *et al.*, 2003; Quesenberry *et al.*, 2005). *In vivo* experiments with blocking antibodies (data not shown) demonstrated that tumor homing of CD34-TRAIL$^+$ cells involves VLA-4/VCAM-1 and CXCR4/SDF-1 interactions (Burger and Kipps, 2006; Jin *et al.*, 2006).

4.2. *Hemorrhagic Necrosis/Apoptosis of TRAIL-Sensitive/Resistant Tumors*

Tumor homing of CD34-TRAIL$^+$ cells is followed by a direct tumor cell-killing effect due to apoptosis triggering. However, several lines of evidence suggest that CD34-TRAIL$^+$ cells also act through an indirect tumor cell-killing mechanism, most likely consisting in a potent vascular-disrupting activity. In fact, tumor vascular endothelium expresses TRAIL-R2 at a high level. Moreover, as early as 30 min after a single intravenous injection of CD34-TRAIL$^+$ cells, tumor cell apoptosis and necrosis exhibited a characteristic perivascular distribution along the coronal vessels at the periphery of the tumor nodule. Thus, binding of CD34-TRAIL$^+$ cells to vascular endothelial TRAIL-R2 might be the first step in a complex cascade involving intravascular tumor homing of mTRAIL-armed cells, followed by endothelial cell binding, which in turn induces extensive vascular damage and hemorrhagic necrosis of the tumor.

On reacting with either TRAIL-R1– and/or TRAIL-R2–expressing tumor cells, CD34-TRAIL$^+$ cells exhibited high killing activity even at a low effector/target ratio on a variety of tumor cell types. Target cell death occurred by apoptosis and did not correlate with the level of surface expression of TRAIL receptors. Finally, and most important, CD34-armed cells were highly cytotoxic against tumor cells resistant to soluble TRAIL. CD34-TRAIL$^+$ cells also showed potent *in vivo* tumoricidal efficacy. *In vivo*, repeated dosing with mTRAIL-armed cells resulted in a significant survival prolongation of tumor-bearing mice. Interestingly, in mice with an advanced-stage KMS-11 xenograft, the antitumor activity of mTRAIL-armed cells was higher than that induced by soluble TRAIL. A therapeutic activity of mTRAIL-expressing cells could also be detected in mice xenografted with TRAIL-resistant cell lines, suggesting that transduced cells might at least partially overcome resistance to soluble TRAIL.

干细胞之家www.stemcell8.cn ←点击进入

4.3. CD34-TRAIL+ Cells as a p53-Independent Apoptotic Death Strategy

Since death receptor activation can instruct malignant cells to undergo apoptosis independent of p53, targeting death receptors with TRAIL-targeting therapeutics represents a rational therapeutic strategy against cancer. According to experimental data obtained thus far, TRAIL-targeting therapeutics with the soluble ligand or monoclonal antibodies possess considerable and specific anti-tumor activity, both when used alone as well as in combination with nonspecific cytotoxic agents, radiation and other target-based therapeutics. Gene therapy approaches using adenoviral-transduced CD34+ cells for the delivery of membrane-bound TRAIL might result in a significant enhancement of the therapeutic potential of TRAIL. These approaches represent a challenging strategy that might achieve systemic tumor targeting and efficient intratumor delivery of the therapeutic agent.

Acknowledgments

This work was supported in part by grants from Ministero dell'Università e della Ricerca (MUR, Rome, Italy), Ministero della Salute (Rome, Italy) and Michelangelo Foundation for Advances in Cancer Research and Treatment (Milano, Italy).

References

Almasan A, Ashkenazi A. (2003) Apo2L/TRAIL: apoptosis signaling, biology, and potential for cancer therapy. *Cytokine Growth Factor Rev* **14**: 337–48.

Armeanu S, Lauer UM, Smirnow I, *et al.* (2003) Adenoviral gene transfer of tumor necrosis factor-related apoptosis-inducing ligand overcomes an impaired response of hepatoma cells but causes severe apoptosis in primary human hepatocytes. *Cancer Res* **63**: 2369–72.

Ashkenazi A. (2002) Targeting death and decoy receptors of the tumour-necrosis factor superfamily. *Nat Rev Cancer* **2**: 420–30.

Ashkenazi A, Pai RC, Fong S, *et al.* (1999) Safety and antitumor activity of recombinant soluble Apo2 ligand. *J Clin Invest* **104**: 155–62.

Bregni M, Shammah S, Malaffo F, *et al.* (1998) Adenovirus vectors for gene transduction into mobilized blood CD34+ cells. *Gene Ther* **5**: 465–72.

Buchsbaum DJ, Zhou T, Lobuglio AF. (2006) TRAIL receptor-targeted therapy. *Future Oncol* **2**: 493–508.

Burger JA, Kipps TJ. (2006) CXCR4: a key receptor in the crosstalk between tumor cells and their microenvironment. *Blood* **107**: 1761–67.

干细胞之家www.stemcell8.cn ←点击进入

Carlo-Stella C, Di Nicola M, Turco MC, *et al.* (2006a) The anti-human leukocyte antigen-DR monoclonal antibody 1D09C3 activates the mitochondrial cell death pathway and exerts a potent antitumor activity in lymphoma-bearing nonobese diabetic/severe combined immunodeficient mice. *Cancer Res* **66**: 1799–808.

Carlo-Stella C, Lavazza C, Di Nicola M, *et al.* (2006b) Antitumor activity of human CD34(+) cells expressing membrane-bound tumor necrosis factor-related apoptosis-inducing ligand. *Hum Gene Ther* **17**: 1225–40.

Chinnaiyan AM, Prasad U, Shankar S, *et al.* (2000) Combined effect of tumor necrosis factor-related apoptosis-inducing ligand and ionizing radiation in breast cancer therapy. *Proc Natl Acad Sci USA* **97**: 1754–59.

Cretney E, Takeda K, Yagita H, *et al.* (2002) Increased susceptibility to tumor initiation and metastasis in TNF-related apoptosis-inducing ligand-deficient mice. *J Immunol* **168**: 1356–61.

De Palma M, Venneri MA, Roca C, Naldini L. (2003) Targeting exogenous genes to tumor angiogenesis by transplantation of genetically modified hematopoietic stem cells. *Nat Med* **9**: 789–95.

Debatin KM, Krammer PH. (2004) Death receptors in chemotherapy and cancer. *Oncogene* **23**: 2950–66.

Degli-Esposti MA, Dougall WC, Smolak PJ, *et al.* (1997) The novel receptor TRAIL-R4 induces NF-kappaB and protects against TRAIL-mediated apoptosis, yet retains an incomplete death domain. *Immunity* **7**: 813–20.

Ehtesham M, Kabos P, Gutierrez MAR, *et al.* (2002a) Induction of glioblastoma apoptosis using neural stem cell-mediated delivery of tumor necrosis factor-related apoptosis-inducing ligand. *Cancer Res* **62**: 7170–74.

Ehtesham M, Kabos P, Kabosova A, *et al.* (2002b) The use of interleukin 12-secreting neural stem cells for the treatment of intracranial glioma. *Cancer Res* **62**: 5657–63.

Emery JG, Mcdonnell P, Burke MB, *et al.* (1998) Osteoprotegerin is a receptor for the cytotoxic ligand TRAIL. *J Biol Chem* **273**: 14363–67.

Fulda S, Wick W, Weller M, Debatin K-M. (2002) Smac agonists sensitize for Apo2L/TRAIL- or anticancer drug-induced apoptosis and induce regression of malignant glioma *in vivo*. *Nat Med* **8**: 808–15.

Gajewski TF. (2007) On the TRAIL toward death receptor-based cancer therapeutics. *J Clin Oncol* **25**: 1305–307.

Griffith TS, Anderson RD, Davidson BL, *et al.* (2000) Adenoviral-mediated transfer of the TNF-related apoptosis-inducing ligand/Apo-2 ligand gene induces tumor cell apoptosis. *J Immunol* **165**: 2886–94.

Griffith TS, Broghammer EL. (2001) Suppression of tumor growth following intralesional therapy with TRAIL recombinant adenovirus. *Mol Ther* **4**: 257–66.

Hao C, Song JH, Hsi B, *et al.* (2004) TRAIL inhibits tumor growth but is nontoxic to human hepatocytes in chimeric mice. *Cancer Res* **64**: 8502–506.

Harrington K, Alvarez-Vallina L, Crittenden M, *et al.* (2002) Cells as vehicles for cancer gene therapy: the missing link between targeted vectors and systemic delivery? *Hum Gene Ther* **13**: 1263–80.

干细胞之家www.stemcell8.cn ←点击进入

Hasegawa H, Yamada Y, Harasawa H, *et al.* (2005) Sensitivity of adult T-cell leukaemia lymphoma cells to tumour necrosis factor-related apoptosis-inducing ligand. *Br J Haematol* **128**: 253–65.

Holen I, Croucher PI, Hamdy FC, Eaton CL. (2002) Osteoprotegerin (OPG) is a survival factor for human prostate cancer cells. *Cancer Res* **62**: 1619–23.

Jin H, Aiyer A, Su J, *et al.* (2006) A homing mechanism for bone marrow-derived progenitor cell recruitment to the neovasculature. *J Clin Invest* **116**: 652–62.

Jo M, Kim TH, Seol DW, *et al.* (2000) Apoptosis induced in normal human hepatocytes by tumor necrosis factor-related apoptosis-inducing ligand. *Nat Med* **6**: 564–67.

Johnson TR, Stone K, Nikrad M, *et al.* (2003) The proteasome inhibitor PS-341 overcomes TRAIL resistance in Bax and caspase 9-negative or Bcl-xL overexpressing cells. *Oncogene* **22**: 4953–63.

Johnston JB, Kabore AF, Strutinsky J, *et al.* (2003) Role of the TRAIL/APO2-L death receptors in chlorambucil- and fludarabine-induced apoptosis in chronic lymphocytic leukemia. *Oncogene* **22**: 8356–69.

Kagawa S, He C, Gu J, *et al.* (2001) Antitumor activity and bystander effects of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) gene. *Cancer Res* **61**: 3330–38.

Kaplan RN, Riba RD, Zacharoulis S, *et al.* (2005) VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. *Nature* **438**: 820–27.

Kaufmann SH, Steensma DP. (2005) On the TRAIL of a new therapy for leukemia. *Leukemia* **19**: 2195–202.

Laconi E, Pani P, Farber E. (2000) The resistance phenotype in the development and treatment of cancer. *Lancet Oncol* **1**: 235–41.

Lamhamedi-Cherradi SE, Zheng SJ, Maguschak KA, *et al.* (2003) Defective thymocyte apoptosis and accelerated autoimmune diseases in TRAIL$^{-/-}$ mice. *Nat Immunol* **4**: 255–60.

Lapidot T, Dar A, Kollet O. (2005) How do stem cells find their way home? *Blood* **106**: 1901–10.

Lawrence D, Shahrokh Z, Marsters S, *et al.* (2001) Differential hepatocyte toxicity of recombinant Apo2L/TRAIL versions. *Nat Med* **7**: 383–85.

Leblanc H, Lawrence D, Varfolomeev E, *et al.* (2002) Tumor-cell resistance to death receptor–induced apoptosis through mutational inactivation of the proapoptotic Bcl-2 homolog Bax. *Nat Med* **8**: 274–81.

Leblanc HN, Ashkenazi A. (2003) Apo2L/TRAIL and its death and decoy receptors. *Cell Death Differ* **10**: 66–75.

Lee J, Elkahloun AG, Messina SA, *et al.* (2003) Cellular and genetic characterization of human adult bone marrow-derived neural stem-like cells: a potential antiglioma cellular vector. *Cancer Res* **63**: 8877–89.

Lee J, Hampl M, Albert P, Fine HA. (2002) Antitumor activity and prolonged expression from a TRAIL-expressing adenoviral vector. *Neoplasia (New York, NY)* **4**: 312–23.

干细胞之家www.stemcell8.cn ←点击进入

Leverkus M, Neumann M, Mengling T, *et al.* (2000) Regulation of tumor necrosis factor-related apoptosis-inducing ligand sensitivity in primary and transformed human keratinocytes. *Cancer Res* **60**: 553–59.

Lucken-Ardjomande S, Martinou JC. (2005) Newcomers in the process of mitochondrial permeabilization. *J Cell Sci* **118**: 473–83.

Mariani SM, Matiba B, Armandola EA, Krammer PH. (1997) Interleukin 1 beta-converting enzyme related proteases/caspases are involved in TRAIL-induced apoptosis of myeloma and leukemia cells. *J Cell Biol* **137**: 221–29.

Marini P. (2006) Drug evaluation: lexatumumab, an intravenous human agonistic mAb targeting TRAIL receptor 2. *Curr Opin Mol Ther* **8**: 539–46.

Mathas S, Lietz A, Anagnostopoulos I, *et al.* (2004) c-FLIP mediates resistance of Hodgkin/Reed-Sternberg cells to death receptor-induced apoptosis. *J Exp Med* **199**: 1041–52.

Mitsiades CS, Treon SP, Mitsiades N, *et al.* (2001) TRAIL/Apo2L ligand selectively induces apoptosis and overcomes drug resistance in multiple myeloma: therapeutic applications. *Blood* **98**: 795–804.

Mouzakiti A, Packham G. (2003) Regulation of tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in Burkitt's lymphoma cell lines. *Br J Haematol* **122**: 61–69.

Muhlenbeck F, Schneider P, Bodmer JL, *et al.* (2000) The tumor necrosis factor-related apoptosis-inducing ligand receptors TRAIL-R1 and TRAIL-R2 have distinct cross-linking requirements for initiation of apoptosis and are non-redundant in JNK activation. *J Biol Chem* **275**: 32208–13.

Nakamizo A, Marini F, Amano T, *et al.* (2005) Human bone marrow-derived mesenchymal stem cells in the treatment of gliomas. *Cancer Res* **65**: 3307–18.

Nakata S, Yoshida T, Horinaka M, *et al.* (2004) Histone deacetylase inhibitors upregulate death receptor 5/TRAIL-R2 and sensitize apoptosis induced by TRAIL/APO2-L in human malignant tumor cells. *Oncogene* **23**: 6261–71.

Nitsch R, Bechmann I, Deisz RA, *et al.* (2000) Human brain-cell death induced by tumour-necrosis-factor-related apoptosis-inducing ligand (TRAIL). *Lancet* **356**: 827–28.

Pan G, Ni J, Wei YF, *et al.* (1997a) An antagonist decoy receptor and a death domain-containing receptor for TRAIL. *Science* **277**: 815–18.

Pan G, O'rourke K, Chinnaiyan AM, *et al.* (1997b) The receptor for the cytotoxic ligand TRAIL. *Science* **276**: 111–13.

Pitti RM, Marsters SA, Ruppert S, *et al.* (1996) Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. *J Biol Chem* **271**: 12687–90.

Pollack IF, Erff M, Ashkenazi A. (2001) Direct stimulation of apoptotic signaling by soluble Apo2L/tumor necrosis factor-related apoptosis-inducing ligand leads to selective killing of glioma cells. *Clin Cancer Res* **7**: 1362–69.

干细胞之家www.stemcell8.cn ←点击进入

Pommier Y, Sordet O, Antony S, *et al.* (2004) Apoptosis defects and chemotherapy resistance: molecular interaction maps and networks. *Oncogene* **23**: 2934–49.

Quesenberry PJ, Colvin G, Abedi M. (2005) Perspective: fundamental and clinical concepts on stem cell homing and engraftment: a journey to niches and beyond. *Exp Hematol* **33**: 9–19.

Ronchetti D, Greco A, Compasso S, *et al.* (2001) Deregulated FGFR3 mutants in multiple myeloma cell lines with t(4;14): comparative analysis of Y373C, K650E and the novel G384D mutations. *Oncogene* **20**: 3553–62.

Rowinsky EK. (2005) Targeted induction of apoptosis in cancer management: the emerging role of tumor necrosis factor-related apoptosis-inducing ligand receptor activating agents. *J Clin Oncol* **23**: 9394–407.

Scaffidi C, Fulda S, Srinivasan A, *et al.* (1998) Two CD95 (APO-1/Fas) signaling pathways. *EMBO J* **17**: 1675–87.

Sheridan JP, Marsters SA, Pitti RM, *et al.* (1997) Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. *Science* **277**: 818–21.

Smyth MJ, Takeda K, Hayakawa Y, *et al.* (2003) Nature's TRAIL — on a path to cancer immunotherapy. *Immunity* **18**: 1–6.

Studeny M, Marini FC, Champlin RE, *et al.* (2002) Bone marrow-derived mesenchymal stem cells as vehicles for interferon-beta delivery into tumors. *Cancer Res* **62**: 3603–608.

Studeny M, Marini FC, Dembinski JL, *et al.* (2004) Mesenchymal stem cells: potential precursors for tumor stroma and targeted-delivery vehicles for anticancer agents. *J Natl Cancer Inst* **96**: 1593–603.

Tolcher AW, Mita M, Meropol NJ, *et al.* (2007) Phase I pharmacokinetic and biologic correlative study of mapatumumab, a fully human monoclonal antibody with agonist activity to tumor necrosis factor-related apoptosis-inducing ligand receptor-1. *J Clin Oncol* **25**: 1390–95.

Verfaillie CM. (1998) Adhesion receptors as regulators of the hematopoietic process. *Blood* **92**: 2609–12.

Verhagen AM, Vaux DL. (2002) Cell death regulation by the mammalian IAP antagonist Diablo/Smac. *Apoptosis* **7**: 163–66.

Wajant H. (2003) Death receptors. *Essays Biochem* **39**: 53–71.

Wajant H, Moosmayer D, Wuest T, *et al.* (2001) Differential activation of TRAIL-R1 and -2 by soluble and membrane TRAIL allows selective surface antigen-directed activation of TRAIL-R2 by a soluble TRAIL derivative. *Oncogene* **20**: 4101–106.

Walczak H, Degli-Esposti MA, Johnson RS, *et al.* (1997) TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL. *EMBO J* **16**: 5386–97.

Walczak H, Miller RE, Ariail K, *et al.* (1999) Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand *in vivo*. *Nat Med* **5**: 157–63.

Wang S, El-Deiry WS. (2003) TRAIL and apoptosis induction by TNF-family death receptors. *Oncogene* **22**: 8628–33.

干细胞之家www.stemcell8.cn ←点击进入

Waxman DJ, Schwartz PS. (2003) Harnessing apoptosis for improved anticancer gene therapy. *Cancer Res* **63**: 8563–72.

Wen J, Ramadevi N, Nguyen D, *et al.* (2000) Antileukemic drugs increase death receptor 5 levels and enhance Apo-2L-induced apoptosis of human acute leukemia cells. *Blood* **96:** 3900–906.

Wiley SR, Schooley K, Smolak PJ, *et al.* (1995) Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity* **3**: 673–82.

Zhang L, Fang B. (2005) Mechanisms of resistance to TRAIL-induced apoptosis in cancer. *Cancer Gene Ther* **12:** 228–37.

干细胞之家www.stemcell8.cn ←点击进入

Chapter 15

Mesenchymal Stem Cells as Vehicles for Delivering Therapeutics and Oncolytic Viruses

Larisa Pereboeva*,†,‡ and Justin Roth†

Advanced understanding of adult stem cell biology as well as refined isolation and culturing techniques have led to an increased use of stem cells in clinical applications, particularly for tissue repair and regeneration. Stem cells have also demonstrated a great utility in disease settings as cell-based vehicles for delivering therapeutics. Among stem cell types, mesenchymal stem cells (MSCs) have great appeal for use in tissue engineering and other therapeutic applications because they are easily isolated from several tissues, are usually multipotent and have an inherent tropism for injured/healing foci as well as tumor bed. This review highlights the current state of MSC knowledge relevant to their application as cell vehicles, including their isolation, characterization and biodistribution. Examples of MSC-based delivery in different experimental settings are described, including their utility as vehicles for targeted delivery of anti-tumor agents such as toxic genes and oncolytic viruses.

Keywords: Mesenchymal stem cells; mesenchymal stromal cells; cell vehicles; oncolytic viruses; CRAds; tumor-targeted delivery.

1. Introduction: Cells as Vectors for Therapeutic Delivery

Delivery of therapeutics is a vast field embracing different areas of biological and medical science. In general, therapeutic agents can be introduced

*Corresponding author. Larisa.Pereboeva@ccc.uab.edu
†Division of Human Gene Therapy, Departments of Medicine, Obstetrics and Gynecology, Pathology, and Surgery, and the Gene Therapy Center, University of Alabama at Birmingham, Birmingham, AL 35294-2172, USA
‡Division of Molecular and Cellular Pathology, Department of Pathology, University of Alabama at Birmingham, Birmingham, AL 35294, USA

干细胞之家www.stemcell8.cn ←点击进入

either directly or with the help of a vehicle that serves to enhance delivery. Cell-based delivery of therapeutics has been proposed based on several unique features of cell carriers: 1) the possibility to utilize autologous cells to avoid an immune response to the carrier; 2) the possibility of reducing the toxicity associated with systemic administration of biotherapeutics; 3) the possibility for *ex vivo* manipulations (isolation and expansion of a specific cell type/subtype and/or genetic modifications); 4) the possibility to serve as intermediate carriers to preserve a therapeutic load (e.g., cytokines, viruses) from degradation by the body's defense systems; and 5) the opportunity to utilize native cellular-specific homing properties for targeted delivery of therapeutics including intrinsic secretions and oncolytic viruses, thereby releasing the therapeutics locally for maximized activity.

The feasibility of utilizing various cell types as vehicles for gene delivery has been widely demonstrated. However, several important parameters have to be considered in order to translate cell-based protocols from the laboratory to a clinical setting (Fig. 1). First, the cells should be accessible using simple and non-invasive procedures. Once isolated, the cells should have the capacity for *ex vivo* expansion, without a loss of phenotype. Finally, the cells should be amenable to genetic manipulations, or efficiently loaded with the therapeutic agent to be delivered. Despite discrepancies in the definition, isolation procedures, and characterization of true mesenchymal stem cells, which will be described below, there is common agreement that multipotent mesenchymal stromal or stem cells have several properties which advocate their growing use in cell and gene therapy applications. Mesenechymal stem cells (MSCs) are easy to isolate and culture, and usually have a great proliferative capacity. Thus, for the most part, MSCs meet the criteria established for clinically relevant cell types used for cell-based therapies.

2. Mesenchymal Stem Cells (MSCs)

2.1. *Definition: Mesenchymal Stem Cells or Multipotent Mesenchymal Stromal Cells?*

The nonhematopoietic cellular compartment of the bone marrow (BM) has been the subject of intensive investigation over the past decade. These cells are variously designated in different studies as mesenchymal stromal cells, marrow stromal cells, or mesenchymal stem cells, which are all abbreviated as "MSCs." The variety of names and definitions that describe this cell type reflects our limited understanding of MSC biology. MSC characteristics differ among laboratories and species, which hampers data comparison. This

干细胞之家www.stemcell8.cn ←点击进入

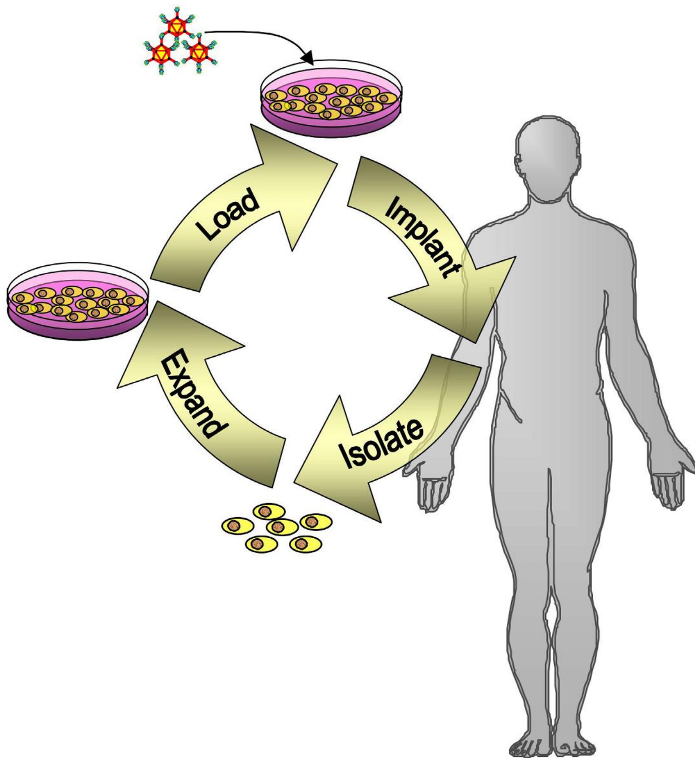


Fig. 1. Cells as therapeutic delivery vehicles. Cells can be used as therapeutic delivery vehicles. This strategy relies on the ability to isolate, expand and load a cell with a therapeutic agent. The loaded cells are then re-infused into the patient for localized delivery of the therapeutic payload.

is often caused by variations in isolation and culturing conditions (Wagner *et al.*, 2006). To date, there is no specific marker or combination of markers that is uniformly applied to characterize or assess the presence of true MSCs in any given population. Several attempts have been undertaken to outline the minimal criteria to define human MSCs (Horwitz *et al.*, 2005; Dominici *et al.*, 2006). The International Society for Cellular Therapy (ISCT) has recently clarified the nomenclature, to some extent, by designating the cells as "multipotent mesenchymal stromal cells" (still MSCs), and recognizes that not all MSCs are stem cells (Dominici *et al.*, 2006). The minimum criteria for defining multipotent mesenchymal stromal cells are: (1) plastic-adherent cells under standard culture conditions; (2) express, CD105, CD73 and CD90, and lack of expression of CD45, CD34, CD14 or CD11b, CD79alpha or CD19, and HLA-DR surface molecules; and (3) differentiation into osteoblasts, adipocytes and chondroblasts *in vitro*. The source tissue from which the

干细胞之家www.stemcell8.cn ←点击进入

MSCs were isolated should also be included in the terminology. Therefore, MSCs are currently defined by a combination of physical, morphological and functional properties. We favor the term "multipotent mesenchymal stromal cells" in accordance with the current ISCT recommendations, and with acknowledgment of the fact that a majority of the published studies have utilized this cell population. However, for the purpose of cohesiveness throughout the review, we will broadly label both mesenchymal stromal and mesenchymal stem cells as "MSCs," regardless of the name given in the referenced study.

2.2. *Methods of Isolation and Culturing*

Several distinct protocols are used for MSC isolation and culturing, aiming at maximizing cell proliferation without losing multipotency. The protocols vary based on the techniques used for isolation, the selection of basic medium, and the source and concentration of fetal bovine serum (FBS). The general growth medium is typically Dulbecco's modified Eagle's medium (DMEM) or α-minimum essential medium (α-MEM) with 10% to 20% fetal bovine serum (FBS) (Pittenger *et al.*, 1999; Digirolamo *et al.*, 1999). Most isolation protocols involve density centrifugation of bone marrow aspirates, followed by plastic adherence-based selection of MSCs (Owen, 1988; Friedenstein *et al.*, 1992; Caplan *et al.*, 1998). However, several other cell types, such as macrophages, endothelial cells, lymphocytes, and smooth muscle cells, can also adhere to plastic and contaminate early MSC cultures (Clark and Keating, 1995; Deans and Moseley, 2000). Elimination of such contaminants can be achieved by using well-established protocols (Digirolamo *et al.*, 1999). In some cases, MSCs are further purified based on the expression of the primitive MSC marker, STRO-1 (Gronthos *et al.*, 2003). Variations in initial plating densities have also been reported, ranging from 1–1000 cells/cm^2 (Prockop *et al.*, 2001; Sekiya *et al.*, 2002) to 1–4 $\times$ 10^6 cells/cm^2 (Pittenger *et al.*, 1999), which may affect the properties of the resulting culture. MSCs in culture are typically described as small, adherent, spindle-shaped cells. However, morphologically heterogeneous cultures containing polygonal and fibroblast-like cells are also commonly observed.

MSCs represent a very small fraction of BM cells, ranging from 0.001–0.01% of the total population of nucleated BM cells. However, cells can be expanded from a few milliliters of bone aspirate up to millions after several passages, with little differentiation for at least 6–10 passages (Pittenger *et al.*, 1999; Spees *et al.*, 2004). MSCs can grow up to 24–40 population doublings

干细胞之家www.stemcell8.cn ←点击进入

in vitro under widely described culture conditions, but the proliferative capacity is inversely proportional to the age of the donor (Stenderup *et al.*, 2003; Sethe *et al.*, 2006). Different MSC populations demonstrate varying propensities toward senescence. For instance, human MSCs senesce after approximately 40 population doublings, whereas senescence of rat MSCs has not been observed (Digirolamo *et al.*, 1999; Javazon *et al.*, 2001). Changes in MSC characteristics occur with increased time in culture, reducing their therapeutic potential. During culture expansion, MSCs undergo an aging process that involves a gradual loss of their early progenitor characteristics, such as proliferation, homing capability (Banfi *et al.*, 2000; Rombouts and Ploemacher, 2003) and telomere length (Baxter *et al.*, 2004; Bonab *et al.*, 2006).

2.3. *MSC Markers*

Cultured MSCs have been extensively characterized in regard to surface and molecular markers. MSCs are generally negative for hematopoietic surface markers including CD34, CD45, CD14, CD31 and CD133 (Pittenger *et al.*, 1999). The CD34 expression status in murine MSCs varies (Javazon *et al.*, 2001; Peister *et al.*, 2004), but human and rat MSCs have been shown to be $CD34^-$ negative. MSCs also express numerous surface adhesion molecules such as CD29, CD44, CD49e, CD58, CD62 and CD106 (VCAM-1), and are typically positive for MHC I, CD90 (Thy-1) and Sca-1 (Majumdar *et al.*, 2003; Deans and Moseley, 2000). However, due to heterogeneity between species and within cultures, variable expression of CD90 (Thy1.1), CD117 (c-kit), SH2 (CD105 or endoglin), SH3, SH4 (CD73) and STRO-1 have been observed (Haynesworth *et al.*, 1992a and 1992b; Pittenger *et al.*, 1999). Antibodies against SH2, SH3, SH4 and STRO-1 are most specific for BM-derived MSCs, and do not react with BM-derived hematopoietic cells, osteoblasts or osteocytes (Barry *et al.*, 2001). Isolation of MSCs by specific antigens offers the potential for more accurate isolation methods, compared to plastic adherence. However, there is currently no consensus over a single marker or a combination of markers that allows for a pure MSC population to be isolated, besides the minimal criteria proposed by ISCT mentioned above. Phenotypic criteria alone are not sufficient to specifically identify MSCs and, therefore, have to be assessed along with their functional properties. The capacity for *in vitro* differentiation into several cell types, such as osteoblasts, adipocytes and chondrocytes (Pittenger *et al.*, 1999) is the most stringent method for characterizing an MSC population. The typical default differentiation pathway for most MSCs in culture is osteogenesis (Jaiswal

干细胞之家www.stemcell8.cn ←点击进入

et al., 1997; Phinney *et al.*, 1999a); however, depending on the culture conditions, they can also be induced to differentiate into non-mesoderm-type cells (Zhao *et al.*, 2002; Minguell *et al.*, 2005; Sanchez-Ramos *et al.*, 2000). The multipotentiality of MSCs can also be demonstrated *in vivo* by transplanting the cells subcutaneously into immunodeficient mice and confirming their ability to form bone, adipocytes and hematopoietic BM-supporting stroma (Friedenstein *et al.*, 1987; Haynesworth *et al.*, 1992b).

2.4. *Subtypes*

Different isolation methods have been attempted in order to obtain more homogeneous MSC populations. Gronthos *et al.* developed a monoclonal antibody, Stro-1, which has been used to isolate a population of cells with characteristics similar to MSCs (Gronthos *et al.*, 1994). Rare multipotent adult progenitor cells (MAPCs) can also be obtained from human BM (Jiang *et al.*, 2002; Reyes and Verfaillie, 2001). MAPCs can be identified in cultured MSCs only after the cells undergo approximately 30 or more population doublings. The MAPC population is characterized by rapid proliferation and by their ability to differentiate into multiple mesenchymal and hematopoietic cell types, which demonstrates that they are a truly primitive stem cell population. MAPC cells are selected from BM mononuclear cells by depletion of CD45 and glycophorin-A–expressing cells (Gly-A). MAPCs can be cultured for more than 70 doublings and have long telomeres that do not shorten during culture, in contrast to MSCs. To date, the relationship between the two cell types has not been determined and it is not clear whether MAPCs are a small rare subpopulation of MSCs or a new cell population developed during its purification.

Finally, MIAMI (marrow-isolated adult multilineage inducible) cells were isolated from whole BM cells by selective adhesion to fibronectin-coated plates in the presence of reduced serum conditions and under low oxygen tension (D'Ippolito *et al.*, 2006). These specific culture conditions were used to mimic the microenvironmental conditions in which the more primitive cells are expected to be found. The MIAMI cell population is consistently obtained from donors ranging from 3 to 72 years old. In addition, MIAMI cells have been expanded *in vitro* for more than 50 population doublings without detectable changes in their molecular profile. Furthermore, this cell population has the capacity to differentiate into progeny cells with mesodermal, neuroectodermal and endodermal characteristics.

干细胞之家www.stemcell8.cn ←点击进入

2.5. *Species Variations*

Nonhuman mesenchymal stem cells are required for the development of disease models and for the evaluation of MSC-based therapies. Isolation of MSCs has been attempted with variable difficulty from several species, including mice (Short *et al.*, 2001; Peister *et al.*, 2004; Phinney *et al.*, 1999a; Tropel *et al.*, 2004), rats (Javazon *et al.*, 2001), guinea pig (Friedenstein *et al.*, 1970), humans (Pittenger *et al.*, 1999 and 2000), cats (Martin *et al.*, 2002), dogs (Kadiyala *et al.*, 1997), rabbits (Park *et al.*, 2007), pigs (Shake *et al.*, 2002; Makkar *et al.*, 2005), baboons (Bartholomew *et al.*, 2001) and rhesus macaques (Izadpanah *et al.*, 2005). MSCs derived from human, rat, dog and baboon BM are relatively easy to isolate, based on their adherence to plastic. Cells from these species are easily expanded in culture and have been the most extensively characterized for their capacity to differentiate into multiple cell phenotypes, including bone, fat, and cartilage. In contrast, murine MSCs are far more difficult to isolate from bone marrow and to expand in culture, compared to human or rat MSCs (Peister *et al.*, 2004; Phinney *et al.*, 1999b). Unlike human or rat MSCs, murine MSC cultures are frequently contaminated by hematopoietic progenitors that overgrow the cultures. Even after immunodepletion, the cultures expand poorly, possibly due to the accumulation of large amounts of extracellular matrix (Phinney *et al.*, 1999b). This greatly limits the ability to test murine MSCs *in vivo*, which requires large cell numbers. To this end, several methods to improve murine MSC isolation and expansion have been reported (Short *et al.*, 2001; Peister *et al.*, 2004; Phinney *et al.*, 1999a; Tropel *et al.*, 2004; Schrepfer *et al.*, 2007a). Despite the overall improvement in these protocols, isolation and expansion of murine MSCs remains problematic compared to other species. Variations in the growth rate and surface marker profile of murine MSCs from different mouse strains have also been noted (Phinney *et al.*, 1999a; Baddoo *et al.*, 2003). We have encountered difficulties attempting to isolate hamster MSCs (unpublished data). These species-specific differences in MSC biology preclude our ability to develop a standard isolation protocol. However, MSCs from a number of species tested are amenable to conventional adherence-based isolation procedures. This was shown in a study that described the MSC isolation from eight different species: human, baboon, canine, sheep, goat, pig, rabbit and rat (Mosca *et al.*, 2000).

Derivation of MSCs from bone marrow appears to be the most frequently published strategy. However, similar MSC populations have been obtained from alternative sources such as lipoaspirates (Zuk *et al.*, 2002), peripheral and cord blood (Kern *et al.*, 2006; Bieback *et al.*, 2004), and

干细胞之家www.stemcell8.cn ←点击进入

fetal tissues (Charbord *et al.*, 2002). The *in vitro* characteristics of these cell types are similar to those of BM MSCs, including plastic adherence, fibroblast-like morphology, colony-forming unit fibroblast (CFU-F) content, phenotypic characteristics and tridifferentiation potential. Newer studies have also demonstrated the possibility of isolating MSC-like cells from the "stroma" of a number of organs including synovial membranes (De Bari *et al.*, 2004) and deciduous teeth (Miura *et al.*, 2003). It has not been established whether these cells are truly MSCs or whether the application of similar isolation and culture techniques induce them to appear the same *in vitro*.

3. MSC as a Delivery Platform: Biodistribution, Homing and Engraftment

The fate of cells that are introduced into the body for therapeutic purposes will depend on their biodistribution, homing and survival rate. The terms "biodistribution" and "homing" are not clearly defined, and are often used interchangably to describe the same process of cell destination after introduction into the living organism. Whereas biodistribution is mostly a mechanical process of dispersing cell particles throughout the body homing is a rather active process of cell movement in response to certain stimuli or gradients. Cell therapy applications, in addition to these events, rely on the capacity of MSCs to survive long-term by engrafting and proliferating in the appropriate target tissue(s). In such applications, MSCs potentially replenish or substitute local stem cell function, provided that they are delivered to the appropriate niche in the desired tissue. Applied use of MSCs as vehicles for delivering gene therapy products may or may not require actual engraftment, but would still rely on specific biodistribution or homing to deliver the therapeutic payload. Regardless of the possible applications, MSC biodistribution and homing patterns are worthy topics for investigation. This information will be important for considering whether or not these cells have utility for specific therapeutic applications. In this section, we will concentrate mostly on short-term MSC homing and biodistribution in different models to address the key question of cell-based therapeutic delivery. Although the long-term engraftment and site-specific differentiation of MSCs are important topics, they will mostly remain out of scope of this review and can be found elsewhere (Javazon *et al.*, 2004; Minguell *et al.*, 2001; Bianco *et al.*, 2001; Bianco and Gehron Robey, 2000; Baksh *et al.*, 2004).

干细胞之家www.stemcell8.cn ←点击进入

3.1. *Detection and Quantification of Transplanted MSCs*

Tracking implanted cells, especially after intravenous administration, is an essential component for studying cellular biodistribution. Most tracking experiments are carried out after the cells are labeled with reporter genes *ex vivo* (*GFP, luciferase*) (Devine *et al.*, 2003; Barbash *et al.*, 2003; Komarova *et al.*, 2006). Reverse transcription–polymerase chain reaction (RT-PCR) amplification of a transgene has also been used to quantify transgene-labeled MSC engraftment (Pereira *et al.*, 1995). Quantitative PCR detection of allogeneic DNA is also common in experiments, when allogeneic donor cells are infused in recipients (Meyerrose *et al.*, 2007; Brouard *et al.*, 2000; Allers *et al.*, 2004). Although each technique may have specific advantages, they each have specific limitations. PCR-based quantification of transduced MSCs is limited when the number of engrafted cells is low. In techniques that require histological analysis to quantify the donor cells in recipient tissues, the index of homing or engraftment is calculated by simply counting the number of labeled cells in a representative sample of high-powered fields. This methodology is hindered by the necessity of counting multiple cell layers and, therefore, is not exact. Moreover, a range of quantitative techniques, including PCR, can only be performed as a single "snapshot" either postmortem or in limited tissue obtained after biopsy. A technique for continuous and noninvasive monitoring of transplanted cell biodistribution, migration and fate is crucial for understanding the major events after cell infusion. The recent ability to label MSCs with radiotracers has allowed homing and biodistribution to be assessed using radionuclide imaging in living organisms. Combining the different tracking strategies may compensate for the limitations of each individual method. Dual labeling of MSCs may help to combine different methods of cell detection.

A dual cell labeling strategy was applied in a study of Barbash *et al.*, (2003) to monitor the kinetics of MSC distribution in the body after intravenous or intraventricular injection in a rat model of myocardial infarction. The cells were labeled with technetium 99m (^{99m}Tc) for gamma camera-based *in vivo* imaging and with BrdU for immunohistochemical detection. In addition, a gene encoding LacZ was delivered for histological detection. Imaging scans taken 4 h after intravenous injection showed a significant amount of MSCs trapped in the lungs, with a lower amount present in the heart and other organs. Intraventricular administration significantly augmented cardiac engraftment and attenuated pulmonary entrapment. One week after intravenous delivery of BM MSCs into infarcted rat myocardium, the labeled

干细胞之家www.stemcell8.cn ←点击进入

cells were identified either in the infarct or infarct border zone, but not in remote myocardium.

Magnetic resonance imaging (MRI) of iron-labeled cells is another technique that has recently been used to track the distribution of MSCs after intramyocardial injection into pigs (Kraitchman *et al.*, 2003; Hill *et al.*, 2003). The iron label can also be conjugated to a fluorescent particle for subsequent histological detection of MSCs. Single photon emission computed tomography (SPECT) was used to track ^{111}In oxine-labeled MSCs after intravenous administration into a porcine model of myocardial infarction (Chin *et al.*, 2003). ^{111}In oxine is a commercially available radioactive tracer, and is clinically used to label leukocytes for monitoring inflammation and other types of cells. High-quality SPECT images, obtained throughout 2 weeks of imaging, confirmed the feasibility of this approach for sequential monitoring of cell trafficking. Initial detection of MSCs demonstrated elevated localization in the lungs; however, there was no appreciable accumulation in the myocardium. It is likely that the elevated signal detected in the lungs obscured the assessment of less prominent trafficking to the myocardium, which may be a limitation for this method of detection.

In this regard, an attempt at improved MSC detection was reported in a study by Kraitchman *et al.* (2005). Dynamic imaging of allogeneic cell trafficking to the sites of myocardial infarction was performed using a canine model of acute myocardial infarction. This study used a radiotracer (^{111}In oxine) and an MRI contrasting agent (ferumoxides–poly-L-lysine) to label MSCs, which were then systemically injected and detected using a single-photon emission CT (SPECT/CT) and MRI, respectively. Importantly, focal and diffuse uptake of MSCs in the infarcted myocardium was already visible as a cardiac "hot spot" in SPECT/CT images in the first 24 h after injection, and persisted 6 additional days after injection. This was further validated by assessing the radioactivity counts in the tissues. In contrast, MRI was unable to detect MSC cardiac localization in part because of the lower sensitivity of MRI. Thus, this study not only dynamically tracked the overall cell biodistribution, but also demonstrated the feasibility to non-invasively detect specific homing of infused cells to the target organ, which in previous studies were assessed primarily by postmortem histology. Monitoring stem cell trafficking by radiolabeling may prove to be a more sensitive strategy compared to immunofluorescent or PCR-based quantitative techniques.

In general, different methods of MSC detection help to analyze different aspects of cell homing and biodistribution, such as the efficiency of therapeutics delivery versus the efficiency of cell engraftment.

干细胞之家www.stemcell8.cn ←点击进入

3.2. *Mode of Cell Introduction: Local Versus Systemic Injection and Lung Trapping*

Different routes of MSC infusion have been tested. Gao *et al.* (2001) reported one of the first studies aimed at investigating the kinetics of cell biodistribution by whole-body scanning and real-time tracing of labeled MSCs. Rat bone marrow-derived MSCs were labeled with ^{111}In oxine and infused into syngeneic rats by three routes: intra-arterially (ia), intravenously (iv) or intraperitoneally (ip). The MSC distribution was monitored immediately or 48 h post-infusion, after which the radioactivity in excised organs was measured. After both ia and iv infusion, most MSC-associated radioactivity was detected in the lungs and a small percentage of the radioactivity accumulated in the liver, spleen and other organs. To maximize systemic cell distribution, a vasodilating agent — sodium nitroprusside — was administered prior to the ia and iv cell infusions. In these conditions, more labeled MSCs cleared the lungs, resulting in a larger proportion of radioactivity detected in the liver. The radioactivity in the lungs and liver after iv and ia infusions, calculated as a percentage of the total infused radioactivity related to the organ mass, comprised about 50% of the infused radioactivity. The use of a vasodilator increased the count in the liver by about 10% and decreased that in the lungs by 15%. These results indicate that lungs represent a significant obstacle for MSC distribution; however, the secondary distribution of MSCs to different organs suggests the possibility that at least a fraction of the cells circulate in the blood or lymphatic flow and eventually home to different organs. Moreover, cells that were allowed to escape the lungs, via vasodilators, demonstrated increased homing to the bone marrow.

It is not surprising that MSCs are initially trapped in the lung vasculature. The lung represents a first-pass mechanical barrier to different cell types after they are injected systemically. However, immune cells can be cleared from the lungs in as little as 2–8 h (Kuppen *et al.*, 1992), while the majority of MSCs are still detected in the lungs as long as 48 h after injection (Gao *et al.*, 2001). The extent of mechanical cell trapping in the lungs is largely dependent on the size of the cells injected, relative to the size of the lung capillaries. Schrepfer *et al.* (2007b) reported the mean size of a suspended mouse MSC to be 15 μm to 19 μm, which is larger than the diameter of murine pulmonary capillaries (10–15 μm). Because of their large size, most iv-injected MSCs are trapped within the pulmonary capillaries, limiting their access to other organs and causing pulmonary and hemodynamic alterations that are often detected in animals receiving intravenous cell injections (Anjos-Afonso *et al.*, 2004). The average size of rat

干细胞之家www.stemcell8.cn ←点击进入

MSCs at the first passage of culture was estimated to be between 20 μm and 24 μm in diameter, while the diameter of the rat lung capillaries range from 10–15 μm. MSCs are naturally located within the bone marrow and, under certain stimuli, occasionally enter circulation. Therefore, their size is usually not a barrier to their physiological role. However, the unnatural introduction of MSCs creates a mismatch between MSCs and the capillary diameters. Therefore, alternative strategies are needed to enhance MSC escape from lung capillaries following iv administration of MSCs for cell-based therapy. In this regard, vasodilating agents offer a better chance for the cells to pass through the lung vasculature and to diminish the symptoms of pulmonary embolism. Although it has been shown that the mechanical barrier of the lung can be overcome to some extent with vasodilation (Gao *et al.*, 2001; Schrepfer *et al.*, 2007b), the kinetics of cell clearance in this context was not addressed and would be of considerable interest.

Another issue that may influence the degree of MSCs trapped in the lung is the attachment potential of BM-derived MSCs. MSCs normally attach to the plastic surfaces in a fibronectin-facilitated manner *in vitro* (Dennis *et al.*, 2002) and, therefore, may specifically attach to fibronectin-rich endothelial cells in a receptor-dependent manner *in vivo*. It is also likely that culturing MSCs on plastic surfaces for extended periods may change the level of expression of adhesive factors they express, and thereby influence their homing pathway. In accordance with this hypothesis, Rombouts and Ploemacher (2003) have recently demonstrated that MSC engraftment in the bone marrow was severely diminished in response to short periods (24 h) of plastic adherence, and was completely lost when the cultures were extended to 48 h on plastic. Therefore, the level of MSC lodging in the lungs could also result from endothelial attachment and depend on the differentiated state of the infused MSCs.

The extent to which MSCs become entrapped in the lungs after systemic injection has prompted the evaluation of alternative, localized routes of MSC introduction. Barbash *et al.* (2003) compared the beneficial effects of local versus systemic MSC delivery in a rat myocardial infarction (MI) model. Radioactively labeled MSCs were transfused several days after MI either intravenously or locally into the left ventricular cavity. Cell delivery into the left ventricular cavity resulted in drastic reduction in the level of lung uptake and better cell uptake in the heart, specifically in infarcted compared with sham-MI hearts. The authors concluded that direct infusion into the left ventricular cavity enhances migration and colonization of the

干细胞之家www.stemcell8.cn ←点击进入

cells preferentially to the ischemic myocardium. Toma *et al*. showed that human MSCs injected directly into the myocardium of mice can express some cardiac markers possibly through differentiation (Toma *et al*., 2002) or fusion with endogenous cardiomyocytes (Alvarez-Dolado *et al*., 2003). In another study, MSCs were injected into the borders of scar tissue in the left ventricular wall 4 weeks after infarction (Olivares *et al*., 2004). In support of previously reported studies, locally delivered cells appeared to have beneficial effects and improved cardiac performance even after the healing process had occurred.

In another study, a GFP-labeled MSC line immortalized with the human telomerase catalytic subunit (hTERT) was injected into immunodeficient mice to evaluate three important parameters: 1) cell biodistribution following different routes of administration (intracardiac and intravenous), 2) MSC adherence to the endothelium of the organ in question, and 3) transmigration over an intact endothelial lining to engraft (Bentzon *et al*., 2005). The pattern of organ distribution suggested that infused cells were efficiently arrested in microvasculature during the first pass, particularly in the lungs after iv injection. A considerable number of cells were still lodging in the vasculature one week after infusion. Only a fraction of cells were capable of vascular emigration and tissue engraftment. These observations indicated that the MSC line was subjected to endothelial adhesion, but it did not enhance the efficiency of their emigration from the microvasculature.

Although lung trapping of infused cells has been documented and has hampered most studies aimed at investigating MSC homing or biodistribution after systemic injection, long-term engraftment studies are demonstrating the possibility of MSC extravasation (Devine *et al*., 2001 and 2003). The high incidence of MSCs found in tissues such as bone marrow, spleen, cartilage, and muscle after extended post-infusion periods (4–13 months) suggests that mesenchymal progenitors do escape, expand and migrate into distinct mesenchymal tissues (Pereira *et al*., 1995). This activity is crucial for targeting sites of pathology, especially in intravascular applications. The ability of MSCs to transmigrate over the endothelial barrier was investigated by Schmidt *et al*. (2006b). The authors used electron microscopy to analyze direct interactions of gold-labeled MSCs with endothelial monolayer in co-culture and in perfusions of isolated heart. MSCs developed tight cell-cell contacts and became integrated into the endothelial wall of the capillary vessel. The ability of the MSCs to fully pass the endothelial barrier was also estimated. The authors showed that the vast majority of MSCs migrated through the endothelial barrier within 30 min. To explore

干细胞之家www.stemcell8.cn ←点击进入

the initial steps by which transplanted MSCs interact with the vessel wall in the course of extravasation, Ruster *et al.* studied the level of human MSC binding to endothelial cells (Ruster *et al.*, 2006). MSCs displayed coordinated rolling and adhesion behavior on endothelial cells similar to peripheral blood mononuclear cells (PBMCs) or $CD34^+$ hematopoietic progenitors. The authors suggested that circulating MSCs are capable of extravasation at levels potentially comparable with those of PBMCs. These observations still have to be confirmed using more stringent *in vivo* conditions, since MSC extravasation abilities can be very important in clinical settings.

In summary, both cell and gene therapy applications favor systemic infusion as a less invasive and more accessible route of delivery. A majority of the studies evaluating the distribution of systemically infused MSCs found temporary or permanent entrapment of MSCs in the lungs of mice, rats and primates. In order to drive or enhance homing of the transplanted MSCs, strategies that prevent their retention in the lung or enhance their specific recruitment may be required.

Different models, cell modifications and the number of cells injected can likely influence the extent of cell entrapment in the lung the ability of cells to evacuate the lung and their ability to utilize true homing signals to engraft other tissues. It is also possible that direct systemic infusion might not be the best delivery route for this type of stem cell. To this end, different ways to locally introduce MSCs have been tested for treatment of cardio or brain injuries, and have demonstrated improved therapeutic endpoints (Chopp and Li, 2002; Chopp *et al.*, 2000). MSCs injected directly into the organ (i.e., intracardially) are able to migrate from the site of injection and seek the site of pathology (infarction). MSCs implanted in different parts of mouse or rat brains were able to migrate along well-defined neural migration pathways and, possibly, to differentiate through microenvironmental stimuli (Azizi *et al.*, 1998; Kopen *et al.*, 1999) or cell fusion (Alvarez-Dolado *et al.*, 2003). Therefore, localized cell infusion, when feasible, may enhance the efficacy of MSC-based therapy. Although compartmentalized infusion restricts cellular biodistribution to the area of the injected organ or compartment, it avoids lung entrapment where a significant fraction of cells get destroyed. Moreover, the homing activities of MSCs have been demonstrated after localized injection. MSC migration to intraperitoneally disseminated tumors was observed when the MSCs were injected ip in mice bearing ovarian tumor xenografts (Komarova *et al.*, 2006). Such motility greatly contrasts the levels observed for fibroblasts, when used as controls. Fibroblasts demonstrate very modest motility and generally remain at the site of injection (Nakamizo *et al.*, 2005). Several pathways in addition to local injections

干细胞之家www.stemcell8.cn ←点击进入

could be manipulated to improve cell delivery to specific sites. First, lung entrapment can be bypassed by administration to the artery supplying the organ of interest. Second, retention in normal tissues could be blocked by reducing cell adhesion to vessels. Finally, organ-specific or vascular-specific capture could be enhanced by inducing the chemokine molecules that normally recruit MSCs to sites of damage.

3.3. *MSC Biodistribution in Unconditioned Versus Conditioned Injury/Cancer Models*

Several groups have tested the consequences of using xenogeneic or syngeneic MSCs in both unconditioned and conditioned recipients. Significant variations in the initial biodistribution, as well as the fate of infused MSCs, were commonly reported and were greatly dependent on the type of model used (Fig. 2).

3.3.1. *Unconditioned models*

Studies using unconditioned recipients have thus far demonstrated MSC engraftment in various tissues; however, this was generally at only low levels. In one such study, Allers *et al.* investigated the biodistribution of human BM-derived MSCs that were intravenously infused into unconditioned adult nude mice (Allers *et al.*, 2004). The cells were labeled with Tc99m and tracked using gamma camera imaging, PCR, and *in situ* hybridization. Whole-body imaging of scattered radioactivity was obtained at different time points post-cell infusion, indicative of the initial accumulation of signal in the lungs and liver and in several additional organs at longer time points. MSCs that are systemically infused into non-conditioned hosts have been shown to home

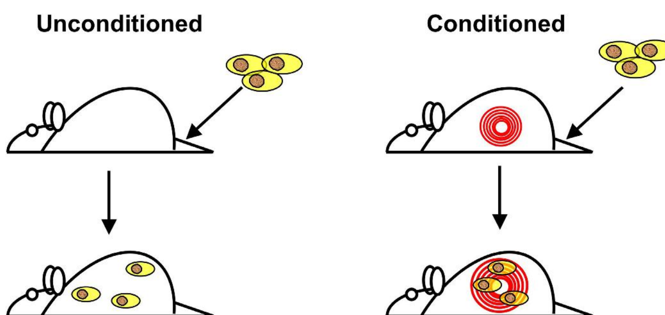


Fig. 2. MSC homing in unconditioned and conditioned animals. MSCs have diffuse and nonspecific homing and engraftment patterns in unconditioned animals, whereas conditioning (injury/tumor) induces cytokine signals that attract and support MSC survival.

干细胞之家www.stemcell8.cn ←点击进入

to hematopoietic tissues (Erices *et al.*, 2003; Brouard *et al.*, 2000). Apparently, the natural capacity of MSCs to adhere to matrix components favors their preferential homing to bone, lung and cartilage. Murine stem cell engraftment into different organs has also been tested in response to a sublethal dose of total body irradiation (Anjos-Afonso *et al.*, 2004). In this study, the donor MSCs were transduced with a lentiviral vector expressing GFP and systemically injected into sublethally irradiated (minimally injured) syngeneic recipients. Rare donor-derived hepatocytes, lung epithelial cells, myofibroblasts, myofibers and renal tubular cells were found in some of the recipient mice. The authors concluded that even in the absence of substantial injury, murine mesenchymal stem cells could contribute to different tissue cell types *in vivo* and acquire tissue-specific morphology. However, extensive levels of MSCs trapped in the lung were also noted, which caused adverse effects in the majority of the experimental animals transplanted. Therefore, in murine recipients, injection of both syngeneic and xenogeneic MSCs results in predominant lung retention, which likely limits the number of MSCs available for homing to other organs (Gao *et al.*, 2001; Barbash *et al.*, 2003). The low level of MSC engraftment observed in unconditioned models may question the reality of the engraftment phenomenon due to possible artifacts in detection, especially when a single method is used to detect the transplanted MSCs. A recent study by Meyerrose *et al.* employed a range of detection techniques, models and routes of cell introduction, and demonstrated the reality of MSC engraftment more clearly (Meyerrose *et al.*, 2007). This study investigated the distribution of human adipose-derived MSCs (AMSCs) following xenotransplantation into three sublethally irradiated immunodeficient mouse models: nonobese diabetic/severe combined immunodeficient (NOD/SCID), nude/NOD/SCID, and NOD/SCID/MPSVII mice. The cells were labeled via transduction with a GFP-expressing retrovirus and were transplanted by iv, ip or subcutaneous injection. Different tissues were evaluated for AMSC engraftment, and persistence of transgene expression was assessed by quantitative duplex PCR and fluorescence at various time points that extended up to 75 days post-transplant. Donor-derived cells were consistently observed in multiple tissues, regardless of the route of administration, and persisted up to the last (75-day) time point. Therefore, the human AMSCs infused were able to migrate to a wide range of tissues and persisted for the duration of the study. The widespread distribution of human cells in mouse tissues suggests that they have the ability to navigate *in vivo*. However, the broad distribution of cells observed in this study may have been influenced by the total body irradiation used for all three

干细胞之家www.stemcell8.cn ←点击进入

models. Irradiation may induce a global inflammatory response affecting cell migration.

In summary, systemically infused MSCs are able to circulate and engraft in specific tissues of unconditioned recipients. This phenomenon represents an interesting observation because a similar mechanism is not known to occur physiologically with other cell types. It should be emphasized that most of the studies described were aimed at investigating long-term engraftment. However, the organs or tissues permitting long-term engraftment may differ from the organs or tissues MSCs are localizing to in short-term biodistribution assays. This is due to the fact that long-term engraftment will rely on initial biodistribution, which is influenced by several factors, as well as secondary survival and proliferation signals, which may or may not be provided by the tissue or organ of initial distribution.

3.3.2. *Injury models*

One of the most interesting characteristics of MSCs is their ability to sense tissue damage or inflammation. It has been noted that various agents or methods of inducing injury promote MSC homing and engraftment. Although the factors responsible for inducing injury-specific MSC migration have yet to be fully elucidated, it is postulated that the MSCs migrate in order to contribute in tissue repair. This remarkable ability of MSCs has been demonstrated in models mimicking widespread or localized damage. Widespread damage is typically modeled using total body irradiation, whereas localized injury is modeled using organ-specific injury such as bone fracture (Mosca *et al.*, 2000; Devine *et al.*, 2002), cerebral ischemia (Chen *et al.*, 2001) and heart infarction (Saito *et al.*, 2002; Price *et al.*, 2006). Apparently, signals from the damaged site are required for recruiting MSCs to engraft and proliferate in the niche. Thus, the ability to home and deliver therapeutics to disease sites represents an additional attractive feature of these cells favorable for cell-based strategies.

The effect of total body irradiation (TBI) on MSC homing has been evaluated (Francois *et al.*, 2006). In this study, human MSCs were infused into NOD/SCID mice exposed to TBI. The impact of localized irradiation (ALI) to the abdomen or leg was superimposed with TBI, as a model of accidental irradiation. Quantitative and spatial hMSC distribution was studied 15 days after irradiation by quantitative PCR-based detection of human DNA. The hMSCs homed to specific tissues (lung, bone marrow and muscle) at a very low level, and no significant engraftment was found in other organs. TBI increased hMSC engraftment levels in the brain, heart, bone marrow and

干细胞之家www.stemcell8.cn ←点击进入

muscles. Local irradiation increased hMSC engraftment specifically in the areas exposed; abdominal irradiation resulted in gut, liver and spleen localization, whereas MSCs localized in the skin, quadriceps and muscles in response to hind leg irradiation. However, increased levels of hMSC engraftment in organs outside the fields of ALI were also observed. The authors concluded that irradiation damage promotes homing of MSCs to injured tissues, and that local irradiation not only induces homing of human MSCs at exposed sites but also promotes their widespread engraftment to multiple organs.

Cardio-injury. Stem cell transplantation for myocardial infarction has captured much attention as a new approach to repair injured myocardium or to boost the limited regenerative capacity of the heart. This has prompted additional studies aimed at evaluating MSC biodistribution following systemic or localized injections. It has also stimulated the application of *in vivo* monitoring techniques, as discussed above. Saito *et al.* were the first to demonstrate that intravenously administered MSCs engraft within regions of myocardial infarction (Saito *et al.*, 2002). This study utilized a xenogeneic transplantation model in which murine MSCs transduced with a *LacZ* reporter gene were injected intravenously into rats (in the absence of immunosuppression). In healthy (noninjured) animals, the MSCs preferentially engrafted in the bone marrow cavity and were detected for at least 13 weeks after transplantation. However, in rats subjected to ischemia/reperfusion, a significant number of the labeled MSCs could be identified in circulation and, subsequently, in the infarcted region of the heart. Another observation of MSC persistence following implantation into acute myocardial infarct models was reported in a subsequent study by the same group (MacDonald *et al.*, 2005). This study confirmed the observation that xenogeneic MSCs can survive and differentiate after immediate implantation into the peri-infarct area of the left ventricle, and this attenuates deterioration of left ventricular function. Functionally, all parameters tested for ventricular function were significantly higher in the animal cohort that received MSCs. Importantly, no improved functions were detected in groups transplanted with *lacZ*-labeled mouse skin fibroblasts. None of the labeled fibroblasts survived after 4 days. These studies showed that xenogeneic bone marrow stromal cells delivered by both systemic and local injection could engraft into the acutely ischemic myocardium. Cells were immunologically tolerated, survived and differentiated, resulting in a cardiac chimera with improved left ventricular function. The remarkable ability of systemically injected MSCs to home to regions of injury, with essentially

干细胞之家www.stemcell8.cn ←点击进入

no engraftment in normal tissue, was illustrated by Pittenger and Martin (2004). To determine the specificity of homing to regions of infarction, rat MSCs labeled with fluorescent dyes were injected iv into rats, followed by an induced infarction. Histological detection of the fluorescently labeled MSCs were clearly identified and restricted to an infarct zone. Essentially, no cells were identified in regions of viable myocardium or noncardiac tissues.

Interestingly, the time window for MSC homing to an infarcted heart appeared to be limited. MSC homing to the injured heart occurred within days following myocardial infarction, and its limited duration may explain the incomplete healing of infarcted myocardium observed in other studies (Bittira *et al.*, 2003). Enhanced homing has also been demonstrated in a case of acute infarction, but not when the cells were administered 2 weeks after infarction (Pittenger and Martin, 2004). These studies suggest that there are molecular pathways which are upregulated immediately following a myocardial infarction that cause stem cells to home to the heart. The transient nature of this phenomenon may be similar to the transient infiltration of injured tissues with macrophages and neutrophils in response to inflammatory mediators. The degree to which species differences account for these varying results requires further investigation.

Although the engrafted cells can be detected, it should be noted that the overall level of MSC engraftment in the heart was only modest in each of these studies. Some experiments have indicated that $<3\%$ of MSCs administered by direct injection persist after 2 weeks. Administration of higher numbers of cells results in only modest augmentation of long-term engraftment.

Lung injury. Enhanced engraftment of MSCs has also been described in injured lungs. Ortiz *et al.* purified MSCs from male bleomycin (BLM)-resistant BALB/c mice and transplanted them into female BLM-sensitive C57BL/6 recipients (Ortiz *et al.*, 2003). Engraftment levels were quantified in recipient lungs by real-time PCR in the presence or absence of bleomycin-induced damage. Engraftment of male DNA in the lungs was 23-fold higher in animals with lung injury than in animals without injury. Fluorescence *in situ* hybridization revealed that male cells which engrafted were localized to areas of BLM-induced injury and exhibited an epithelium-like morphology. Rojas *et al.* also demonstrated participation of bone marrow-derived mesenchymal stem cells in repair of the bleomycin-induced lung injury. MSCs localized to the injured lung and assumed lung cell phenotypes (Rojas *et al.*, 2005).

干细胞之家www.stemcell8.cn ←点击进入

Brain injury (stroke, traumatic injury). There is increasing evidence that stem cells transplanted into the CNS extensively migrate into damaged tissue, and have the potential to improve neurological function and possibly to differentiate into neural cells (Deng *et al.*, 2006; Urdzikova *et al.*, 2006). Most of the studies investigating MSCs for treatment of traumatic or ischemic brain injury have detected MSC migration and accumulation at the sites of brain injury followed by enhanced therapeutic effects (Satake *et al.*, 2004). Chen *et al.* investigated the possibility of using intravenously administered autologous MSCs as a strategy for stroke treatment (Satake *et al.*, 2004; Chen *et al.*, 2003). The donor rat MSCs were found to survive and preferentially localized to the ipsilateral ischemic hemisphere of rats with induced cerebral ischemia. Significant behavioral recovery was found in MSC recipient rats compared to control animals. In another study by Mahmood *et al.*, MSCs were evaluated as a therapy to improve the functional outcome of traumatic brain injury (TBIn) (Mahmood *et al.*, 2004b). Systemically injected cells successfully migrated into the rat brain following TBIn, selectively concentrated around the injury sites, and partially expressed the immature neuronal marker, Tuj1, and an astrocytic marker, GFAP. Functional improvement was evident at 7 days post-infusion and increased with time. The researchers concluded that the functional recovery may have been the result of neurotrophic growth factor production, rather than the replacement of damaged neurons. A later study continued the investigation to determine the mechanism of functional recovery observed in the TBIn rat model after intravenous administration of rat BM MSCs (Mahmood *et al.*, 2004a). In addition to functional outcomes, the level of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and basic fibroblast growth factor (bFGF) were measured, since they are thought to play a part in functional recovery. Immunohistochemical analysis implied that the transplanted BM MSCs likely produced the trophic factors and induced their production in the surrounding glial tissue.

Urdzikova *et al.* investigated the potential of MSCs for therapeutic improvement of spinal cord compression lesions in a rat model (Urdzikova *et al.*, 2006). Seven days after induced injury, rats were intravenously injected with iron-oxide–labeled MSCs. Five weeks post-injection, MR images of the spinal cords were taken *ex vivo*. Engrafted MSCs were evident in the lesions, as confirmed histologically by a large number of iron-containing and PKH 26-positive cells in the lesion site. In attempts at identifying potential candidates for the treatment of CNS injuries, the migration and fate

干细胞之家www.stemcell8.cn ←点击进入

of several stem cell types were compared by Sykova *et al.* (2006). Mouse embryonic stem cells, rat and human mesenchymal stem cells labeled with iron oxide nanoparticles, and human CD34$^+$ stem cells labeled with magnetic MicroBeads were injected into rats containing cortical or spinal cord lesions. Labeled cells were grafted intracerebrally or contralaterally to a cortical photochemical lesion, or were injected intravenously. The authors confirmed that transplanted cells migrated to the cortical lesion by several detection methods. This was visible on MR images as early as 2 h after induction and persisted for more than 30 days. These data correlated with electron microscopy and histological cell detection of other cell labels.

There is significant evidence that systemically or locally administered MSCs migrate to sites of pathology. The question that has yet to be answered is what signals direct and promote the migration of MSCs to the injured tissue. Chemokines are likely to be the chief determinants of MSC migration. SDF-1 was identified as one of the myocardial homing factors (Askari *et al.*, 2003). In another study, monocyte chemotactic protein-3 (MCP3) was identified as a potential myocardial mesenchymal stem cell homing factor (Schenk *et al.*, 2007). There is growing interest in the spectrum of biologically active substances that MSC normally secret, as well as the substances that attract MSCs to sites of pathology. Marrow-derived stromal cells secrete a broad spectrum of cytokines and other biologically important molecules including interleukins 6, 7, 8, 11, 12, 14 and 15, M-CSF, Flt-3 ligand, SCF, LIF, bFGF, VEGF, PlGF and MCP-1 (Kinnaird *et al.*, 2004; Potapova *et al.*, 2007; Haynesworth *et al.*, 1996). Croitoru-Lamoury *et al.* assessed the functionality of the chemokines and chemokine receptor expression in human MSCs (Croitoru-Lamoury *et al.*, 2007). Using chemotaxis assays, they showed that SDF-l, alpha, fractalkine, and interferon-gamma (IFN-γ)–inducible protein (IP-10) lead to significant levels of MSC migration. Additionally, tumor necrosis factor-alpha (TNF-α) and IFN-γ were determined to be major regulators of chemokine and/or cytokine receptor expression in MSCs. In another study, MSC chemotaxis towards bFGF, erythropoietin, interleukin-6, stromal cell-derived factor-beta and vascular endothelial growth factor was examined using a Boyden chamber assay (Schmidt *et al.*, 2006a). The addition of bFGF was found to enhance the migratory activity of MSCs through activation of the Akt/protein kinase B (PKB) pathway. The most comprehensive analysis of chemotactic factors active on MSCs was recently described by Ozaki

干细胞之家www.stemcell8.cn ←点击进入

Y *et al.* (2007). The effects of 26 growth factors/cytokines were evaluated to determine which growth factors or cytokines affect the migration of MSCs to injured tissues. The migration activity of rabbit and human MSCs were analyzed using a microchemotaxis chamber. Among them, platelet-derived growth factor (PDGF)-BB, as well as various combinations of growth factors/cytokines, showed the greatest effect on migration. Several studies indicated that the Wnt signaling pathway is critically involved in the regulation of the migration/invasion capacity of hMSCs as well as cell proliferation (Neth *et al.*, 2006). Despite great interest in this issue, the data from different studies are still contradictory and have likely only scratched the surface of the complex signaling pathways involved in this process.

A growing number of studies are aimed at using MSC transplantation for cell and gene therapy applications. This has promoted further investigation into the homing properties and homing mechanisms of transplanted MSCs. A deeper understanding into the mechanisms associated with stem cell trafficking to injured tissues is needed. This includes the identification of the factors involved, the time course in which these factors are active, the specificity of each factor in relation to the different stem cell lineages, and the co-factors that may be needed in order to optimize cell engraftment. Elucidation of the homing mechanisms will allow attempts at enhancing gene-modified MSC homing to locations of clinical interest. Despite all of the issues that need further investigation, the endogenous homing ability of MSCs is intriguing and enhances their potential as cell vehicles.

3.3.3. *Cancer models*

As shown above, MSCs contribute to tissue regeneration and wound healing at the sites of injury. The formation of tumor stroma closely resembles wound healing and is so tightly associated to cancer pathological growth that tumors are seen as “never-healing wounds” (Dvorak, 1985). Increased turnover and proliferation of connective stromal cells in tumors appear thus to rely, at least in part, on MSC regenerative potential, thereby paving the way for MSC-mediated targeted cancer therapy (Studeny *et al.*, 2002). The propensity of MSCs to home into the tumor bed is the very drive of an emerging MSC-based strategy for targeted delivery of therapeutics and oncolytic viruses to primary and metastatic tumors, and is discussed below.

干细胞之家www.stemcell8.cn ←点击进入

4. MSC-based Delivery of Anti-Cancer Therapeutics

Genetically engineered MSCs are being evaluated as vehicles for delivering chemotherapeutic payloads to tumors. These studies aim to capitalize on the inherent tumor-homing activity of MSCs. A pioneering study by Studeny *et al.* demonstrated that cultured MSCs can serve as a platform for the delivery of biological agents to tumors (Studeny *et al.*, 2002). MSCs were engineered to express IFN-beta in order to generate a localized anticancer response at tumor sites. These MSCs were injected systemically to evaluate the effect on the growth of pulmonary metastases and murine survival, compared to the effect of IFN-beta delivered by intravenous injection. IFN-β–expressing MSCs prolonged mouse survival compared with untreated mice, and the mice intravenously infused with recombinant IFN-β. The authors also documented that intravenous injection of the IFN-β–expressing MSCs led to incorporation of MSCs in the tumor architecture, and that the tumor microenvironment preferentially promoted the engraftment of MSCs, compared to engraftment levels in other tissues. Systemically delivered IFN-β and IFN-β produced by MSCs at a site distant from the tumors, both failed to suppress tumor growth. These data suggested that tumor inhibition was the result of IFN-β expressed by MSCs which were detected in the tumor microenvironment.

Although this study was the first to demonstrate the fact that MSCs engraft and proliferate in tumors, subsequent concern was raised over the use of a xenogeneic model (Wolf *et al.*, 2005). The concern was over the fact that human MSCs may preferentially target human cells when evaluated in murine models, and not necessarily due to the fact that the human cells were "tumor" in origin (i.e., a species-specific rather than an MSC tumor-specific attraction). To address this issue, several studies included fibroblast controls along with MSCs and showed that only the injected MSCs targeted tumors (Nakamizo *et al.*, 2005; Xin *et al.*, 2007). Importantly, in the study of Xin *et al.*, the tumor homing properties of MSCs were documented in a syngeneic system where mouse MSCs were used to target mouse tumors. These data confirmed that, although most of the cell homing phenomena are described using xenograft models, MSCs do in fact demonstrate tumor-specific homing activity. Another study by Hung *et al.* attempted to assess the efficacy of human MSC engraftment in tumors (Hung *et al.*, 2005). MSCs expressing "tracer genes" *HSV1-TK* and *eGFP* were transplanted systemically into immunodeficient mice bearing sc xenografts of human colon cancer. The resulting tumors were examined for the specificity and magnitude of HSV1-TK$^+$, eGFP$^+$ stem cell engraftment as well as for their proliferation in tumor

干细胞之家www.stemcell8.cn ←点击进入

stroma by *in vivo* positron emission tomography (PET). The authors confirmed the notion that hMSCs can target microscopic tumors, where they subsequently proliferate and differentiate, to contribute to the formation of a significant portion of tumor stroma. GFP-positive cells were detected by PCR and flow cytometry only in the stromal cell fraction, but were not detected in the loose tumor cell fraction. To quantitate the number of MSCs that reached sc tumors, partially digested and minced tumors were fractionated and the cell fractions were subjected to flow cytometry. Donor-derived GFP-positive cells were found to represent 11.5% of total cells in the stromal cell fraction.

The tumor-targeting property of MSCs has been exploited in several studies to deliver different therapeutics to tumors. MSC-based delivery of the immunostimulatory chemokines IFN-β (Nakamizo *et al.*, 2005) and CX3CL1 (Xin *et al.*, 2007) were tested in tumor models. Intravenous injection of CX3CL1-expressing MSCs into mice bearing C26 or B16F10 lung metastasis inhibited the development of tumors and prolonged the survival of treated animals via enhancement of both innate and adaptive immunity.

A study by Stagg *et al.* evaluated MSC-mediated tumor delivery of IL-2 in order to enhance immune responses against a poorly immunogenic murine melanoma model (Stagg *et al.*, 2004). A similar study by Elzaouk *et al.* utilized MSCs to deliver IL-12 to murine melanomas (Elzaouk *et al.*, 2006). MSCs expressing IL-12 strongly reduced the formation of B16F10 lung metastases, compared to control MSCs, and this effect was dependent on the presence of natural killer (NK) cells. Furthermore, when injected into established subcutaneous melanomas, the IL-12–expressing MSCs elicited a pronounced tumor growth delay and led to prolonged survival. MSC-mediated tumor suppression has also been evaluated in a murine model of Burkitt's lymphoma (Kyriakou *et al.*, 2006). MSCs stably expressing tsFlk-1 were injected to evaluate the effects on subcutaneous tumor growth in immunodeficient mice. Tumors in these mice were established as an injection of premixed Raji Burkitt's lymphoma cells and MSCs. Tumor growth was significantly impaired in the cohort of mice transplanted with MSCs expressing tsFlk-1, compared to controls with either unmanipulated MSCs or GFP-expressing MSCs.

MSCs have also been tested for engraftment into brain tumors. Nakamizo *et al.* tested human bone marrow-derived MSCs for brain tumor tropism and potential therapy for gliomas (Nakamizo *et al.*, 2005). Fluorescently labeled MSCs injected into the carotid artery of mice bearing human glioma xenografts were detected exclusively within the brain tumors. In

干细胞之家www.stemcell8.cn ←点击进入

contrast, intracarotid injections of fibroblasts or U87 glioma cells resulted in a widespread distribution of cells without tumor specificity. Furthermore, hMSCs injected directly into the cerebral hemisphere opposite to an established human glioma were capable of migrating into the xenograft. Several factors were evaluated for their influence on hMSC migration in this study. MSC migration was enhanced by glioma-conditioned media and by several growth factors that were tested separately, but not by media derived from fibroblasts or astrocytes. MSCs expressing IFN-β were also given to mice containing human U87 intracranial glioma xenografts. Compared to control MSCs, mice injected with the IFN-β–MSCs had a survival advantage. A similar approach was tested by Nakamura *et al.* using a syngeneic glioma model in rats (Nakamura *et al.*, 2004). Primary rat MSCs were shown to migrate towards rat glioma cells *in vitro* and *in vivo*. MSCs injected into the contralateral hemisphere migrated towards glioma cells through the corpus callosum, and MSCs implanted directly into the tumor localized mainly at the border between the tumor cells and normal brain parenchyma. Intratumoral injection of MSCs caused significant inhibition of tumor growth and increased the survival of the glioma-bearing rats. MSCs transduced with an adenoviral vector encoding human interleukin-2 (IL-2) augmented the antitumor effect and further prolonged the survival of tumor-bearing rats. Thus, gene therapy employing MSCs as a targeting vehicle would be promising as a new therapeutic approach for refractory brain tumors.

Little is known about the factors involved in regulating MSC motility. Solid tumor tissues express and secrete growth factors, cytokines and chemokines that are similar to those secreted in response to injury and are, therefore, likely involved in promoting MSC migration. Some investigators are beginning to study this issue, although the conclusions drawn from the different studies can be contradictory. For example, in a study by Schichor *et al.*, vascular endothelial growth factor A (VEGF-A) was found to contribute to glioma-induced migration of human MSCs (Schichor *et al.*, 2006). In this study, human MSC migration and invasiveness in response to VEGF-A, or glioma-conditioned medium, was analyzed *in vitro* using a modified Boyden chamber assay. Glioma-conditioned medium significantly increased directional migration and invasion of MSCs. VEGF-A induced similar effects, but to a lesser extent than glioma-conditioned medium. This is in contrast to the study of Nakamizo *et al.* where VEGF did not show any *in vitro* effect on MSC migration. Sordi *et al.* showed that MSCs chemotactically migrate in response to CX3CL1 and CXCL12 (Sordi *et al.*, 2005). Since injured or inflammatory tissues upregulate CXCL12 and CX3CL1, the migration

干细胞之家www.stemcell8.cn ←点击进入

of MSCs to the injured tissues as well as to tumors may be regulated by CXCR4/CXCL12 and CX3CR1/CX3CL1 interactions. MSCs were recently demonstrated to express functional c-met and exhibit chemotactic migration towards hepatocyte growth factor (HGF). CXCL12 was found to enhance HGF-mediated MSC migration activity *in vitro* (Son *et al.*, 2006). Although the precise molecular mechanisms by which MSCs migrate to tumor tissues are still unknown, the mechanisms of migration of MSCs to injured organs are beginning to be understood, thereby opening the way to cross-checking experiments in tumor settings.

In summary, the utility of MSCs for treatment of cancer has been demonstrated using models that exploit the tumor-homing activity of these cells to deliver therapeutic products. However, the question of MSC homing specifically to tumors still requires further investigation. New approaches that increase MSC homing to specific sites, especially tumors, will be needed to realize the full therapeutic potential of the transplanted cells.

5. Cells as Carriers of Oncolytic Viruses: Examples of Strategies

5.1. *Problems Related to Direct Virus Introduction*

Virotherapy describes the use of viruses to treat illness. The use of viruses to treat malignancies dates back to the 1950s, when wild-type adenovirus was used to treat cervical cancer (Huebner *et al.*, 1956). Since that time, our knowledge of viral biology has led to tremendous advances in this field and has resulted in viral vectors that are specifically intended for use in therapy, bringing a new spin to the old idea of virotherapy. Many viruses have now been engineered or selected as oncolytic agents that capitalize on unique features of viral or tumor biology to selectively kill tumor cells. Oncolytic viruses entering the clinic include herpes simplex virus, adenovirus, Newcastle disease virus, measles virus, reovirus, vesicular stomatitis virus and others. These viruses selectively replicate in cancer cells, albeit by different mechanisms (Roberts *et al.*, 2006). Additional tumor cell selectivity can be engineered by placing an essential viral gene under the control of a tumor-specific promoter (Nettelbeck, 2003). The most straightforward approach for tumor-related virotherapy involves the direct injection of purified virions, either locally or systemically. Indeed, direct intratumoral administration of many oncolytic viruses has been shown to be effective in a variety of tumor models when the tumors are easily accessible (Liu and Deisseroth, 2006; Taki *et al.*, 2005). However, systemic

干细胞之家www.stemcell8.cn ←点击进入

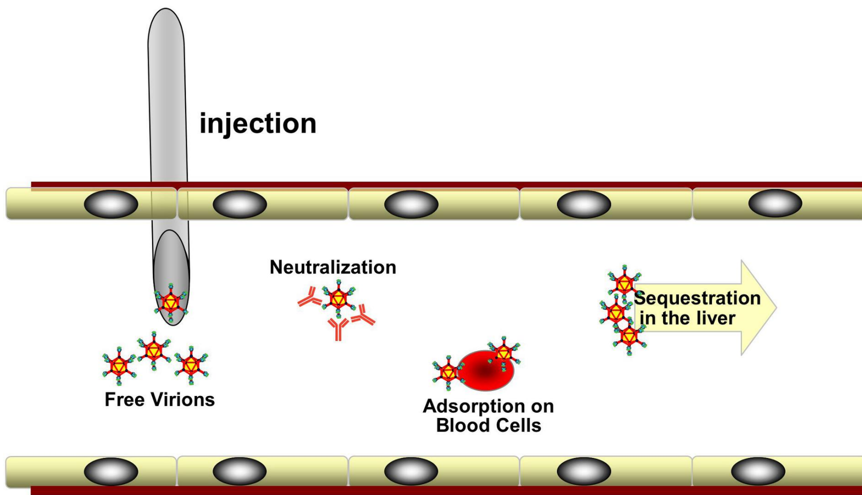


Fig. 3. Problems associated with systemically-injected virions. A virus injected directly into blood circulation is susceptible to inactivation by immunological effector molecules, such as neutralizing antibodies. The virus is also lost due to nonspecific adsorption onto blood cells or sequestration in the liver.

injection of viruses for treatment of disseminated or inaccessible tumors has resulted in difficulties. Different arms of the host immune system — including neutralizing antibodies (Chen *et al.*, 2000; Tsai *et al.*, 2004), inactivation by serum proteins or blood cells (Baker *et al.*, 2007; Seymour, 2006; Lyons *et al.*, 2006), or uptake by the reticuloendothelial system (Worgall *et al.*, 1997a and 1997b; Ye *et al.*, 2000) — have limited the effectiveness of systemically delivered viruses (Fig. 3). In addition, infection of non-target cells results in only a small fraction of virus remaining to reach the tumor. Therefore, even though the viruses are engineered with selective tumor-restricted replication mechanisms, their systemic delivery is still compromised. Thus, alternative strategies for viral introduction will have to be developed in order for virotherapy to be utilized as an effective treatment.

5.2. *Requirements for Cell-based Virus Delivery*

The idea of hiding viruses from the immune system appears to be obvious, and several strategies have been implemented that utilize this approach. One approach that has demonstrated some efficacy involves the use of polymers to coat virions. This strategy has been applied to capsid- and membrane-bound viruses, and helps to conceal the virus from immune clearance and prevent uptake in non-target cells. Moreover, targeting moieties

干细胞之家www.stemcell8.cn ←点击进入

can be introduced in polymer coating to redirect the virus to tumor cells (Stevenson *et al.*, 2007).

Alternatively, the idea of intermediate "stealthy" virus carriers has recently become an attractive option for viral delivery. To this end, different cell types offer the potential to serve as a sort of "Trojan horse" for delivering viral particles to tumors. This strategy appears reasonable, since cells often serve as natural hosts for viruses and in some cases promote viral dissemination (Digel *et al.*, 2006; Morrow *et al.*, 2007). Cells are normally overlooked by the immune system, especially by the humoral arm, until the onset of antigen expression at the later stages of infection. Therefore, viruses hidden in the cells may get a chance to survive longer in circulation. Cell carriers offer additional advantages in that the viral load can be amplified via replication as the cell travels to its destination. Furthermore, the cells can be manipulated to confer or enhance endogenous tumor-homing activities. This approach involves infection of the cell carriers *in vitro*, after which they are systemically injected to reach the tumor sites.

In order for cells to be utilized as cell carriers, they must themselves be susceptible to virus infection and maintain *de novo* production and release of progeny virions. Therefore, the search for an appropriate cell vehicle requires preliminary investigation of the viral life cycle in each particular cell type. Although these requirements may seem obvious, some primary cell types possess natural mechanisms of viral defense that might either block viral entry or alter further steps of viral replication and release. Ideally, the virus must efficiently infect the cells, but the viral life cycle should be sufficiently slow or delayed enough to allow the cell carriers time to reach the tumor sites before the virus is released. An even better solution would allow viral replication or release to be controlled by exogenous factors that are triggered when cells reach the tumors. Several strategies have been utilized to control virus replication, and are typically based on the nature of the virus being delivered. For example, Herrlinger *et al.* temporarily arrested HSV1 vector replication in cell carriers by treating the cells with mimosine, reversibly arresting cell cycle progression of the cell carriers (Herrlinger *et al.*, 2000). This prevented rapid HSV-mediated cell killing and allowed time for cell migration to achieve localized virus delivery. Controlled replication of retroviruses has also been achieved in T-cells (Crittenden *et al.*, 2003; Yotnda *et al.*, 2004). The cytotoxicity of adenoviruses for cell carriers can also be regulated by the introduction of regulatory elements that enable viral replication only under specific conditions. The most straightforward regulatory mechanism to control adenoviral replication would be to use the Tet-off system

干细胞之家www.stemcell8.cn ←点击进入

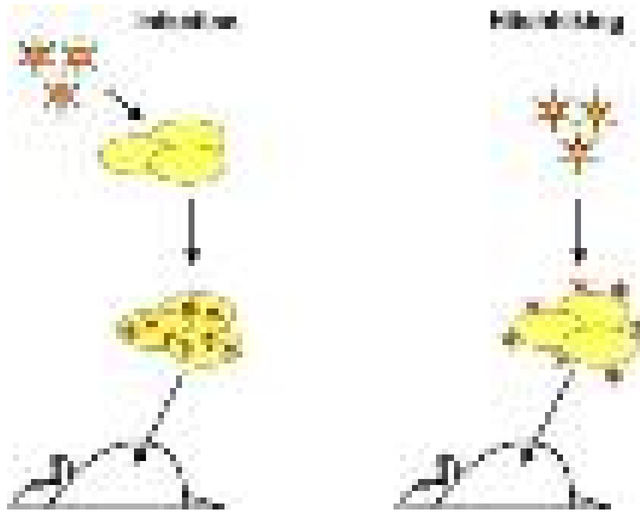


Fig. 4. Strategies for loading carrier cells with virus. Viruses can either infect carrier cells or specifically/non-specifically bind to the cell surface to "hitchhike" on the cell carriers. Infected cell carriers replicate the virus, resulting in amplification of the initial viral dose, and also may serve to temporarily hide the virus. Hitchhiking is limited by the initial viral dose used for coating the carrier cell surface, but is not limited to cell populations that support productive infections.

(Fechner *et al.*, 2007; Sipo *et al.*, 2006) to control early adenoviral (Ad) gene genes expression. Another promising way is to use tumor-specific promoters, which have attenuated activity in non-cancerous cell carriers, but show high transcriptional activity in tumor cells.

Some of the cell vehicle requirements related to virus infectivity and replication rates can be avoided using a viral "hitchhiking" (Fig. 4) strategy, which utilizes nonspecific virus–cell interactions to coat the surface of the cell vehicle with a virus (Cole *et al.*, 2005). The virus-coated vehicle is then infused and can "hand off" the virus to target cells expressing the appropriate virus receptor. Thus, depending on the target, virus and vehicle used, direct infection of the cell vehicle may not be required. Several viruses have been shown to bind to the cell surface in a receptor-independent manner (Pizzato *et al.*, 1999; Schlegel *et al.*, 1982; Pan *et al.*, 2007). Furthermore, the viral "hand off" can occur weeks after the vehicles are loaded (Pan *et al.*, 2007). The disadvantage of this strategy is that the virus is not amplified in the cell vehicle and, therefore, the virus load delivered is dependent on the initial amount of virus used to coat the cells and on the number of cells infused. The surface-bound virus might also be susceptible to inactivation by immune factors.

干细胞之家www.stemcell8.cn ←点击进入

5.3. *Cell Type Tested*

Different cell types have been evaluated as vehicles for delivering oncolytic viruses to tumors. The inherent tumor-homing and tumor-fighting activities of the cell will depend on the specific cell type used. The importance of choosing an appropriate carrier cell type for therapeutic delivery was demonstrated in cell carrier studies for glioma treatment (Li *et al.*, 2007; Namba *et al.*, 1998 and 2000). These studies demonstrated that, among the carriers tested, the best therapeutic efficacy was achieved with neural progenitor cells (Li *et al.*, 2005 and 2007).

The infectability of the vehicle and the level of viral replication within that vehicle will also vary, depending on the cell type used. Although the benefits of cells as viral carriers have been recognized, only initial steps have been undertaken towards a practical implementation of this approach.

5.3.1. *Immune cells (primary T lymphocytes, CIK cells)*

Immune cells are logical candidates as carriers for systemic virus delivery, since they naturally circulate and have the capacity to infiltrate tumors. In addition, many immune cell types have natural tumor-killing activities that have been widely exploited for cancer immunotherapy. Therefore, these cells may exhibit synergistic tumor cell-killing activities when combined with oncolytic viruses. T lymphocytes were utilized as carriers of conditionally replicative retroviruses in a study by Chester *et al.* (2002). In this investigation, the T lymphocytes were equipped with a chimeric receptor against the colorectal tumor-associated antigen, carcinoma embryonic antigen (CEA), and were then used to produce and deliver retroviruses to CEA-positive tumor xenografts. The key aspect of this strategy lies in the controlled viral production, which was specifically triggered in response to the recombinant T-cell receptor binding to the tumor antigens. Multiple levels of targeting control were incorporated in this delivery system at the stages of surface targeting, viral production and gene expression (Chester *et al.*, 2002; Crittenden *et al.*, 2003).

A method of controlled viral release in response to specific binding has also been proposed in a study by Yotnda *et al.* (2004). This study utilized cytotoxic T-lymphocytes (CTLs) directed against Epstein–Barr virus (EBV) antigens. These CTLs were derived from mice transgenic for the adenoviral E1 gene under the control of the cell activation-dependent CD40 ligand (CD40L) promoter. Following transduction with E1-deficient adenoviral vectors, the CTLs produced infectious virus only when exposed to

干细胞之家www.stemcell8.cn ←点击进入

HLA-matched EBV-expressing targets, but not when exposed to major histocompatibility complex (MHC)-mismatched or otherwise irrelevant cells.

Ong *et al.* utilized T-cells as vehicles for delivering oncolytic measles viruses to tumors (Ong *et al.*, 2007). In this study, the authors initially examined freshly isolated human peripheral blood lymphocytes (PBLs), but they found that T-cells activated with interleukin-2 and phytohemagglutinin were the primary population susceptible to viral infection. Interestingly, the viral replication cycle appeared to be abortive even in these pre-activated T cells. Nevertheless, infected T cells were still able to transfer the measles virus to tumor cells via a cell-cell fusion mechanism. This "heterofusion" process led to successful delivery of measles viruses and tumor infection when the infected T cells were administered to mice by intravenous injection.

Since T-cells are typically refractory to viral infection, Cole *et al.* developed the viral hitchhiking strategy to coat tumor-specific T-cells with a retrovirus vector expressing the immuno-stimulatory molecule IL-12 or the suicide gene *HSV-TK* (Cole *et al.*, 2005). These cells were then evaluated for treatment of a murine lung metastasis model. Although the T cells were able to mediate complete tumor regression alone, a protective memory response was only generated in mice infused with T-cells that were coated with the IL-12–expressing virus. T-cells coated with HSV-TK expression vectors were also shown to protect animals challenged with a greater tumor burden, following ganciclovir treatment. These findings indicate that arming tumor effector cells with an oncolytic virus serves to combine and complement the individual properties of cellular and viral therapeutics.

Cytokine-induced killer (CIK) cells are another potential virus carrier with effector properties. CIK cells are obtained by *in vitro* treatment of human peripheral blood lymphocytes or murine splenocytes with interferon-γ, interleukin-2 and an antibody that cross-links the T-cell receptor (Baker *et al.*, 2001). The resultant cells bear phenotypic markers of natural killer (NK) and T cells, mediate NKG2D-dependent, non-major histocompatibility complex-restricted lysis of a variety of transformed cell types and traffic to tumors *in vivo* (Verneris *et al.*, 2004; Negrin *et al.*, 2002). Thorne *et al.* used CIK cells to deliver an oncolytic strain of the Vaccinia virus. These cells supported Vaccinia virus infection and, more importantly, were able to produce viral titers comparable to those generated by transformed cells (Thorne *et al.*, 2006). In addition, viral release was delayed by several days. This rate of replication supports the cell-based virus delivery strategy, as it allows a time window for the infected CIK cells to traffic to tumors before the viral payload is released. In this study, the systemically administered

干细胞之家www.stemcell8.cn ←点击进入

CIK cells took up to 72 h to traffic to tumors, which corresponded to the peak timing for Vaccinia virus production in CIK cells. Non-invasive imaging techniques were used to demonstrate that the Vaccinia-infected CIK cells were present in tumors following intravenous administration in mice, and this strategy resulted in powerful antitumor efficacy in several murine cancer models. These findings demonstrate that some immunological killer cells can be loaded with oncolytic viruses to achieve potentially synergistic gains in antitumor activity.

5.3.2. *Tumor cell lines*

Although the introduction of tumor cell lines in patients would raise significant safety concerns, the ease of handling these cells and the fact that they can be readily infected with most oncolytic viruses make them attractive candidates as cell-based viral delivery vehicles. One of the first examples of a strategy utilizing tumor cells for delivering viruses used a carcinoma cell line (PA-1) as a vehicle for delivering a replication-competent attenuated herpes simplex virus (HSV-1716) to treat human epithelial ovarian cancer *in vivo* (Coukos *et al.*, 1999). In a separate study by Garcia-Castro *et al*. (2005), irradiated human breast carcinoma (MDA-MB-231) cells were used in an attempt to deliver adenovirus to lung metastases in a murine model. Both studies utilized immuno-compromised murine xenograft models to evaluate their cell-based delivery strategies. The use of immunocompetent models represents a more stringent system for evaluating cell-based delivery strategies, as the immune system is responsible for clearing virus-infected cells. A variety of tumor cell lines have been tested in immunocompetent mouse models for their ability to deliver oncolytic parvovirus (Raykov *et al.*, 2004), measles virus (Iankov *et al.*, 2007) and vesicular stomatitis virus (Power *et al.*, 2007). Tumor cell lines support high levels of viral replication and, therefore, greatly increase the initial viral load that can be delivered to the tumors. However, tumor cells do not necessarily have tumor-homing activity and represent an additional neoplastic threat when introduced into the body. In the clinical context, it will be critical to ensure that uninfected cells cannot establish *de novo* metastatic growth following systemic administration. This would require a supplemental mechanism by which the transferred cells are killed after fulfilling their transient function. This could be accomplished by irradiating the tumor cells before administration, which ablates tumorigenicity but preserves the metabolic activity required for virus replication. Alternatively, suicide genes could be used that could be activated

干细胞之家www.stemcell8.cn ←点击进入

after the time whereby the virus would have been released to ensure elimination of residual cells. Tumor cells may offer potential as cell carriers in cancer settings; however, translation to clinical practice might present more problems than benefits.

5.3.3. *Progenitor/stem cells*

Progress in the fields of developmental and cellular biology has led to a significant number of progenitor or stem cell types available as potential therapeutic vehicles. So far, neural, mesenchymal and endothelial precursor cells have been evaluated for use in cell-based approaches to cancer treatment and, more specifically, in approaches using these cells as viral delivery vehicles. While immune cell vehicles offer the potential for an inherent tumor-killing effect, stem cell populations offer alternative properties that could also be advantageous in tumor settings.

Neural progenitor/stem cells (NSCs). The native tumor-tropic and migratory capacities of neural stem cells (NSCs) were recently published by several groups (Aboody *et al.*, 2000; Yip *et al.*, 2003). These capacities have been exploited lately to deliver therapeutics to the brain. Cell-mediated vector delivery to brain tumors has been explored previously using retrovirus packaging cell lines, as well as other cell types (Culver *et al.*, 1992; Ram *et al.*, 1997). However, the tumor-homing ability of these cell populations was minimal. In this regard, the NSCs ideally filled the missing homing requirement for efficient cell vehicle-mediated delivery of viral vectors. Herrlinger *et al.* used NSCs infected with an HSV-1–based virus to target pre-established intracerebral CNS-1 gliomas in nude mice (Herrlinger *et al.*, 2000). The cells were injected directly into the tumors, but migrated extensively throughout the tumor and into the surrounding parenchyma over the following 3 days. After intratumoral injection, HSV-1 replication was also detected in the tumor and the surrounding parenchyma. This study demonstrated the potential of cell-based delivery of HSV-1 vectors to tumor cells in the brain.

Endothelial progenitor/stem cells (EPCs). It is widely accepted that tumor growth is dependent on the formation of supportive blood vessels. The existence of bone marrow-derived endothelial progenitor cells (EPCs) led to the suggestion that bone marrow-derived stem cells might be a systemic source of endothelial cells supporting neovascularization (Asahara *et al.*, 1997). Recently, studies have convincingly demonstrated that circulating EPCs are recruited to the tumor neovasculature and that genetically

干细胞之家www.stemcell8.cn ←点击进入

modified EPCs can serve as angiogenesis-targeting vectors to deliver anti-cancer genes (Lyden *et al.*, 2001; Davidoff *et al.*, 2001). The potential of EPCs as viral carriers has been investigated; notably, these cells supported infection with non-replicating retroviral and adenoviral vectors, and were also able to deliver retrovirus to tumors upon systemic administration (Jevremovic *et al.*, 2004).

Mesenchymal progenitor/stem cells (MSCs). MSCs have recently gained increased attention in tumor-related applications and have been tested as carriers of replication-competent adenoviruses, as will be discussed in more detail below.

5.4. *Carrier Cell Immune Evasion*

Although one of the main advantages proposed for the use of cells as viral delivery vehicles was immune evasion, only a few recent studies have addressed this therapeutic strategy in the context of host immunity. This is largely due to the fact that most oncolytic viruses are based on human viruses. These viruses usually demonstrate host (human)–restricted replication and oncolysis (adenovirus or measles virus) or may only have weak infectivity in murine tissues (adenovirus). These issues have limited the use of animal models for evaluating active virus production in the setting of a functional immune system. Nevertheless, initial attempts to use cells as viral carriers to evade anti-viral immunity have been undertaken for several oncolytic viruses.

Iankov *et al.* have shown that an immortalized monocyte cell line can deliver measles virus to tumors despite the presence of high titers of human antibodies passively transferred to mice (Iankov *et al.*, 2007). This model was used to recreate the high anti-measles antibody titers situation found in patients. In contrast to direct ip injection of naked virions, infected cell carriers transferred the virus to tumor cells by heterofusion, which was more resistant to antibody neutralization. Repeated injection of monocytic cell carriers significantly improved survival of mice with human ovarian cancer xenografts.

The negative impact the humoral immune response has on the systemic delivery of oncolytic vesicular stomatitis virus (VSV) was demonstrated in a study of Power *et al.* (2007). As a better alternative to direct virus administration, the authors tested different tumor cell lines as cell carriers. This study confirmed that a wide variety of established, nonautologous cell lines, particularly those of hematological origin, could serve as vehicles for delivery of

干细胞之家www.stemcell8.cn ←点击进入

oncolytic viruses. It has been convincingly demonstrated that cellular vehicles can shield oncolytic virus from neutralizing antibodies during delivery, providing a simple and effective means to improve therapeutic outcomes in the context of antiviral immunity.

CIK-mediated delivery of Vaccinia virus was tested in both immunodeficient and immunocompetent mouse models with established tumors (Thorne *et al.*, 2006). Although a direct assessment of an immune response was not carried out, a survival benefit was seen in groups with cell-based viral delivery compared to groups treated with cells or virus alone, suggesting that infected cell carriers indeed protect viral agents from being inactivated by the immune system.

An immunocompetent rat model was also used to investigate cell-based delivery of an oncolytic parvovirus to tumor metastases (Raykov *et al.*, 2004). The Morris hepatoma (MH3924A) tumor cell line was used as a parvovirus vehicle to target and suppress metastases formed by the same cells. The question of a neutralizing antibody response was also addressed in regard to carrier cell-mediated or direct administration of virus. The presence of neutralizing antiviral antibodies was quantified by measuring the ability of rat serum to protect indicator cells from the killing effect of the parvovirus. The sera of rats injected with either carrier cells or free virus had a clear neutralizing activity *in vitro*. However, it was shown that the infected cell carriers elicited less of a neutralizing antibody response, compared to systemically administered virions.

In contrast, a study by Ong *et al.* demonstrated that both infected cell vehicles and viruses can be inactivated by anti-viral antibodies in passively immunized mice (Ong *et al.*, 2007). In this study, T-cells were evaluated for delivering oncolytic measles virus (MV) to multiple myeloma xenografts in the presence of anti-MV antibodies. Although the feasibility of viral delivery was established using the T-cell vehicles, neither delivery strategy was able to overcome high titers of anti-viral antibodies. However, the cell-based delivery was effective over a broader range of antibody titers that mitigated infection with cell-free virus. The authors further demonstrated that the extent of tumor infection was dependent on the titer of neutralizing antibodies present, whether the therapeutic was administered as naked virions or within infected T cells. Based on imaging analysis, this study determined that 1–2% of the total T-cells injected reached tumor nodules.

Although these studies provide a novel strategy for systemic delivery of oncolytic viruses, further work is now required to determine how the immune response will affect the vehicles carrying the virus. Additional

干细胞之家www.stemcell8.cn ←点击进入

investigations are also needed to evaluate the extent to which consecutive viral vehicle administrations will elicit an immune response, and whether multi-dose treatment regimens are feasible in a clinical setting.

6. Mesenchymal Stromal Cells as Carriers for Oncolytic Adenoviruses

The inherent properties of MSCs that favor their use as clinically relevant cell carriers has led to practical attempts at using these cells for delivering anti-tumor therapeutics such as interleukins and toxic genes (see above). However, it is questionable whether or not cell vehicles expressing cytotoxic genes as tumor payloads will provide an effective tumor-killing bystander response. Although the tumor-targeting aspect has been added via cell vehicles, the requirement of a bystander effect limits the overall efficacy of the strategy.

The ability of oncolytic viruses to replicate offers the potential for exponential amplification of the killing effect. This demonstrates a clear advantage in using these agents, compared to toxic gene therapy strategies. Conditionally replicative adenoviruses (CRAds) have been developed for a broad range of tumors and are rapidly transitioning to clinical settings. However, it is apparent that direct introduction of replication competent Ads would face the same problems as non-replicative Ad vectors, which have previously been tested in a range of gene therapy applications. To this end, viral delivery could also make use of vehicles with endogenous tumor-targeting activity, allowing viral replication in the vicinity or directly inside the tumor. In this regard, MSCs represent a promising cell population for use as a viral delivery platform. We first investigated the properties of MSCs as cell vehicles for delivering anti-cancer therapeutics (Pereboeva *et al.*, 2003) and subsequently hypothesized that MSCs would serve as efficient CRAd delivery vehicles. Therefore, we established the key feasibilities determining whether MSC-based CRAd delivery was a viable strategy (Komarova *et al.*, 2006). First, we investigated the ability of MSCs to home to growing tumors. An ovarian cancer xenograft model and several detection methods were used to demonstrate that ip-injected MSCs preferentially localize in the vicinity or within the growing ovarian tumors [Fig. 5(A)]. Only a short-term MSC homing effect to ovarian or breast cancer xenografts was investigated, since cell-based delivery of Ad does not rely on long-term survival and proliferation of cell carriers. Imaging signals were used in the ovarian model to demonstrate that the ip-injected cells were initially distributed nonspecifically in the peritoneal cavity (15 min, 2 h). Two days later, a faint

干细胞之家www.stemcell8.cn ←点击进入

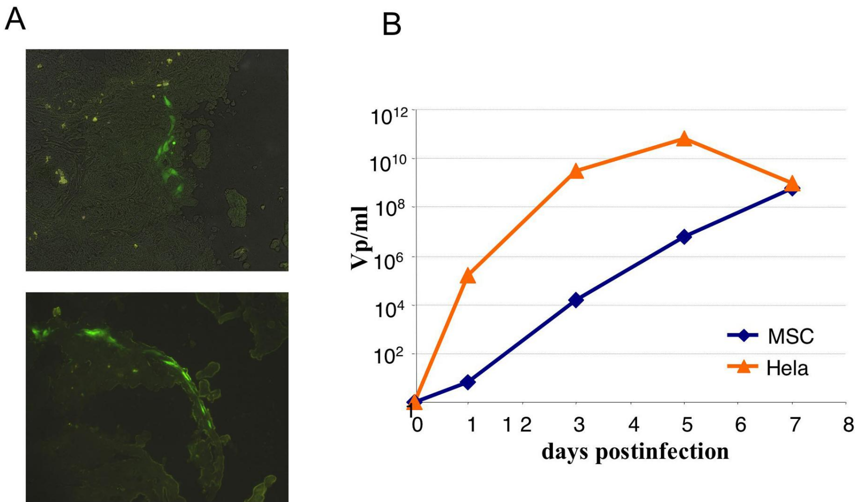


Fig. 5. MSCs as intermediate carriers of oncolytic adenoviruses. (**A**) MSCs home to tumors. MSCs labeled with fluorescent dye CFDA-SE and injected i.p. into mice bearing ovarian tumor xenografts were located in tumor parenchyma or attached to the tumor nodules. Microphotographs represent cryosections of tumor samples. (**B**) MSCs are loaded with adenovirus via infection and produce *de novo* viral particles with delayed kinetics. MSCs and Hela cells (Ad susceptible cell line) were infected with Ad5 at MOI 1 pfu/cell for 2 h. *De novo* viral production in infected cells was estimated at 1, 3, 5 and 7 days by titration of cell lysates on 293 cells. Virus concentration (vp/mL) was calculated as recommended in the manual for AdEasy protocol (TCID50 assay for virus titration).

overall luminescent signal became patchy, indicating that several hours are sufficient for MSCs to travel within the peritoneal cavity to accumulate in specific sites. Imaging of dissected ovarian tumors and other organs of the peritoneal cavity at the time of sacrifice confirmed that the signal was almost exclusively within the tumors.

Next, the ability of MSCs to maintain an adenoviral infection, including viral DNA replication and formation of new infectious viral particles, was examined. Compared to cancer cell lines that are efficiently infected and rapidly produce adenovirus, the ability of different primary cells to support Ad infection was not widely tested. We utilized several methods to demonstrate that MSCs were able to support Ad5 replication, although with slower production kinetics, compared to the permissive HeLa cells [Fig. 5(B)]. In addition, we showed that some of the tropism-modified adenoviruses were more efficiently produced in the MSC vehicles. For instance, the level of Ad5/3 amplification in MSCs almost reached the levels generated in Hela cells. Since adenovirus spreads via lysis of infected cells, the viral life cycle should be slow enough in the cell vehicle to allow sufficient

干细胞之家www.stemcell8.cn ←点击进入

time for the vehicle to reach and establish contact with tumor cells before being destroyed. A panel of replication-competent Ads was tested for an MSC-based cell vehicle approach, which led to the identification of Ad5/3 as the best virus for our experiments. This virus is derived from the wild-type human Ad5 virus, but contains a fiber modification for targeting purposes (Krasnykh *et al.*, 1996). As a viral payload, we used replication-competent Ad5/3 (Komarova *et al.*, 2006) and a conditionally replicative version of this virus with the E1 gene driven by the CXCR4 promoter (Ad5/3CXCR4) (Stoff-Khalili *et al.*, 2007). Both viruses were tested *in vitro* and were demonstrated to have delayed growth kinetics in the vehicle, compared to tumor cells.

The therapeutic efficacy of MSC-based Ad delivery was investigated in human xenograft models using two modes of cell vehicle injection. Infected MSCs were administered locally for an ovarian tumor xenograft model, and systemically for a model of breast cancer metastases of the lung. We wanted to test whether administration of virus-loaded MSCs would improve the therapeutic effect over virus alone. In the ovarian model, the tumor growth was more delayed in animals that received MSC-Ad5/3. The average tumor burden, as measured by total weight of tumor nodules or the intensity of imaging signal at day 21, was significantly less than in tumors collected from control groups treated with PBS, MSCs and Ad5/3. The rate of tumor development correlated with the tumor mass detected by image intensity (Fig. 6). Treatment with MSC-based virus vehicles also resulted in the longest animal survival times. The median survival time was extended to 69 days in groups treated with MSCs-Ad5/3, compared to the control PBS-treated group (34 days). However only modest differences in average survival rates were noted, compared to the virus-only Ad5/3-treatment group (59 days). In the model of pre-established pulmonary metastases derived from MDA-MB-231 cells, MSCs loaded with Ad5/3.CXCR4 also improved the survival of mice with lung tumors, compared to mice treated with either Ad5/3.CXCR4 or hMSCs alone (Stoff-Khalili *et al.*, 2007).

In summary, MSCs loaded with replication-competent Ad were able to exert an oncolytic effect on tumor cells *in vitro* and *in vivo* in both models tested. Delivery of virus by cell carriers with endogenous tumor-targeting properties resulted in a therapeutic benefit, compared to viral injections alone. Although complete tumor regression was not achieved, the cell vehicle-based delivery was superior in terms of delayed tumor development. This effect may result from the amplification of viral load in cell carriers and/or the targeted virus delivery and multifocal release of the virus in the vicinity of tumor cells. Regardless of the mechanism, the rationale for

干细胞之家www.stemcell8.cn ←点击进入

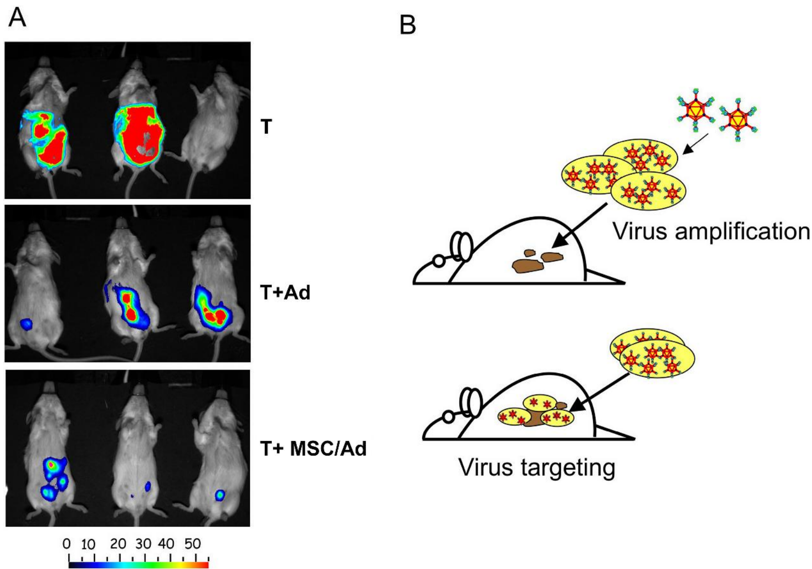


Fig. 6. Effect of oncolytic Ad5/3 delivered directly or via MSC cell carriers on human ovarian xenograft growth. **(A)** Ovarian tumor xenografts were established ip in CB17 SCID mice by injection of SKOV3luc cells. Animals received three injections of PBS only (T), direct injections of Ad5/3 (T + Ad) at 1×10^{10} vp/injection, or MSCs preloaded with Ad5/3 (T + MSC/Ad) at 1×10^{6} cells/injection. Live image of mice taken at day 28 demonstrate tumor development in three experimental groups. The bioluminescence intensity of the image corresponds to the luc-expressing tumor burden (black, least intense; and red, most intense). One mouse in the tumor-only group did not develop tumor and served as a negative control. **(B)** Potential attributes of MSC-Ad delivery: amplification of initial viral load and local release of viral load in vicinity of tumors. (A) Adapted from Fig. 6(A) of: Komarova *et al.*, *Mol Cancer Ther* **5**: 755–766, 2006 with permission from the American Association for Cancer Research.

using MSCs as adenoviral vehicles is valid, and calls for further investigation and optimization.

Acknowledgments

This work was supported by the NIH grant 1 R21 CA115568 (Dr. Larisa Pereboeva). We thank Svetlana Komarova for excellent assistance with experimental studies and contribution to the manuscript.

References

Aboody KS, Brown A, Rainov NG, *et al*. (2000) Neural stem cells display extensive tropism for pathology in adult brain: evidence from intracranial gliomas. *Proc Natl Acad Sci USA* **97**: 12846–51.

干细胞之家www.stemcell8.cn ←点击进入

Allers C, Sierralta WD, Neubauer S, *et al*. (2004) Dynamic of distribution of human bone marrow-derived mesenchymal stem cells after transplantation into adult unconditioned mice. *Transplantation* **78**: 503–508.

Alvarez-Dolado M, Pardal R, Garcia-Verdugo JM, *et al*. (2003) Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. *Nature* **425**: 968–73.

Anjos-Afonso F, Siapati EK, Bonnet D. (2004) *In vivo* contribution of murine mesenchymal stem cells into multiple cell-types under minimal damage conditions. *J Cell Sci* **117**: 5655–64.

Asahara T, Murohara T, Sullivan A, *et al*. (1997) Isolation of putative progenitor endothelial cells for angiogenesis. *Science* **275**: 964–67.

Askari AT, Unzek S, Popovic ZB, *et al*. (2003) *Lancet* **362**: 697–703.

Azizi SA, Stokes D, Augelli BJ, *et al*. (1998) Engraftment and migration of human bone marrow stromal cells implanted in the brains of albino rats–similarities to astrocyte grafts. *Proc Natl Acad Sci USA* **95**: 3908–13.

Baddoo M, Hill K, Wilkinson R, *et al*. (2003) Characterization of mesenchymal stem cells isolated from murine bone marrow by negative selection. *J Cell Biochem* **89**: 1235–49.

Baker AH, McVey JH, Waddington SN, *et al*. (2007) The influence of blood on *in vivo* adenovirus bio-distribution and transduction. *Mol Ther* **15**: 1410–16.

Baker J, Verneris MR, Ito M, *et al*. (2001) Expansion of cytolytic CD8(+) natural killer T cells with limited capacity for graft-versus-host disease induction due to interferon gamma production. *Blood* **97**: 2923–31.

Baksh D, Song L, Tuan RS. (2004) Adult mesenchymal stem cells: characterization, differentiation, and application in cell and gene therapy. *J Cell Mol Med* **8**: 301–16.

Banfi A, Muraglia A, Dozin B, *et al*. (2000) Proliferation kinetics and differentiation potential of *ex vivo* expanded human bone marrow stromal cells: implications for their use in cell therapy. *Exp Hematol* **28**: 707–15.

Barbash IM, Chouraqui P, Baron J, *et al*. (2003) Systemic delivery of bone marrow-derived mesenchymal stem cells to the infarcted myocardium: feasibility, cell migration, and body distribution. *Circulation* **108**: 863–68.

Barry F, Boynton R, Murphy M, *et al*. (2001) The SH-3 and SH-4 antibodies recognize distinct epitopes on CD73 from human mesenchymal stem cells. *Biochem Biophys Res Commun* **289**: 519–24.

Bartholomew A, Patil S, Mackay A, *et al*. (2001) Baboon mesenchymal stem cells can be genetically modified to secrete human erythropoietin *in vivo*. *Hum Gene Ther* **12**: 1527–41.

Baxter MA, Wynn RF, Jowitt SN, *et al*. (2004) Study of telomere length reveals rapid aging of human marrow stromal cells following *in vitro* expansion. *Stem Cells* **22**: 675–82.

干细胞之家www.stemcell8.cn ←点击进入

Bentzon JF, Stenderup K, Hansen FD, *et al*. (2005) Tissue distribution and engraftment of human mesenchymal stem cells immortalized by human telomerase reverse transcriptase gene. *Biochem Biophys Res Commun* **330**: 633–40.

Bianco P, Gehron Robey P. (2000) Marrow stromal stem cells. *J Clin Invest* **105**: 1663–68.

Bianco P, Riminucci M, Gronthos S, Robey PG. (2001) Bone marrow stromal stem cells: nature, biology, and potential applications. *Stem Cells* **19**: 180–92.

Bieback K, Kern S, Kluter H, Eichler H. (2004) Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood. *Stem Cells* **22**: 625–34.

Bittira B, Shum-Tim D, Al-Khaldi A, Chiu RC. (2003) Mobilization and homing of bone marrow stromal cells in myocardial infarction. *Eur J Cardiothorac Surg* **24**: 393–98.

Bonab MM, Alimoghaddam K, Talebian F, *et al*. (2006) Aging of mesenchymal stem cell *in vitro*. *BMC Cell Biol* **7**: 14.

Brouard N, Chapel A, Thierry D, *et al*. (2000) Transplantation of gene-modified human bone marrow stromal cells into mouse-human bone chimeras. *J Hematother Stem Cell Res* **9**: 175–81.

Caplan AI, Reuben D, Haynesworth SE. (1998) Cell-based tissue engineering therapies: the influence of whole body physiology. *Adv Drug Deliv Rev* **33**: 3–14.

Charbord P, Oostendorp R, Pang W, *et al*. (2002) Comparative study of stromal cell lines derived from embryonic, fetal, and postnatal mouse blood-forming tissues. *Exp Hematol* **30**: 1202–10.

Chen J, Li Y, Katakowski M, *et al*. (2003) Intravenous bone marrow stromal cell therapy reduces apoptosis and promotes endogenous cell proliferation after stroke in female rat. *J Neurosci Res* **73**: 778–86.

Chen J, Li Y, Wang L, *et al*. (2001) Therapeutic benefit of intravenous administration of bone marrow stromal cells after cerebral ischemia in rats. *Stroke* **32**: 1005–11.

Chen Y, Yu DC, Charlton D, Henderson DR. (2000) Pre-existent adenovirus antibody inhibits systemic toxicity and antitumor activity of CN706 in the nude mouse LNCaP xenograft model: implications and proposals for human therapy. *Hum Gene Ther* **11**: 1553–67.

Chester J, Ruchatz A, Gough M, *et al*. (2002) Tumor antigen-specific induction of transcriptionally targeted retroviral vectors from chimeric immune receptor-modified T cells. *Nat Biotechnol* **20**: 256–63.

Chin BB, Nakamoto Y, Bulte JW, *et al*. (2003) 111In oxine labelled mesenchymal stem cell SPECT after intravenous administration in myocardial infarction. *Nucl Med Commun* **24**: 1149–54.

Chopp M, Li Y. (2002) Treatment of neural injury with marrow stromal cells. *Lancet Neurol* **1**: 92–100.

Chopp M, Zhang XH, Li Y, *et al*. (2000) Spinal cord injury in rat: treatment with bone marrow stromal cell transplantation. *Neuroreport* **11**: 3001–3005.

干细胞之家www.stemcell8.cn ←点击进入

Clark BR, Keating A. (1995) Biology of bone marrow stroma. *Ann N Y Acad Sci* **770**: 70–78.
Cole C, Qiao J, Kottke T, *et al*. (2005) Tumor-targeted, systemic delivery of therapeutic viral vectors using hitchhiking on antigen-specific T cells. *Nat Med* **11**: 1073–81.
Coukos G, Makrigiannakis A, Kang EH, *et al*. (1999) Use of carrier cells to deliver a replication-selective herpes simplex virus-1 mutant for the intraperitoneal therapy of epithelial ovarian cancer. *Clin Cancer Res* **5**: 1523–37.
Crittenden M, Gough M, Chester J, *et al*. (2003) Pharmacologically regulated production of targeted retrovirus from T cells for systemic antitumor gene therapy. *Cancer Res* **63**: 3173–80.
Croitoru-Lamoury J, Lamoury FM, Zaunders JJ, *et al*. (2007) Human mesenchymal stem cells constitutively express chemokines and chemokine receptors that can be upregulated by cytokines, IFN-beta, and copaxone. *J Interferon Cytokine Res* **27**: 53–64.
Culver KW, Ram Z, Wallbridge S, *et al*. (1992) *In vivo* gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors. *Science* **256**: 1550–52.
Davidoff AM, Ng CY, Brown P, *et al*. (2001) Bone marrow-derived cells contribute to tumor neovasculature and, when modified to express an angiogenesis inhibitor, can restrict tumor growth in mice. *Clin Cancer Res* **7**: 2870–79.
De Bari C, Dell'Accio F, Luyten FP. (2004) Failure of *in vitro* differentiated mesenchymal stem cells from the synovial membrane to form ectopic stable cartilage *in vivo*. *Arthritis Rheum* **50**: 142–50.
Deans RJ, Moseley AB. (2000) Mesenchymal stem cells: biology and potential clinical uses. *Exp Hematol* **28**: 875–84.
Deng YB, Liu XG, Liu ZG, *et al*. (2006) Implantation of BM mesenchymal stem cells into injured spinal cord elicits *de novo* neurogenesis and functional recovery: evidence from a study in rhesus monkeys. *Cytotherapy* **8**: 210–14.
Dennis JE, Carbillet JP, Caplan AI, Charbord P. (2002) The STRO-1+ marrow cell population is multipotential. *Cells Tissues Organs* **170**: 73–82.
Devine MJ, Mierisch CM, Jang E, *et al*. (2002) Transplanted bone marrow cells localize to fracture callus in a mouse model. *J Orthop Res* **20**: 1232–39.
Devine SM, Bartholomew AM, Mahmud N, *et al*. (2001) Mesenchymal stem cells are capable of homing to the bone marrow of non-human primates following systemic infusion. *Exp Hematol* **29**: 244–55.
Devine SM, Cobbs C, Jennings M, *et al*. (2003) Mesenchymal stem cells distribute to a wide range of tissues following systemic infusion into nonhuman primates. *Blood* **101**: 2999–3001.
Digel M, Sampaio KL, Jahn G, Sinzger C. (2006) Evidence for direct transfer of cytoplasmic material from infected to uninfected cells during cell-associated spread of human cytomegalovirus. *J Clin Virol* **37**: 10–20.

干细胞之家www.stemcell8.cn ←点击进入

Digirolamo CM, Stokes D, Colter D, *et al*. (1999) Propagation and senescence of human marrow stromal cells in culture: a simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate. *Br J Haematol* **107**: 275–81.

D'Ippolito G, Howard GA, Roos BA, Schiller PC. (2006) Isolation and characterization of marrow-isolated adult multilineage inducible (MIAMI) cells. *Exp Hematol* **34**: 1608–10.

Dominici M, Le Blanc K, Mueller I, *et al*. (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* **8**: 315–17.

Dvorak HF. (1986) Tumors: wounds that do not heal: similarities between tumor stroma generation and wound healing. *N Engl J Med* **315**: 1650–59.

Elzaouk L, Moelling K, Pavlovic J. (2006) Anti-tumor activity of mesenchymal stem cells producing IL-12 in a mouse melanoma model. *Exp Dermatol* **15**: 865–74.

Erices AA, Allers CI, Conget PA, *et al*. (2003) Human cord blood-derived mesenchymal stem cells home and survive in the marrow of immunodeficient mice after systemic infusion. *Cell Transplant* **12**: 555–61.

Fechner H, Wang X, Pico AH, *et al*. (2007) A bidirectional Tet-dependent promotor construct regulating the expression of E1A for tight control of oncolytic adenovirus replication. *J Biotechnol* **127**: 560–74.

Francois S, Bensidhoum M, Mouiseddine M, *et al*. (2006) Local irradiation not only induces homing of human mesenchymal stem cells at exposed sites but promotes their widespread engraftment to multiple organs: a study of their quantitative distribution after irradiation damage. *Stem Cells* **24**: 1020–29.

Friedenstein AJ, Chailakhjan RK, Lalykina KS. (1970) The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell Tissue Kinet* **3**: 393–403.

Friedenstein AJ, Chailakhyan RK, Gerasimov UV. (1987) Bone marrow osteogenic stem cells: *in vitro* cultivation and transplantation in diffusion chambers. *Cell Tissue Kinet* **20**: 263–72.

Friedenstein AJ, Latzinik NV, Gorskaya Yu F, *et al*. (1992) Bone marrow stromal colony formation requires stimulation by haemopoietic cells. *Bone Miner* **18**: 199–213.

Gao J, Dennis JE, Muzic RF, *et al*. (2001) The dynamic *in vivo* distribution of bone marrow-derived mesenchymal stem cells after infusion. *Cells Tissues Organs* **169**: 12–20.

Garcia-Castro J, Martinez-Palacio J, Lillo R, *et al*. (2005) Tumor cells as cellular vehicles to deliver gene therapies to metastatic tumors. *Cancer Gene Ther* **12**: 341–49.

Gronthos S, Graves SE, Ohta S, Simmons PJ. (1994) The STRO-1+ fraction of adult human bone marrow contains the osteogenic precursors. *Blood* **84**: 4164–73.

干细胞之家www.stemcell8.cn ←点击进入

Gronthos S, Zannettino AC, Hay SJ, *et al*. (2003) Molecular and cellular characterisation of highly purified stromal stem cells derived from human bone marrow. *J Cell Sci* **116**: 1827–35.
Haynesworth SE, Baber MA, Caplan AI. (1992a) Cell surface antigens on human marrow-derived mesenchymal cells are detected by monoclonal antibodies. *Bone* **13**: 69–80.
Haynesworth SE, Baber MA, Caplan AI. (1996) Cytokine expression by human marrow-derived mesenchymal progenitor cells *in vitro*: effects of dexamethasone and IL-1 alpha. *J Cell Physiol* **166**: 585–92.
Haynesworth SE, Goshima J, Goldberg VM, Caplan AI. (1992b) Characterization of cells with osteogenic potential from human marrow. *Bone* **13**: 81–88.
Herrlinger U, Woiciechowski C, Sena-Esteves M, *et al*. (2000) Neural precursor cells for delivery of replication-conditional HSV-1 vectors to intracerebral gliomas. *Mol Ther* **1**: 347–57.
Hill JM, Dick AJ, Raman VK, *et al*. (2003) Serial cardiac magnetic resonance imaging of injected mesenchymal stem cells. *Circulation* **108**: 1009–14.
Horwitz EM, Le Blanc K, Dominici M, *et al*. (2005) Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement. *Cytotherapy* **7**: 393–95.
Huebner RJ, Rowe WP, Schatten WE, *et al*. (1956) Studies on the use of viruses in the treatment of carcinoma of the cervix. *Cancer* **9**:1211–18.
Hung SC, Deng WP, Yang WK, *et al*. (2005) Mesenchymal stem cell targeting of microscopic tumors and tumor stroma development monitored by noninvasive *in vivo* positron emission tomography imaging. *Clin Cancer Res* **11**: 7749–56.
Iankov ID, Blechacz B, Liu C, *et al*. (2007) Infected cell carriers: a new strategy for systemic delivery of oncolytic measles viruses in cancer virotherapy. *Mol Ther* **15**: 114–22.
Izadpanah R, Joswig T, Tsien F, *et al*. (2005) Characterization of multipotent mesenchymal stem cells from the bone marrow of rhesus macaques. *Stem Cells Dev* **14**: 440–51.
Jaiswal N, Haynesworth SE, Caplan AI, Bruder SP. (1997) Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells *in vitro*. *J Cell Biochem* **64**: 295–312.
Javazon EH, Beggs KJ, Flake AW. (2004) Mesenchymal stem cells: paradoxes of passaging. *Exp Hematol* **32**: 414–25.
Javazon EH, Colter DC, Schwarz EJ, Prockop DJ. (2001) Rat marrow stromal cells are more sensitive to plating density and expand more rapidly from single-cell-derived colonies than human marrow stromal cells. *Stem Cells* **19**: 219–25.
Jevremovic D, Gulati R, Hennig I, *et al*. (2004) Use of blood outgrowth endothelial cells as virus-producing vectors for gene delivery to tumors. *Am J Physiol Heart Circ Physiol* **287**: H494–H500.

干细胞之家www.stemcell8.cn ←点击进入

Jiang Y, Vaessen B, Lenvik T, *et al*. (2002) Multipotent progenitor cells can be isolated from postnatal murine bone marrow, muscle, and brain. *Exp Hematol* **30**: 896–904.

Kadiyala S, Young RG, Thiede MA, Bruder SP. (1997) Culture expanded canine mesenchymal stem cells possess osteochondrogenic potential *in vivo* and *in vitro*. *Cell Transplant* **6**: 125–34.

Kern S, Eichler H, Stoeve J, *et al*. (2006) Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells* **24**: 1294–301.

Kinnaird T, Stabile E, Burnett MS, *et al*. (2004) Local delivery of marrow-derived stromal cells augments collateral perfusion through paracrine mechanisms. *Circulation* **109**: 1543–49.

Komarova S, Kawakami Y, Stoff-Khalili MA, *et al*. (2006) Mesenchymal progenitor cells as cellular vehicles for delivery of oncolytic adenoviruses. *Mol Cancer Ther* **5**: 755–66.

Kopen GC, Prockop DJ, Phinney DG. (1999) Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. *Proc Natl Acad Sci USA* **96**: 10711–16.

Kraitchman DL, Heldman AW, Atalar E, *et al*. (2003) *In vivo* magnetic resonance imaging of mesenchymal stem cells in myocardial infarction. *Circulation* **107**: 2290–93.

Kraitchman DL, Tatsumi M, Gilson WD, *et al*. (2005) Dynamic imaging of allogeneic mesenchymal stem cells trafficking to myocardial infarction. *Circulation* **112**: 1451–61.

Krasnykh VN, Mikheeva GV, Douglas JT, Curiel DT. (1996) Generation of recombinant adenovirus vectors with modified fibers for altering viral tropism. *J Virol* **70**: 6839–46.

Kuppen PJ, Marinelli A, Camps JA, *et al*. (1992) Biodistribution of lymphokine-activated killer (LAK) cells in Wag rats after hepatic-artery or jugular-vein infusion. *Int J Cancer* **52**: 266–70.

Kyriakou CA, Yong KL, Benjamin R, *et al*. (2006) Human mesenchymal stem cells (hMSCs) expressing truncated soluble vascular endothelial growth factor receptor (tsFlk-1) following lentiviral-mediated gene transfer inhibit growth of Burkitt's lymphoma in a murine model. *J Gene Med* **8**: 253–64.

Li S, Tokuyama T, Yamamoto J, *et al*. (2005) Bystander effect-mediated gene therapy of gliomas using genetically engineered neural stem cells. *Cancer Gene Ther* **12**: 600–607.

Li S, Gao Y, Tokuyama T, *et al*. (2007) Genetically engineered neural stem cells migrate and suppress glioma cell growth at distant intracranial sites. *Cancer Lett* **251**: 220–27.

Liu Y, Deisseroth A. (2006) Oncolytic adenoviral vector carrying the cytosine deaminase gene for melanoma gene therapy. *Cancer Gene Ther* **13**: 845–55.

干细胞之家www.stemcell8.cn ←点击进入

Lyden D, Hattori K, Dias S, *et al*. (2001) Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. *Nat Med* **7**: 1194–201.

Lyons M, Onion D, Green NK, *et al*. (2006) Adenovirus type 5 interactions with human blood cells may compromise systemic delivery. *Mol Ther* **14**: 118–28.

MacDonald DJ, Luo J, Saito T, *et al*. (2005) Persistence of marrow stromal cells implanted into acutely infarcted myocardium: observations in a xenotransplant model. *J Thorac Cardiovasc Surg* **130**: 1114–21.

Mahmood A, Lu D, Chopp M. (2004a) Intravenous administration of marrow stromal cells (MSCs) increases the expression of growth factors in rat brain after traumatic brain injury. *J Neurotrauma* **21**: 33–39.

Mahmood A, Lu D, Chopp M. (2004b) Marrow stromal cell transplantation after traumatic brain injury promotes cellular proliferation within the brain. *Neurosurgery* **55**: 1185–93.

Majumdar MK, Keane-Moore M, Buyaner D, *et al*. (2003) Characterization and functionality of cell surface molecules on human mesenchymal stem cells. *J Biomed Sci* **10**: 228–41.

Makkar RR, Price MJ, Lill M, *et al*. (2005) Intramyocardial injection of allogenic bone marrow-derived mesenchymal stem cells without immunosuppression preserves cardiac function in a porcine model of myocardial infarction. *J Cardiovasc Pharmacol Ther* **10**: 225–33.

Martin DR, Cox NR, Hathcock TL, *et al*. (2002) Isolation and characterization of multipotential mesenchymal stem cells from feline bone marrow. *Exp Hematol* **30**: 879–86.

Meyerrose TE, De Ugarte DA, Hofling AA, *et al*. (2007) *In vivo* distribution of human adipose-derived mesenchymal stem cells in novel xenotransplantation models. *Stem Cells* **25**: 220–27.

Minguell JJ, Erices A, Conget P. (2001) Mesenchymal stem cells. *Exp Biol Med (Maywood)* **226**: 507–20.

Minguell JJ, Fierro FA, Epunan MJ, *et al*. (2005) Nonstimulated human uncommitted mesenchymal stem cells express cell markers of mesenchymal and neural lineages. *Stem Cells Dev* **14**: 408–14.

Miura M, Gronthos S, Zhao M, *et al*. (2003) SHED: stem cells from human exfoliated deciduous teeth. *Proc Natl Acad Sci USA* **100**: 5807–12.

Morrow G, Vachot L, Vagenas P, Robbiani M. (2007) Current concepts of HIV transmission. *Curr HIV/AIDS Rep* **4**: 29–35.

Mosca JD, Hendricks JK, Buyaner D, *et al*. (2000) Mesenchymal stem cells as vehicles for gene delivery. *Clin Orthop Relat Res*: S71–S90.

Nakamizo A, Marini F, Amano T, *et al*. (2005) Human bone marrow-derived mesenchymal stem cells in the treatment of gliomas. *Cancer Res* **65**: 3307–18.

Nakamura K, Ito Y, Kawano Y, *et al*. (2004) Antitumor effect of genetically engineered mesenchymal stem cells in a rat glioma model. *Gene Ther* **11**: 1155–64.

干细胞之家www.stemcell8.cn ←点击进入

Namba H, Tagawa M, Iwadate Y, *et al*. (1998) Bystander effect-mediated therapy of experimental brain tumor by genetically engineered tumor cells. *Hum Gene Ther* **9**: 5–11.

Namba H, Tagawa M, Miyagawa T, *et al*. (2000) Treatment of rat experimental brain tumors by herpes simplex virus thymidine kinase gene-transduced allogeneic tumor cells and ganciclovir. *Cancer Gene Ther* **7**: 947–53.

Negrin RS, Edinger M, Verneris M, *et al*. (2002) Visualization of tumor growth and response to NK-T cell based immunotherapy using bioluminescence. *Ann Hematol* **81 (**Suppl 2**)**: S44–S45.

Neth P, Ciccarella M, Egea V, *et al*. (2006) Wnt signaling regulates the invasion capacity of human mesenchymal stem cells. *Stem Cells* **24**: 1892–903.

Nettelbeck DM. (2003) Virotherapeutics: conditionally replicative adenoviruses for viral oncolysis. *Anticancer Drugs* **14**: 577–84.

Olivares EL, Ribeiro VP, Werneck de Castro JP, *et al*. (2004) Bone marrow stromal cells improve cardiac performance in healed infarcted rat hearts. *Am J Physiol Heart Circ Physiol* **287**: H464–70.

Ong HT, Hasegawa K, Dietz AB, *et al*. (2007) Evaluation of T cells as carriers for systemic measles virotherapy in the presence of antiviral antibodies. *Gene Ther* **14**: 324–33.

Ortiz LA, Gambelli F, McBride C, *et al*. (2003) Mesenchymal stem cell engraftment in lung is enhanced in response to bleomycin exposure and ameliorates its fibrotic effects. *Proc Natl Acad Sci USA* **100**: 8407–11.

Owen M. (1988) Marrow stromal stem cells. *J Cell Sci Suppl* **10**: 63–76.

Ozaki Y, Nishimura M, Sekiya K, *et al*. (2007) Comprehensive analysis of chemotactic factors for bone marrow mesenchymal stem cells. *Stem Cells Dev* **16**: 119–29.

Pan YW, Scarlett JM, Luoh TT, Kurre P. (2007) Prolonged adherence of human immunodeficiency virus-derived vector particles to hematopoietic target cells leads to secondary transduction *in vitro* and *in vivo*. *J Virol* **81**: 639–49.

Park H, Temenoff JS, Tabata Y, *et al*. (2007) Injectable biodegradable hydrogel composites for rabbit marrow mesenchymal stem cell and growth factor delivery for cartilage tissue engineering. *Biomaterials* **28**: 3217–27.

Peister A, Mellad JA, Larson BL, *et al*. (2004) Adult stem cells from bone marrow (MSCs) isolated from different strains of inbred mice vary in surface epitopes, rates of proliferation, and differentiation potential. *Blood* **103**: 1662–68.

Pereboeva L, Komarova S, Mikheeva G, *et al*. (2003) Approaches to utilize mesenchymal progenitor cells as cellular vehicles. *Stem Cells* **21**: 389–404.

Pereira RF, Halford KW, O'Hara MD, *et al*. (1995) Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice. *Proc Natl Acad Sci USA* **92**: 4857–61.

Phinney DG, Kopen G, Isaacson RL, Prockop DJ. (1999a) Plastic adherent stromal cells from the bone marrow of commonly used strains of inbred mice: variations in yield, growth, and differentiation. *J Cell Biochem* **72**: 570–85.

干细胞之家www.stemcell8.cn ←点击进入

Phinney DG, Kopen G, Righter W, *et al*. (1999b) Donor variation in the growth properties and osteogenic potential of human marrow stromal cells. *J Cell Biochem* **75**: 424–36.

Pittenger MF, Mackay AM, Beck SC, *et al*. (1999) Multilineage potential of adult human mesenchymal stem cells. *Science* **284**: 143–47.

Pittenger MF, Martin BJ. (2004) Mesenchymal stem cells and their potential as cardiac therapeutics. *Circ Res* **95**: 9–20.

Pittenger MF, Mosca JD, McIntosh KR. (2000) Human mesenchymal stem cells: progenitor cells for cartilage, bone, fat and stroma. *Curr Top Microbiol Immunol* **251**: 3–11.

Pizzato M, Marlow SA, Blair ED, Takeuchi Y. (1999) Initial binding of murine leukemia virus particles to cells does not require specific Env-receptor interaction. *J Virol* **73**: 8599–611.

Potapova IA, Gaudette GR, Brink PR, *et al*. (2007) Mesenchymal stem cells support migration, extracellular matrix invasion, proliferation, and survival of endothelial cells *in vitro*. *Stem Cells* **25**: 1761–68.

Power AT, Wang J, Falls TJ, *et al*. (2007) Carrier cell-based delivery of an oncolytic virus circumvents antiviral immunity. *Mol Ther* **15**: 123–30.

Price MJ, Chou CC, Frantzen M, *et al*. (2006) Intravenous mesenchymal stem cell therapy early after reperfused acute myocardial infarction improves left ventricular function and alters electrophysiologic properties. *Int J Cardiol* **111**: 231–39.

Prockop DJ, Sekiya I, Colter DC. (2001) Isolation and characterization of rapidly self-renewing stem cells from cultures of human marrow stromal cells. *Cytotherapy* **3**: 393–96.

Ram Z, Culver KW, Oshiro EM, *et al*. (1997) Therapy of malignant brain tumors by intratumoral implantation of retroviral vector-producing cells. *Nat Med* **3**: 1354–61.

Raykov Z, Balboni G, Aprahamian M, Rommelaere J. (2004) Carrier cell-mediated delivery of oncolytic parvoviruses for targeting metastases. *Int J Cancer* **109**: 742–49.

Reyes M, Verfaillie CM. (2001) Characterization of multipotent adult progenitor cells, a subpopulation of mesenchymal stem cells. *Ann N Y Acad Sci* **938**: 231–33; discussion 233–35.

Roberts MS, Lorence RM, Groene WS, Bamat MK. (2006) Naturally oncolytic viruses. *Curr Opin Mol Ther* **8**: 314–21.

Rojas M, Xu J, Woods CR, *et al*. (2005) Bone marrow-derived mesenchymal stem cells in repair of the injured lung. *Am J Respir Cell Mol Biol* **33**: 145–52.

Rombouts WJ, Ploemacher RE. (2003) Primary murine MSC show highly efficient homing to the bone marrow but lose homing ability following culture. *Leukemia* **17**: 160–70.

Ruster B, Gottig S, Ludwig RJ, *et al*. (2006) Mesenchymal stem cells display coordinated rolling and adhesion behavior on endothelial cells. *Blood* **108**: 3938–44.

干细胞之家www.stemcell8.cn ←点击进入

Saito T, Kuang JQ, Bittira B, *et al*. (2002) Xenotransplant cardiac chimera: immune tolerance of adult stem cells. *Ann Thorac Surg* **74**: 19–24; discussion 24.

Sanchez-Ramos J, Song S, Cardozo-Pelaez F, *et al*. (2000) Adult bone marrow stromal cells differentiate into neural cells *in vitro*. *Exp Neurol* **164**: 247–56.

Satake K, Lou J, Lenke LG. (2004) Migration of mesenchymal stem cells through cerebrospinal fluid into injured spinal cord tissue. *Spine* **29**: 1971–79.

Schenk S, Mal N, Finan A, *et al*. (2007) Monocyte chemotactic protein-3 is a myocardial mesenchymal stem cell homing factor. *Stem Cells* **25**: 245–51.

Schichor C, Birnbaum T, Etminan N, *et al*. (2006) Vascular endothelial growth factor A contributes to glioma-induced migration of human marrow stromal cells (hMSC) *Exp Neurol* **199**: 301–10.

Schlegel R, Willingham MC, Pastan IH. (1982) Saturable binding sites for vesicular stomatitis virus on the surface of Vero cells. *J Virol* **43**: 871–75.

Schmidt A, Ladage D, Schinkothe T, *et al*. (2006a) Basic fibroblast growth factor controls migration in human mesenchymal stem cells. *Stem Cells* **24**: 1750–58.

Schmidt A, Ladage D, Steingen C, *et al*. (2006b) Mesenchymal stem cells transmigrate over the endothelial barrier. *Eur J Cell Biol* **85**: 1179–88.

Schrepfer S, Deuse T, Lange C, *et al*. (2007a) Simplified protocol to isolate, purify, and culture expand mesenchymal stem cells. *Stem Cells Dev* **16**: 105–107.

Schrepfer S, Deuse T, Reichenspurner H, *et al*. (2007b) Stem cell transplantation: the lung barrier. *Transplant Proc* **39**: 573–76.

Sekiya I, Larson BL, Smith JR, *et al*. (2002) Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality. *Stem Cells* **20**: 530–41.

Sethe S, Scutt A, Stolzing A. (2006) Aging of mesenchymal stem cells. *Ageing Res Rev* **5**: 91–116.

Seymour JF. (2006) Extra-pulmonary aspects of acquired pulmonary alveolar proteinosis as predicted by granulocyte-macrophage colony-stimulating factor-deficient mice. *Respirology* **11**(Suppl): S16–S22.

Shake JG, Gruber PJ, Baumgartner WA, *et al*. (2002) Mesenchymal stem cell implantation in a swine myocardial infarct model: engraftment and functional effects. *Ann Thorac Surg* **73**: 1919–25; discussion 1926.

Short B, Brouard N, Driessen R, Simmons PJ. (2001) Prospective isolation of stromal progenitor cells from mouse BM. *Cytotherapy* **3**: 407–408.

Sipo I, Hurtado Pico A, Wang X, *et al*. (2006) An improved Tet-On regulatable FasL-adenovirus vector system for lung cancer therapy. *J Mol Med* **84**: 215–25.

Son BR, Marquez-Curtis LA, Kucia M, *et al*. (2006) Migration of bone marrow and cord blood mesenchymal stem cells *in vitro* is regulated by stromal-derived factor-1-CXCR4 and hepatocyte growth factor-c-met axes and involves matrix metalloproteinases. *Stem Cells* **24**: 1254–64.

干细胞之家www.stemcell8.cn ←点击进入

Sordi V, Malosio ML, Marchesi F, *et al*. (2005) Bone marrow mesenchymal stem cells express a restricted set of functionally active chemokine receptors capable of promoting migration to pancreatic islets. *Blood* **106**: 419–27.

Spees JL, Gregory CA, Singh H, *et al*. (2004) Internalized antigens must be removed to prepare hypoimmunogenic mesenchymal stem cells for cell and gene therapy. *Mol Ther* **9**: 747–56.

Stagg J, Lejeune L, Paquin A, Galipeau J. (2004) Marrow stromal cells for interleukin-2 delivery in cancer immunotherapy. *Hum Gene Ther* **15**: 597–608.

Stenderup K, Justesen J, Clausen C, Kassem M. (2003) Aging is associated with decreased maximal life span and accelerated senescence of bone marrow stromal cells. *Bone* **33**: 919–26.

Stevenson M, Hale AB, Hale SJ, *et al*. (2007) Incorporation of a laminin-derived peptide (SIKVAV) on polymer-modified adenovirus permits tumor-specific targeting via alpha6-integrins. *Cancer Gene Ther* **14**: 335–45.

Stoff-Khalili MA, Rivera AA, Mathis JM, *et al*. (2007) Mesenchymal stem cells as a vehicle for targeted delivery of CRAds to lung metastases of breast carcinoma. *Breast Cancer Res Treat*, Epub ahead of print.

Studeny M, Marini FC, Champlin RE, *et al*. (2002) Bone marrow-derived mesenchymal stem cells as vehicles for interferon-beta delivery into tumors. *Cancer Res* **62**: 3603–608.

Sykova E, Jendelova P, Urdzikova L, *et al*. (2006) Bone marrow stem cells and polymer hydrogels — two strategies for spinal cord injury repair. *Cell Mol Neurobiol* **26**: 1113–29.

Taki M, Kagawa S, Nishizaki M, *et al*. (2005) Enhanced oncolysis by a tropism-modified telomerase-specific replication-selective adenoviral agent OBP-405 ('Telomelysin-RGD') *Oncogene* **24**: 3130–40.

Thorne SH, Negrin RS, Contag CH. (2006) Synergistic antitumor effects of immune cell-viral biotherapy. *Science* **311**: 1780–84.

Toma C, Pittenger MF, Cahill KS, *et al*. (2002) Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. *Circulation* **105**: 93–98.

Tropel P, Noel D, Platet N, *et al*. (2004) Isolation and characterisation of mesenchymal stem cells from adult mouse bone marrow. *Exp Cell Res* **295**: 395–406.

Tsai V, Johnson DE, Rahman A, *et al*. (2004) Impact of human neutralizing antibodies on antitumor efficacy of an oncolytic adenovirus in a murine model. *Clin Cancer Res* **10**: 7199–206.

Urdzikova L, Jendelova P, Glogarova K, *et al*. (2006) Transplantation of bone marrow stem cells as well as mobilization by granulocyte-colony stimulating factor promotes recovery after spinal cord injury in rats. *J Neurotrauma* **23**: 1379–91.

Verneris MR, Karami M, Baker J, *et al*. (2004) Role of NKG2D signaling in the cytotoxicity of activated and expanded CD8+ T cells. *Blood* **103**: 3065–72.

干细胞之家www.stemcell8.cn ←点击进入

Wagner W, Feldmann RE Jr, Seckinger A, *et al*. (2006) The heterogeneity of human mesenchymal stem cell preparations — evidence from simultaneous analysis of proteomes and transcriptomes. *Exp Hematol* **34**: 536–48.

Wolf D, Rumpold H, Koeck R, Gunsilius E. (2005) Re: Mesenchymal stem cells: potential precursors for tumor stroma and targeted-delivery vehicles for anticancer agents. *J Natl Cancer Inst* **97**: 540–41; author reply 541–42.

Worgall S, Leopold PL, Wolff G, *et al*. (1997a) Role of alveolar macrophages in rapid elimination of adenovirus vectors administered to the epithelial surface of the respiratory tract. *Hum Gene Ther* **8**: 1675–84.

Worgall S, Wolff G, Falck-Pedersen E, Crystal RG. (1997b) Innate immune mechanisms dominate elimination of adenoviral vectors following *in vivo* administration. *Hum Gene Ther* **8**: 37–44.

Xin H, Kanehira M, Mizuguchi H, *et al*. (2007) Targeted delivery of CX3CL1 to multiple lung tumors by mesenchymal stem cells. *Stem Cells* **25**: 1618–26.

Ye X, Jerebtsova M, Ray PE. (2000) Liver bypass significantly increases the transduction efficiency of recombinant adenoviral vectors in the lung, intestine, and kidney. *Hum Gene Ther* **11**: 621–27.

Yip S, Aboody KS, Burns M, *et al*. (2003) Neural stem cell biology may be well suited for improving brain tumor therapies. *Cancer J* **9**: 189–204.

Yotnda P, Savoldo B, Charlet-Berguerand N, *et al*. (2004) Targeted delivery of adenoviral vectors by cytotoxic T cells. *Blood* **104**: 2272–80.

Zhao LR, Duan WM, Reyes M, *et al*. (2002) *Exp Neurol* **174**: 11–20.

Zuk PA, Zhu M, Ashjian P, *et al*. (2002) Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* **13**: 4279–95.

干细胞之家www.stemcell8.cn ←点击进入

This page intentionally left blank

干细胞之家www.stemcell8.cn ←点击进入

INDEX

in vitro, 115, 118–123, 127, 128
5-FC, 340–342, 347, 348, 355, 356, 358–360
5-FU, 342, 347, 348, 355
5-fluorocytosine, 342, 347, 348
5-fluorouracil, 342, 347, 348

AAVS1 locus, 19, 21, 24, 26, 27, 34
Ad-TRAIL, 373, 378, 379, 382, 383
adeno-associated virus, 19, 21, 47, 48
adenosine deaminase, 1, 2
autologous pluripotent stem cells, xxix, xxxiv, 89, 98, 104

bone morphogenetic proteins, 299, 307
brain tumors, 299, 301–307, 309, 311, 312
breast cancer, 262, 264, 280–286

cancer stem cells (CSCs), xxii, xxvi–xxviii, 203, 213, 285, 286, 317, 318, 321, 322, 326
cancer therapy, 351, 354
carboxylesterase, 339, 342, 348, 349, 361, 362
CD24, 274–276, 280, 285, 286
CD29/β1-integrin, 274
CD34^{+} cells, 373, 379–387
CD44, 317–328
CD49f/α6-integrin, 274
cell vehicles, 393, 414, 425, 427, 428
clinical trials, 2–5, 8, 9
CPT-11, 339, 342, 348, 349, 361–364
CRAds, 428
custom site-specific endonucleases, 169, 180, 183
cybrid stem cells, xxix, xxxii, 100–102, 104
cytosine deaminase, 339, 342, 347, 348, 353

DNA recognition, 135, 140, 145, 146, 150, 151, 156, 158–160

emulsion, 119–121, 123, 125, 128
endonuclease-boosted gene targeting, xxi, xxii, xxviii, xxx, xxxi, 81, 84, 86, 90, 103
estrogen receptor, 275, 284
expression, 115, 116, 118–123, 125–128

gene correction, 135, 139, 140
gene repair, 169, 172, 179, 182
gene repair/alteration, xxi, xxix, xxxi, 84, 86, 96, 104
gene therapy, 1, 2, 4–6, 8, 10–13, 136, 139, 169, 170, 178–180, 183, 187–191, 195, 197, 336, 337, 339, 354, 373, 378, 379, 387
genome engineering, 136–140, 145, 150, 161
glioma, 335–338, 340–342, 344, 350–358, 365

helper-dependent adenoviral vectors, 169, 173, 174
hematopoietic stem cells (HSC), 1, 2, 204–209, 211, 212, 215, 217–220, 222, 225, 226, 232, 234–238, 240–242
Herpes simplex virus type 1, 47, 48
homing, 319, 320, 327, 328
homing endonuclease, 135, 140–145, 147, 149–161
homologous recombination, 169–171
HSV/AAV hybrid vector, 47, 49, 54, 60–66, 68, 69

irinotecan, 342, 348, 361

LAGLIDADG, 135, 141, 142, 144–149, 151, 153, 160
leukemic stem cells (LSCs), 203, 204, 214, 215, 229, 233
long-term and transient gene therapy, xxi, xxii, xxiv, xxvii, xxix, xxxii, xxxiii

干细胞之家www.stemcell8.cn ←点击进入

long-term gene expression, 48, 59, 60
long-term gene therapy, 81, 103, 105

mammary stem cell, 261, 262, 267–269, 271, 273–276, 278–281, 284, 286
medulloblastoma, 335, 336, 338, 342, 344, 358
melanoma, 335, 336, 338, 341, 358–360
mesenchymal stem cells, 393, 394, 396, 399, 408, 411, 413, 426
mesenchymal stromal cells, 394–396, 428
metastasis, 317–319, 321–323, 326–328
methyltransferase, 115, 124, 126, 127
MMTV-wnt-1, 286
multipotency, 187
myelopreparative conditioning, 1, 2, 5, 8

neu, 283
neural stem cells, 335, 337, 345, 346, 349, 352, 353, 356, 361, 362, 364
neuroblastoma, 335, 336, 338, 341, 342, 344, 348, 352, 353, 360–364
neurosphere assay, 302, 303, 306
normal and cancer stem cells, 300, 305, 314

oncolytic viruses, 393, 394, 414, 418, 422–424, 426–428

plasticity, 192
prodrug-activating enzyme, 346, 347, 353, 355, 365
protein engineering, 144, 151, 152

recombination, 135, 137–140, 142, 143, 149, 153, 154, 157, 158, 161
Rep protein, 19, 22–30, 32, 34–37

selection, 115, 117–122, 124–128
self-renewal, 204, 206–208, 213, 214, 216–224, 237–239, 242
severe combined immunodeficiency, 1–3, 8
site-specific integration, 47, 59–63, 66–69
Site-specific transgene integration, 19
SN-38, 339, 348, 349, 362, 364
stem cells, 169, 180–183, 187–190, 192, 193, 195, 196

targeted therapy, 363, 365
targeted transgene integration, xxxi, 81, 94
tissue- and tumor-homing, xxiv
TRAIL, 373–387
TRAIL receptors, 373–376, 382, 386
transient regenerative gene therapy, 81, 102, 104
tumor homing, 373, 379, 383, 384, 386
tumor progenitors, 324
tumor-initiating cells, 317, 318, 321
tumor-targeted delivery, 393
tumorigenicity, 305, 310, 313

wnt-1, 280, 284

xenotransplantation models, 207

zinc finger, 115–117, 122, 124, 127