FOREWORD

Stem cell biology is emerging as a field in biology with tremendous therapeutic potential. Making this potential a reality requires an international effort. The recognition that such a promising yet multifaceted discipline needs fostering led to the establishment of the International Society for Stem Cell Research (ISSCR). Central to the efforts of the ISSCR is the development of tools to ensure the success of stem cell researchers. What better way to do this than to collaborate with *Current Protocols* to develop this valuable compendium of protocols in stem cell biology?

Stem cell researchers have developed a number of breakthrough techniques, from the derivation and manipulation of pluripotent embryonic stem cells to purification and transplantation of tissue-restricted stem cells from adult organs. A number of laboratories have become leaders in the field as a result of developing such techniques. The more efficient scientists are at implementing new and powerful methodologies in their own laboratories, the faster stem cell biology will advance our understanding of normal development and lead to the development of therapies. Thus, the availability of quality protocols will have a major impact on the success of the entire field. *Current Protocols* has long been the premier volume for proven in-depth protocols regarding many aspects of biology, and this volume on stem cell biology will prove a valuable addition to researchers worldwide.

Experiments in stem cell biology must be interpreted with great caution as well as openness to alternative explanations. For example, the recently discovered phenomenon of cell fusion in vivo or the existence of tissue-restricted blood stem cells in peripheral tissues were initially misinterpreted as evidence for stem cell trans-differentiation.

It is very important that this compendium of protocols highlight potential pitfalls as well as maintain the opportunity for clarification and correction when the need arises. The fact that these protocols will be provided online will help ensure that researchers always have the latest, most up-to-date protocols available to them.

The stem cell field is burgeoning, and, as I have seen within the ISSCR, there is a genuine push to share information and interact so that the field can move forward quickly. There is a drive to develop not only excellent basic research skills but to bring the findings to clinical use so that patients can benefit. As a Hematology Attending Physician at Children's Hospital Boston, I treat children who have pediatric blood diseases or leukemia and am drawn by the need to translate our research findings into therapies to help treat a number of diseases. The promise is great, but we need to deliver, and I believe *Current Protocols in Stem Cell Biology* will help tremendously.

Leonard I. Zon

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PREFACE

The concept of tissue regeneration was already present in ancient Greece, reflected by the mythological stories of Prometheus or the Hydra, and described by Aristotle. The first scientific studies of the phenomenon were performed around 1740 by Abraham Trembley on the cnidarian polyp Hydra. Yet, it took another 150 years until the idea emerged that tissue maintenance, turnover, and regeneration may be rooted in rare cells with unique properties: stem cells.

During the past 50 years, the development and improvement of techniques to isolate, track, manipulate, culture, characterize, and transplant cells has led to the discovery of stem cells in many different tissues. The power of the hematopoietic stem cell to repopulate the entire blood system, first demonstrated by Till and McCulloch in 1963, has long since been harnessed for clinical use. More recently, the identification of the even more versatile pluripotent embryonic stem cell by Evans and Kaufman (1981) and Martin (1981) has revolutionized our ability to probe mammalian developmental biology and to model human diseases.

In recent years the fascination of scientists with stem cells has spilled over into the public domain, and many share the hope that the 21st century will see a revolution in regenerative medicine as novel therapies are derived from stem cells. Continued scientific study of the biology of stem cells will be critical for this prospect to become a reality. It is the goal of the editors, in developing this manual, to facilitate this endeavor by providing scientists with a compendium of well established protocols in stem cell biology. Along with the continued progress of the field of stem cell biology, this collection of protocols will expand. The manual is written such that even a seasoned stem cell biologist will find many novel and useful ideas, but with enough detail provided to also guide those with less experience.

This product is not intended to substitute for a graduate course in stem cell biology or for a comprehensive textbook in the field. Introductory texts on stem cells and cell and developmental biology that we recommend include *Handbook of Stem Cells* (Lanza et al., 2004), *Developmental Biology* (Gilbert, 2006), and *Molecular Cell Biology* (Lodish et al., 2004) or *Molecular Biology of the Cell* (Alberts et al., 2002).

We also strongly recommend that readers gain first-hand experience in basic laboratory techniques and safety procedures by training in a well established laboratory. Finally, with the great promise and potential of stem cells, come ethical concerns. We urge stem cell biologists to reflect on these issues and to respect internationally accepted ethical guidelines and limitations such as those developed by the International Society for Stem Cell Research on the Conduct of Human Embryonic Stem Cell Research (ISSCR; see *APPENDIX A1.1*).

HOW TO USE THIS MANUAL

Format and Organization

This publication is available online, with monthly supplements.

Subjects in this manual are organized by chapters, which are subdivided into sections that contain protocols organized in units. Protocol units, which constitute the bulk of the title, generally describe a method and include one or more protocols with listings of materials, steps and annotations, recipes for unique reagents and solutions, and commentaries on the

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"hows" and "whys" of the method. Other units present more general information in the form of explanatory text with no protocols. Overview units contain theoretical discussions that lay the foundation for subsequent protocols, while discussion units present more general information. Page numbering in the PDF version reflects the modular arrangement by unit; for example, page 1A.2.3 refers to Chapter 1 (Embryonic and Extraembryonic Stem Cells), Section A (Isolation of Embryonic Stem Cells, *UNIT 1.2* (Derivation of hESCs from Intact Blastocysts), and page 3 of that particular unit.

Although many reagents and procedures are employed repeatedly throughout the manual, we have opted to retain individual authors' recipes or supplier designations because of the importance of using a particular reagent or procedure for successful stem cell experiments. Cross-referencing among the units is used for very basic procedures that do not vary from laboratory to laboratory.

Introductory and Explanatory Information

Because this publication is first and foremost a compilation of laboratory techniques in stem cell biology, we have included explanatory information where required to help readers gain an intuitive grasp of the procedures. Some sections begin with special overview units that describe the state of the art of the topic matter and provide a context for the procedures that follow. Section and unit introductions describe how the protocols that follow connect to one another, and annotations to the actual protocol steps describe what is happening as a procedure is carried out. Finally, the Commentary that closes each protocol unit describes background information regarding the historical and theoretical development of the method, as well as alternative approaches, critical parameters, troubleshooting guidelines, anticipated results, and time considerations. All units contain cited references and many indicate key references to inform users of particularly useful background reading, original descriptions, or applications of a technique.

Protocols

Many units in the manual contain groups of protocols, each presented with a series of steps. One or more *basic* protocols are presented first in each unit and generally cover the recommended or most universally applicable approaches. *Alternate* protocols are provided where different equipment or reagents can be employed to achieve similar ends, where the starting material requires a variation in approach, or where requirements for the end product differ from those in the basic protocol. *Support* protocols describe additional steps that are required to perform the basic or alternate protocols; these steps are separated from the core protocol because they might be applicable to other uses in the manual or because they are performed in a time frame separate from the basic protocol steps.

Reagents and Solutions

Reagents required for a protocol are itemized in the materials list before the procedure begins. Many are common stock solutions, others are commonly used buffers or media, while others are solutions unique to a particular protocol. Recipes for solutions are provided in each unit, following the protocols (and before the commentary) under the heading Reagents and Solutions. It is important to note that the *names* of some of these special solutions might be similar from unit to unit (e.g., RIPA buffer) while the *recipes* differ; thus, make certain that reagents are prepared from the proper recipes.

Commercial Suppliers

Throughout the manual, the authors have recommended commercial suppliers of chemicals, biological materials, and equipment. It is recommended that the user follow the author's designations; often those are the products that the author, after considerable experimentation, has found will work under the particular conditions. In other cases, the experience of the author of that protocol is limited to that brand. In the latter situation, recommendations are offered as an aid to the novice in obtaining the tools of the trade.

Phone numbers, facsimile numbers, and URLs of all suppliers mentioned in this manual are provided in the *SUPPLIERS APPENDIX*.

Safety Considerations

Anyone carrying out these protocols may encounter the following hazardous or potentially hazardous materials: (1) radioactive substances, (2) toxic chemicals and carcinogenic or teratogenic reagents, and (3) pathogenic and infectious biological agents. Check the guidelines of your particular institution with regard to use and disposal of these hazardous materials. Although cautionary statements are included in the appropriate units, we emphasize that users must proceed with the prudence and precaution associated with good laboratory practice, and that all materials must be used in strict accordance with local and national regulations.

Animal Handling

Many protocols call for use of live animals (usually rats or mice) for experiments. Prior to conducting any laboratory procedures with live subjects, the experimental approach must be submitted in writing to the appropriate Institutional Animal Care and Use Committee (IACUC) or must conform to appropriate governmental regulations regarding the care and use of laboratory animals. Written approval from the IACUC (or equivalent) committee is absolutely required prior to undertaking any live-animal studies. Some specific animal care and handling guidelines are provided in the protocols where live subjects are used, but check with your IACUC or governmental guidelines to obtain more extensive information.

Human Material

See the International Society for Stem Cell Research "Guidelines for the Conduct of Human Embryonic Stem Cell Research," reproduced in *APPENDIX A1.1*. Research using human tissues must be reviewed and approved by the independent institutional ethics review panel, and donated material must be provided voluntarily with informed consent.

Reader Response

Most of the protocols included in this manual are used routinely in the authors' laboratories. These protocols work for them; to make them work for you the authors have annotated critical steps and included critical parameters and troubleshooting guides in the commentaries to most units. However, the successful evolution of this manual depends upon readers' observations and suggestions. Consequently, we encourage readers to send in their comments (kmorgan@wiley.com).

ACKNOWLEDGMENTS

This manual is the product of dedicated efforts by many of our scientific colleagues who are acknowledged in each unit and by the hard work by the Current Protocols editorial staff at John Wiley and Sons. We are extremely grateful for the critical contributions by Kathy Morgan (Series Editor), who kept the editors and the contributors on track and played a key role in bringing the entire project into existence. Other skilled members of the Current Protocols staff who contributed to the project include Joseph White, Tom Cannon, and Sheila Kaminsky. The extensive copyediting required to produce an accurate protocols manual was ably handled by Allen Ranz, Susan Lieberman, Marianne Huntley, and Sylvia de Hombre. Typesetting and electronic illustrations were prepared by Aptara.

RECOMMENDED BACKGROUND READING

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Mick Bhatia, Andrew Elefanty, Susan J. Fisher, Roger Patient, Thorsten M. Schlaeger, and Evan Y. Snyder

SECTION 1A
Isolation of Embryonic Stem Cells

INTRODUCTION

Derivation of first primate and soon thereafter human embryonic stem cells set the stage for the next exciting chapters in the stem cell field, in which we are beginning to learn the extent to which lessons learned from studying model systems apply to primate species. The commonalities will certainly be easier to discern than the unique aspects. However, before either is apparent, investigators need access to high-quality primate embryonic stem cell lines that are the truest in vitro representatives of their in vivo counterparts. In the case of mouse embryonic stem cells, it took decades for the field to establish criteria for their evaluation and produce lines that met them. In the context of work on model systems, it is virtually certain that many more primate embryonic stem cell lines must be produced before we know that we have the tools needed to delve deeper into major questions regarding the cells' capacity for self-renewal as well as for differentiation.

For these reasons this section includes detailed protocols for producing embryonic stem cells from both nonhuman primates (*UNIT 1A.1*) as well as humans (*UNIT 1A.2*). Not surprisingly, the methods are not dramatically different. However, special considerations apply in each case. For example, in nonhuman primates, complement-mediated lysis of the trophectoderm layer is deemed preferable to remove these cells before the stem cell derivation process begins. In contrast, many investigators who are producing new human embryonic stem cell lines wish to avoid their exposure to animal products such as antibodies. Thus, they opt to use intact embryos for derivation purposes and allow the trophectoderm layer to die during generation of the stem cell lines. Eventually, we will want to know if the presence or absence of trophoblasts, which contribute to the placenta, is a positive, negative, or neutral factor with regard to influencing embryonic stem cells quality.

It is interesting to note from the numerous details that both groups include in their protocols, the complexity of the derivation process and the commitment this work requires. It takes a great deal of expertise to grow and manipulate human and nonhuman primate embryos. It requires vigilant monitoring of the cultures as the initial outgrowths form. A crucial step is making decisions about when the cultures should be divided. Although the authors have attempted to give as much specific information as possible about these steps, qualitative aspects of decision making remain that are subject to individual judgments best made based on experience.

Finally, we note that the protocols focus on laboratory methods rather than ethical considerations, such as how to properly describe these studies to institutional review boards and how to obtain informed consent from donors. The enormity of these issues, which are handled in different ways by different institutions, are beyond the scope of this section but are of primary consideration to all investigators who are involved in both the derivation and use of new human and nonhuman primate embryonic stem cell lines.

Susan J. Fisher

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Derivation and Characterization of Nonhuman Primate Embryonic Stem Cells

Christopher S. Navara,[1] Carrie Redinger,[1] Jocelyn Mich-Basso,[1] Stacie Oliver,[1] Ahmi Ben-Yehudah,[1] Carlos Castro,[1] and Calvin Simerly[1]

[1]University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania

ABSTRACT

Embryonic stem (ES) cells are a powerful research tool enabling the generation of mice with custom genetics, the study of the earliest stages of mammalian differentiation in vitro and, with the isolation of human ES cells, the potential of cell based therapies to a number of diseases including Parkinson's and Type 1 diabetes. ES cells isolated from non-human primates offer the opportunity to ethically test the developmental potential of primate ES cells in chimeric offspring. If these cells have similar potency to mouse ES cells we may open a new era of primate models of human disease. Non-human primates are the perfect model system for the preclinical testing of ES cell–derived therapies. In this unit we describe methods for the derivation and characterization of non-human primate ES cells. With these protocols the investigator will be able to isolate nhpES cells and perform the necessary tests to confirm the pluripotent phenotype. *Curr. Protoc. Stem Cell Biol.* 1:1A.1.1-1A.1.21. © 2007 by John Wiley & Sons, Inc.

Keywords: nonhuman primate • embryonic stem cells • Oct-4 • Nanog • karyotype • teratoma

INTRODUCTION

The use of murine embryonic stem (mES) cells has revolutionized the production of transgenic knockout, knockin, and knockdown mice, and has furthered biomedical research perhaps more than any other technological advance. Murine embryonic stem cells are stably growing cell lines that retain the ability to be recombined with cleavage-stage embryos to produce animals with tissues derived from both the embryo and the stem cells. Alternatively, in a very elegant experimental procedure, embryonic stem cells can be combined with an experimentally derived tetraploid embryo. Tetraploid mouse embryos only form trophectoderm and extra-embryonic tissues during development. In these experiments, the resulting animal, including the germ line, is completely derived from the embryonic stem cells (Maatman et al., 2003). The overriding superiority of this technology is that transfection can be carried out on the mES cells using highly efficient techniques optimized for cultured cell lines. Selection of expression characteristics and stability of the transgene can be analyzed in vitro prior to generating transgenic animals. As the embryonic stem cells can be propagated, a large number of transgenic animals can be made in the F1 generation.

Human embryonic stem cells (hESC), first isolated in 1998 (Thomson et al., 1998), hold great promise for cell-mediated therapies for debilitating diseases such as diabetes and Parkinson's disease. These cells appear to be immortal in culture and retain the ability to form all tissues of the adult even through more than 100 passages. Due to obvious ethical concerns, the ability of these cells to contribute to chimeric offspring and the germ line has not, and should not, be tested; consequently, it is unknown if these cells share that important developmental property with mouse embryonic stem cells.

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Nonhuman primate embryonic stem cells (nhpESC) were first isolated in 1995 from in vivo–fertilized rhesus embryos (*Macaca mulatta*; Thomson et al., 1995) and in 1996 from marmosets (*Callithrix jacchus*; Thomson et al., 1996). They have also been isolated from in vitro–fertilized (IVF) and intracytoplasmic sperm injection (ICSI)–fertilized (Suemori et al., 2001) and parthenogenetic cynomolgus monkeys (Cibelli et al., 2002; Vrana et al., 2003). These cells may prove invaluable in several ways. First, they serve as a preclinical model for testing the efficacy and safety of embryonic stem cell–derived therapies (Sanchez-Pernaute et al., 2005; Takagi et al., 2005). Secondly, they may enable the generation of nonhuman primates (NHP) expressing disease conditions as preclinical models of human disease. Some contribution of nhpESC to chimeric embryos has been shown, but no chimeric offspring have been generated (Takada et al., 2002; Mitalipov et al., 2006) to date. It is well established with regard to murine embryonic stem cells that some lines are able to contribute to fetal tissues but are deficient in their ability to contribute to the germ line. Therefore demonstrating that nhpES cells have this ability may require the derivation and testing of dozens of embryonic stem cell lines. In this unit, protocols are described for the high-efficiency derivation of embryonic stem cells from rhesus monkey embryos (Basic Protocol) and for the characterization of the pluripotent phenotype using immunocytochemistry (Support Protocol 1), RT-PCR (Support Protocol 2), and teratoma formation (Support Protocol 4). Additionally, as the generation of aneuploid cell lines is a recurring problem, a protocol is included for karyotyping nonhuman primate ES cells (Support Protocol 3).

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO2 incubator unless otherwise specified.

BASIC PROTOCOL

DERIVING NONHUMAN PRIMATE EMBRYONIC STEM CELLS

Two basic techniques have been used for the isolation of embryonic stem cells. The first, described below, involves removing the outer trophectodermal cells of the expanded blastocyst using an antibody/complement reaction ("immunosurgery"). The tight junctions between trophectodermal cells prevent diffusion of the antibody into the inner cell mass (ICM), ensuring that only the trophectodermal cells bind antibody, and thus that they are the only cells lysed by the addition of complement. An alternative technique involves direct plating of the blastocyst without removal of the trophectoderm. This procedure also works successfully, but requires the investigator to later passage the inner cell mass (ICM) cells away from the trophectodermal cells in vitro. The former technique is included in this unit because it results in a cleaner embryonic stem cell preparation.

Materials

0.1% (w/v) gelatin in DPBS (Invitrogen, cat. no. 14190-144)
Inactivated mouse embryonic fibroblasts (MEFs; Specialty Media, *http://www.specialtymedia.com*; also available from ATCC, cat. no. SCRC-1040.2)
MEF medium (see recipe)
nhpES cell medium (see recipe)
Expanded non-human primate blastocysts (Hewitson, 2004)
Acidified Tyrode's medium (Chemicon)
TALP-HEPES medium (see recipe)
Anti-monkey serum produced in rabbit (Sigma, cat. no. M-0278)
Mineral or silicon oil, embryo quality (Cooper Medical)
Guinea pig complement, lyophilized (Biomeda; store at –20°C until use)
Embryo-quality H_2O (Sigma)

Fetal bovine serum (FBS; Invitrogen, cat. no. 16000-044)
Dimethylsulfoxide (DMSO)
Liquid nitrogen

6-well tissue culture plate
Hamilton syringe with 20-µl Unopette tip (Becton Dickinson) attached
37°C slide warmer
30-mm organ culture dish (Fisher)
Dissecting microscope
60-mm non–tissue culture–treated petri dishes
Stripping pipet: "Stripper" pipetting instrument (Fig. 1A.2.6) and 125-µm inner diameter plastic tips (MidAtlantic Diagnostics, *http://www.midatlanticdiagnostics.com*; cat. no. MXL3-125)
Fine glass needle for passaging ES cells: pull a Pasteur pipet as thinly as possible while heating over Bunsen burner, such that a pair of needles with pointed sealed ends (mandatory) are produced, and bend according to preference for optimal access to the wells; alternatively, use commercially available stem cell knives (Swemed cat. no. 25111-109M; *http://www.swemed.com*)
Cell scrapers
15-ml conical centrifuge tubes
1-ml cryovials
Mr. Frosty freezing containers (Fisher)

Prepare MEF plates

1. At a time point 48 hr prior to immunosurgery, prepare a gelatin-coated 6-well plate by placing 3 ml of 0.1% gelatin in PBS into each well and incubating in a sterile environment 1 to 2 hr at room temperature.

2. Rinse wells with MEF medium and plate 150,000 mitotically inactivated MEF cells/cm^2 in 3 ml MEF medium. Return cells to incubator.

   The authors purchase MEFs from Specialty Media, but they are also available from ATCC; protocols exist for preparing them in one's own laboratory, as well (Schatten et al., 2005). Plates containing MEFs are ready to use 24 to 48 hr after plating and should be used within 5 days. It is best to test MEFs before use, by culturing existing embryonic stem cell lines to determine that they support pluripotency

3. The day of the immunosurgery, remove the MEF medium and rinse each well with 2 ml nhpES cell medium. Discard rinse and add 3 ml of nhpES cell medium to each well. Return cells to incubator.

   This step should be performed well in advance of the immunosurgery (~1 hr before), so that the medium is completely equilibrated before ICM plating.

Perform immunosurgery

Embryos are always transferred using a Hamilton syringe with a 20-µl Unopette attached to the end. Monkey tissues are BSL-2 and should not be pipetted by mouth, as is common with mouse tissues. All immunosurgery steps are performed at 37°C on a prewarmed slide warmer.

4. Transfer rhesus expanded blastocysts (see Fig. 1A.1.1A) to 1 ml acidified Tyrode's medium in a 30-mm organ culture dish. Observe under a dissecting microscope until the zona pellucida is removed (also see *UNIT 1A.2*).

   In very expanded blastocysts the zona is observed as a smooth, shiny region surrounding the embryo (Fig. 1A.1.1A); when it is successfully removed, the trophectoderm will become much more cellular.

5. Immediately after zona removal, transfer blastocysts into 3 to 5 ml TALP-HEPES medium and let stand for 5 min to wash.

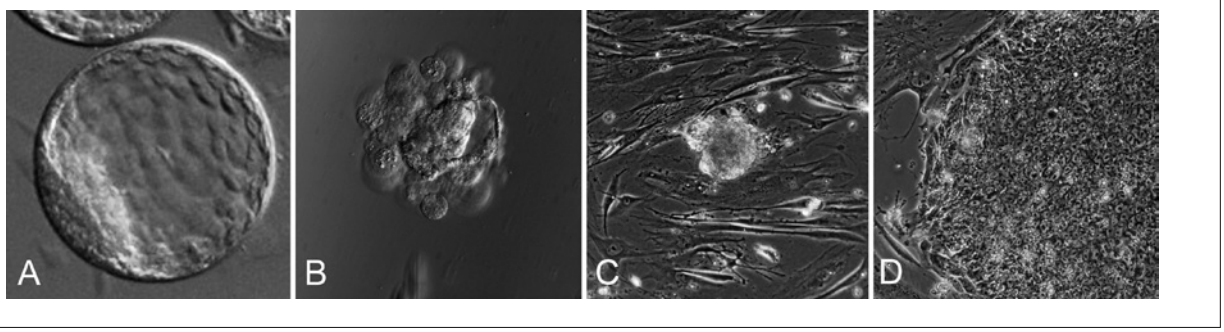

Figure 1A.1.1 Embryo to embryonic stem cells. (**A**) Nonhuman primate blastocysts should be fully expanded with a large distinct inner cell mass (ICM) prior to use for embryonic stem cell derivation. (**B**) After the complement is added the trophectoderm, cells are lysed, and the blastocyst will collapse. Lysed trophectodermal cells are only loosely associated with the ICM. (**C**) Isolated inner cell mass plated onto mouse embryonic feeder cells. Early passage nhpES cells for passaging should have very tightly packed cells with prominent nucleoli.

6. Prepare a 1:3 dilution of anti-monkey serum in TALP-HEPES medium (for a final concentration of 25% anti-monkey serum). In a 60-mm non–tissue culture–treated petri dish, place five to ten 10-µl drops of the diluted anti-monkey serum (number of drops will depend on number of blastocysts to be processed), then add just enough embryo-quality mineral or silicon oil to completely cover the drops. Warm to 37°C.

7. Transfer zona-free blastocysts to the drops of diluted anti-monkey serum and incubate on a 37°C slide warmer for 15 min.

8. Resuspend lyophilized guinea pig complement in 10 ml 4°C embryo-quality water, then prepare a 1:3 dilution of the reconstituted complement in TALP-HEPES medium (for a final concentration of 25% reconstituted complement) and keep on ice. Just prior to use, warm to 37°C and place 1 ml in an organ culture dish. Transfer embryos from anti-monkey serum directly into the complement solution. Incubate in the complement 15 min at 37°C.

9. Prepare a petri dish containing 50-µl drops of nhpES medium under oil, using the technique described in step 6. Briefly rinse the blastocysts with TALP-HEPES medium using the technique described in step 5 (but let stand only ~30 sec instead of 5 min), transfer the blastocysts to the 50-µl drops of medium under oil, and return the blastocysts to the incubator for 30 min.

   The success of immunosurgery depends heavily on the blastocyst. Using the exact same conditions described here, the authors have observed classic lysis of the trophectodermal cells (Fig 1A.1.1B) and also almost no lysis of the trophectoderm. ES cells were successfully derived from both kinds of blastocysts.

   The dilution factor above applies to the lyophilized complement from Biomeda; other formulations may require different dilutions.

10. Draw the blastocyst into a stripping pipet with an inner diameter of 125 µm to remove the lysed cells and plate immediately (step 10).

    The diameter of the pipet is big enough to let the inner cell mass through but will strip the lysed cells from the ICM.

Plate isolated ICM on MEFs

11. Add 3 ml of nhpES cell medium to each well of a 6-well tissue culture dish containing an MEF feeder layer (prepared as in steps 1 to 3, above). Plate one isolated ICM (from step 9) or embryo (in case of failed immunosurgery or isolation without immunosurgery) into each well of the dish using the Hamilton syringe with 20-µl Unopette.

    The 6-well plate should only be opened in a biological safety cabinet.

12. Return plated embryos to the incubator. Do not disturb for at least 24 hr and preferably 48 hr.

13. After 48 hr, check the wells to determine if the ICM has firmly attached to the substrate (Fig. 1A.1.1C). If the ICM is firmly attached, replace 80% of the medium with 3 ml nhpES cell medium that has been preincubated at least 1 hr in a 37°C, 5% CO_2 incubator in order to equilibrate it with the gas mixture and prewarm it to 37°C.

    If the ICM has not yet attached, add 3 ml of nhpES cell medium to the well. It is helpful at this point to use an objective marker to circle the location of the plated ICM.

14. Every 48 hr replace the medium with fresh nhpES cell medium. Continue for ~14 days, until it becomes necessary to perform the first passage of the putative cell line.

    The cells should not be carried past 14 days without passaging, because the quality of the feeder cells will diminish and it is important to transfer the ES cells to fresh feeder cells.

    At this stage, cells that are promising will have a very large cell mass that may or may not look like embryonic stem cells.

    Prior to passaging on day 14, cell masses should be carefully watched for signs of retraction from the feeder layer. If this is observed, cell masses should be passaged immediately.

15. Manually passage any cells with proper embryonic stem cell morphology (high nuclei/cytoplasm ratio and prominent nucleoli; Fig. 1A.1.1D), cutting the cell masses into pieces containing 10 to 15 cells with a fine glass needle, and transferring the pieces to newly plated MEFs in a 6-well plate, as described above. Also cut and passage cell masses that do not resemble embryonic stem cells, if possible.

    If it is not possible to cut them manually, cell masses should be treated with 1 ml of 1 mg/ml collagenase and passaged.

    The authors have derived several stem cell lines from cell masses that did not initially have canonical embryonic stem cell morphology, so it is also advisable to attempt to culture these.

16. Maintain the initial culture plates for at least 1 week, changing medium every 48 hr, to determine if any other nhpES cell colonies begin to grow.

17. After the initial passage, passage cell lines approximately weekly using manual passaging, being sure to select only cells with proper ES cell morphology.

Freeze cells

As soon as cultures are large enough to be split into three wells (day 6 or 7 after mechanical passaging), one well should be frozen.

18. Remove 6-well plate from incubator. Using a cell scraper, gently release ESCs and feeder layer from the bottom of the well.

19. Aspirate the cell suspension and place in a 15-ml conical tube. Rinse the well with 3 ml nhpES cell medium to resuspend any remaining cells and transfer to the same 15-ml tube.

20. Centrifuge 5 min at 200 × *g*, room temperature. During the centrifugation, prepare the freezing medium (90% v/v FBS containing 10% v/v DMSO).

21. When centrifugation is complete, remove and discard the supernatant. Resuspend pellet in 1 ml freezing medium.

22. Transfer resuspended cells to 1-ml cryovial. Place cryovial into a Mr. Frosty freezing container at room temperature. Place the Mr. Frosty freezing container in a –80°C freezer for 24 hr, then transfer to liquid nitrogen.

Confirm and characterize the pluripotent phenotype

23. Once putative embryonic stem cells have been isolated, characterize them for the pluripotency markers SSEA-4, TRA160, and TRA 181 (Support Protocols 1 and/or 2), stable correct karyotype (Support Protocol 3), and ability to differentiate into tissues from all three germ layers (Support Protocol 4).

The authors traditionally prefer to use immunocytochemistry (Support Protocol 1) whenever possible, as this allows for determining the heterogeneity of stem cell colonies. They also use RT-PCR (Support Protocol 2) to identify expression of genes related to pluripotency. The final criterion of pluripotency is the ability to form tissues derived from all three germ layers (Support Protocol 4). For determining this, the authors prefer teratoma formation, which offers straightforward technique and clear interpretation. Normal and stable karyotype is an important consideration when first deriving nonhuman primate ES cells, and is an ongoing concern while maintaining them. Included in this unit is a protocol for karyotyping nonhuman primate ES cells (Support Protocol 3).

SUPPORT PROTOCOL 1

IMMUNOCYTOCHEMISTRY OF nhpES CELLS

Immunocytochemistry has the advantage of measuring not only expression of a given protein but also the localization of the protein within the cell and within the embryonic stem cell colony. A number of pluripotency markers have been described for human and nonhuman primate embryonic stem cells. The classic markers are the transcription factors Nanog and Oct-4 and the surface antigen stage–specific embryonic antigen 3/4 (SSEA3/4), tumor rejection antigen (TRA) 1-60, and TRA 1-81. Primate embryonic stem cells are negative for the mouse embryonic stem cell marker SSEA1. Nanog and Oct-4 have functional relationships with pluripotency, whereas SSEA3/4, TRA 1-60, and TRA 1-81 are simply surface markers without a known function in embryonic stem cells.

Materials

0.1% (w/v) gelatin in DPBS
Inactivated mouse embryonic fibroblasts (MEFs; Specialty Media, *http://www.specialtymedia.com*; also available from ATCC, cat. no. SCRC-1040.2)
MEF medium (see recipe)
Rhesus ES cells growing in culture (see Basic Protocol)
Dulbecco's phosphate-buffered saline (DPBS, Ca^{2+}- and Mg^{2+}-free; Invitrogen, cat. no. 14190-144), prewarmed
DPBS containing 2% (v/v) formaldehyde
DPBS containing 0.1% (v/v) Triton X-100
DPBS containing 0.3% (w/v) nonfat dry milk and 5% (v/v) normal goat serum
Primary antibodies against desired ES markers (perform all dilutions in DPBS containing 0.1% v/v Triton X-100):
  Mouse Oct-4 [Santa Cruz Biotechnology (sc-5276); use at 1:100 dilution]
  Goat Nanog (R&D Systems; use at 1:20 dilution)
  Mouse SSEA-4 (Developmental Studies Hybridoma Bank; use at 1:5 dilution)
  Mouse TRA-1-81 (Santa Cruz Biotechnology; use at 1:5 dilution)
  Mouse TRA-1-60 (Santa Cruz Biotechnology; use at 1:5 dilution)
Secondary antibody against IgG of species in which primary antibody was raised, labeled with Alexa Fluor 488; use at 100:1 dilution in DPBS containing 0.1% Triton X-100
10 mg/ml RNase in DPBS containing 0.1% Triton X-100
5 μM TOTO-3 (Invitrogen) in DPBS containing 0.1% Triton X-100
Vectashield mounting medium (Vector)
Thermanox plastic coverslips (Ted Pella, Inc.)
6-well tissue culture plate
Humidified chamber (e.g., Tupperware box containing moistened paper towels)
Microscope slides

Prepare ES cells on MEF feeder layers for immunostaining

1. Prepare gelatin-coated Thermanox coverslips in a 6-well tissue culture plate containing one coverslip per well by placing 3 ml of 0.1% gelatin on the correct surface of each coverslip and incubating in a sterile environment 1 to 2 hr at room temperature.

   These coverslips are "sided"; medium will bead on the incorrect side.

2. Rinse coverslips with MEF medium and plate 15,625 MEF cells/cm^2 on the gelatin-coated surface. Incubate for 48 hr.

3. To prepare cells for immunostaining, passage nhpES cells (as described in Basic Protocol 1, step 15) onto the gelatin-coated Thermanox plastic coverslips seeded with MEF feeder cells and incubate ~1 week prior to fixation and processing.

   Passaging and culture of cells is done as in the Basic Protocol, steps 15 to 17, except that in this protocol the wells of the 6-well plate contain coverslips.

Fix cells and block nonspecific binding

4. Prior to fixation, rinse coverslips with 3 ml warm DPBS to remove proteins found in the culture medium.

5. Transfer the coverslip immediately to 5 ml DPBS/2% formaldehyde and fix by incubating 40 min at room temperature.

6. After fixation, rinse cells with 5 to 7 ml DPBS/0.1% Triton X-100.

7. If necessary, block nonspecific binding of the antibodies at this stage using a 20-min incubation in 5 to 7 ml DPBS/0.3% (w/v) nonfat dry milk/5% (v/v) normal goat serum.

   Note that the Nanog antibody from R&D Systems is raised in goats, and blocking in goat serum will result in undesirable generalized staining masking the Nanog signal.

Treat cells with primary and secondary antibodies

8. Incubate sample coverslip with 100 µl primary antibody against the ES cell markers of interest at the appropriate dilution in DPBS/0.1% Triton X-100 for 40 min (except for Oct-4 and Nanog, which are most successfully stained at 4°C overnight) at 37°C in a humidified chamber.

   Alternative antibodies may work and investigators should determine their own optimal dilution.

9. Wash all samples for 15 min with DPBS/0.1% Triton X-100.

10. Add 100 µl fluorescently labeled secondary antibody to the sample coverslip and incubate for 40 min at 37°C in a humidified chamber.

11. Wash secondary antibody–exposed coverslip as described in step 9.

Counterstain and mount

12. Pretreat coverslip with 100 µl of 10 mg/ml RNase for 20 min.

    TOTO-3 will bind both RNA and DNA, so the coverslips are pretreated to remove RNA.

13. Add 5 µM TOTO-3 to the sample for 20 min for DNA counterstaining.

14. Add Vectashield mounting medium to the coverslip and mount on a microscope slide to help prevent photobleaching.

15. Examine samples for immunocytochemical staining.

    It is important to consider the intensity of staining as well as the localization of staining. SSEA-4 and the TRA antigens are all located at the cell surface, and the staining should reflect this. Conversely, Oct-4 and Nanog are both transcription factors and should be

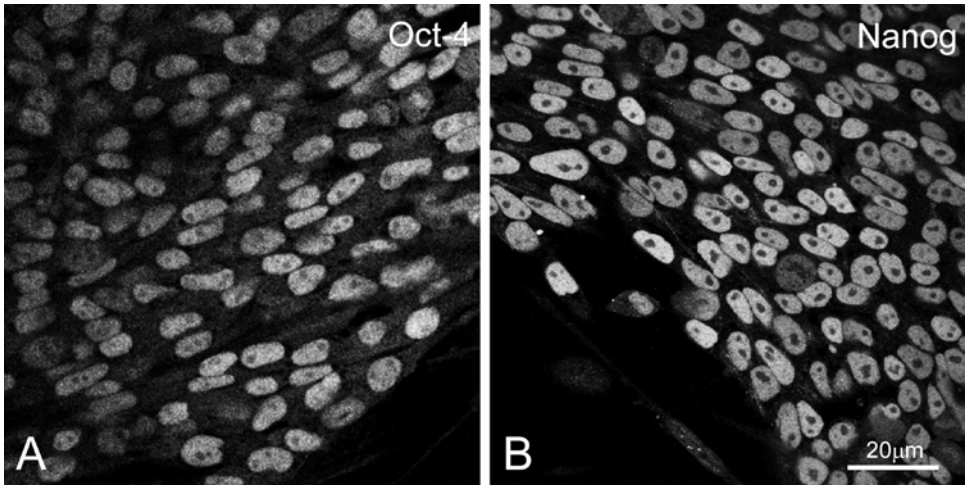

Figure 1A.1.2 Immunocytochemical localization of the pluripotency markers (**A**) Oct-4 and (**B**) Nanog in nhpES cells. Immunocytochemistry allows for the determination of heterogeneity in colonies. (**A**) Oct-4 and (**B**) Nanog are transcription factors and should be localized to the nuclei in healthy pluripotent colonies. This staining also highlights the prominent nucleoli observed in ES cells.

localized to the nucleus (Fig. 1A.1.2). Failure to localize properly could indicate problems in the stem cell culture or the labeling protocol.

SUPPORT PROTOCOL 2

DETECTION OF OCT-4, NANOG, SOX-2, AND REX-1 BY RT-PCR

RT-PCR allows for the rapid screening of expression for a number of proteins in a bulk population of embryonic stem cells. This technique's primary strength, sensitivity, is also a major limitation, as low levels of mRNA can still be amplified, resulting in a positive signal. It is also difficult to measure the expression levels across all embryonic stem cells, as high expression in one population of cells will mask low expression in another population. However it is a quick and cost-efficient means of measuring expression of pluripotent genes and is confirmatory when combined with immunocytochemistry.

Materials

One 70% confluent well of a 6-well plate of nhpES cells (Basic Protocol)
TRIzol Reagent (Invitrogen)
Chloroform (minimum 99%; Sigma)
Isopropanol
75% ethanol in nuclease-free water
Nuclease-free water (ISC Bioexpress; *http://www.bioexpress.com*)
DNA-free Kit (Ambion) containing:
  10× DNase I buffer
  recombinant DNase I (rDNase I)
  DNase Inactivation Reagent
Reverse Transcription System (Promega) containing:
  25 mM $MgCl_2$
  5× reverse transcription buffer
  10 mM dNTP mixture
  Recombinant RNasin ribonuclease inhibitor
  Reverse transcriptase
  Oligo(dT) primer
Biolase PCR Kit (Bioline) containing:
  Biolase *Taq* DNA Polymerase
  10× NH_4 Buffer

50 mM $MgCl_2$ Solution
2× PolyMate Additive
10 mM dNTP mix (Roche Applied Science)
PCR primers for rhesus EC markers:
Oct-4:
forward: 5′-CGACCATCTGCCGCTTTGAG-3′
reverse: 5′-CCCCCTGTCCCCCATTCCTA-3′
Nanog:
forward: 5′-CTGTGATTTGTGGGCCTGAA-3′
reverse: 5′-TGTTTGCCTTTGGGACTGGT-3′
Rex-1:
forward: 5′-GCGTACGCAAATTAAAGTCCAGA-3′
reverse: 5′-CAGCATCCTAAACAGCTCGCAGAAT-3′
Sox2:
forward: 5′-CCCCCGGCGGCAATAGCA-3′
reverse: 5′-TCGGCGCCGGGGAGATACAT-3′

Cell scrapers
15-ml conical tubes
Refrigerated centrifuge
Automatic pipettors and filtered pipet tips designated for RNA work (RNase-free; Molecular BioProducts; *http://www.mbpinc.com/html/index.html*
0.6-ml microcentrifuge tubes, sterile and RNase free (Molecular BioProducts; *http://www.mbpinc.com/html/index.html*)
0.2-ml PCR reaction tubes (ISC Bioexpress, *http://www.bioexpress.com*)
Thermal cycler (e.g., PTC-200 Peltier Thermal Cycler; MJ Research)

Additional reagents and equipment for isolating ES cells (Basic Protocol), nucleic acid quantitation (Gallagher and Desjardins, 2006) and agarose gel electrophoresis (Voytas, 2000)

NOTE: Use nuclease-free water to prepare all reagents. All tubes and pipets must be RNase-free. Always wear gloves while handling samples. Do not leave tubes open any longer than absolutely necessary. Before each use, wipe gloves and pipets with RNase Away (Molecular BioProducts; *http://www.mbpinc.com/html/index.html*).

Isolate RNA

1. Isolate nhpES cells by manual scraping of cell colonies. Transfer cells to a 15-ml conical tube and centrifuge 5 min at 200 × *g*, room temperature.
2. Remove all but ∼100 μl of supernatant, add 1 ml of TRIzol to the cell pellet, and mix by vortexing for 10 sec.
3. Add 200 μl chloroform and vortex for 30 sec.
4. Centrifuge 5 min at 2500 × *g*, 4°C.

Purify RNA

5. Carefully transfer the aqueous phase (∼600 μl) to a sterile RNase-free 0.6 ml microcentrifuge tube. Avoid disturbing the white precipitate layer, which contains DNA and protein) and add 600 μl isopropanol. Incubate at −20°C for at least 2 hr, but preferably overnight.
6. After incubation, centrifuge tube 30 min at 14,000 to 16,000 × *g*, 4°C.
7. Carefully remove the isopropanol, leaving a small amount behind in order to avoid disturbing the pellet, if necessary.
8. Add 600 μl of 75% ethanol and centrifuge 10 min at 14,000 to 16000 × *g*, 4°C.

9. Remove the ethanol, again being careful not to disturb the pellet. Dry the pellet at room temperature (pellet should become transparent).

10. Resuspend pellet in 25 to 50 µl RNase-free water.

Treat sample with DNase

11. Add 0.1 vol of 10× DNase I buffer and 1 µl rDNase I (from the Ambion DNA-free kit) to the RNA, mix gently, and incubate at 37°C for 20 to 30 min.

    We find that we get better results if we first treat the RNA with DNase.

12. Add 2 µl or 0.1 vol (whichever is greater) of resuspended DNase Inactivation Reagent, and mix well.

13. Incubate at room temperature for 2 min, mixing occasionally.

14. Centrifuge 1.5 min at 10,000 × *g*, 4°C, and transfer the supernatant to a new sterile RNase-free tube. Determine RNA concentration by measuring A_{260}/A_{280} (Gallagher and Desjardins, 2006).

Perform reverse transcription

15. To prepare the isolated RNA for the production of cDNA, incubate 1 µg of total RNA for 10 min at 70°C (in thermal cycler), then microcentrifuge briefly at maximum speed and place on ice.

16. In a 0.2-ml PCR reaction tube on ice, prepare a 20-µl RT-PCR reaction by adding the following reagents in the order listed:

    2.4 µl 25 mM $MgCl_2$
    4 µl 5× reverse transcription buffer
    1 µl 10 mM dNTP mixture
    0.5 µl recombinant RNasin ribonuclease inhibitor
    1 µl reverse transcriptase
    1.0 µg Oligo(dT) primer
    1.0 µg total RNA (from step 15)
    Nuclease-free H_2O to final volume of 20 µl.

17. Incubate reaction mixture in the thermal cycler at 42°C for 1 hr, followed by 5 min at 95°C, followed immediately by 5 min at 4°C.

    The cDNA can be stored for long periods of time at −20°C or can be used immediately in the procedures below.

Amplify cDNA and characterize product

The PCR programs described below are for an MJ Research PTC-200 Peltier Thermal Cycler. They can serve as a starting point for researchers employing other thermal cyclers.

18. Prepare a 50-µl amplification reaction by adding the following reagents (from Biolase PCR Kit, except for the 10 mM dNTP mix, which is purchased from Roche Applied Science) in the order listed:

    5.0 µl 10× NH_4 buffer
    1.5 µl 50 mM $MgCl_2$
    1.0 µl 10 mM dNTP mix
    0.5 µl 50 µM forward primer for marker of interest
    0.5 µl 50 µM reverse primer for marker of interest
    0.5 µl 5 U/µl Biolase *Taq* DNA polymerase
    1.0 µg cDNA (from step 17)
    Nuclease-free H_2O to final volume of 50 µl.

19a. *To amplify for Oct-4 (resulting in a product that is 577 bp):* Use the following thermal cycling program:

1 cycle:	5 min	94°C	(initial denaturation)
35 cycles:	30 sec	94°C	(denaturation)
	30 sec	60°C	(annealing)
	45 sec	72°C	(extension)
1 cycle	5 min	72°C	(final extension).

19b. *To amplify for Nanog (resulting in a product that is 152 bp):* Use the following thermal cycling program:

1 cycle:	5 min	94°C	(initial denaturation)
35 cycles:	30 sec	94°C	(denaturation)
	30 sec	62°C	(annealing)
	1 min	72°C	(extension)
1 cycle:	5 min	72°C	(final extension).

19c. *To amplify for Rex-1 (resulting in a product that is 350 bp):* Use the following thermal cycling program:

1 cycle:	5 min	94°C	(initial denaturation)
35 cycles:	30 sec	94°C	(denaturation)
	30 sec	56°C	(annealing)
	45 sec	72°C	(extension)
1 cycle	5 min	72°C	(final extension).

19d. *To amplify for Sox-2 (resulting in a product that is 448 bp):* Use the following thermal cycling program:

1 cycle:	5 min	94°C	(initial denaturation)
35 cycles:	30 sec	94°C	(denaturation)
	30 sec	57.9°C	(annealing)
	1 min	72°C	(extension)
1 cycle:	5 min	72°C	(final extension).

20. To determine presence of product and product size, load 10 μl of each product and 5 μl of a 100-bp DNA size ladder onto a 1.5% agarose gel containing 0.5 μg ethidium bromide and perform electrophoresis in 1× TAE buffer (Voytas, 2000).

KARYOTYPING OF NONHUMAN PRIMATE ES CELL CULTURES

SUPPORT PROTOCOL 3

Human embryonic stem cells have well documented karyotypic instability in culture, and there is evidence suggesting that nonhuman primate ES cells have similar instability. It is therefore imperative that cultures be checked periodically (every 6 months and any time the pattern of cell growth changes). The protocol below is based on a protocol for human ES cells developed by Dr. Maya Mitalipova, Whitehead Institute for Biomedical Research, and modified for nonhuman primates in the authors' laboratory. If the investigator does not have the interest or resources to perform this in the laboratory, samples can be sent to the University of Pittsburgh Cytogenetics Facility under the direction of Dr. Susanne Gollin (*http://www.upci.upmc.edu/facilities/Cytogen/*).

Materials

nhpES cells cultures in log-phase growth in 6-well plates (Basic Protocol)
Dulbecco's phosphate-buffered saline (DPBS, Ca^{2+}- and Mg^{2+}-free; Invitrogen, cat. no. 14190-144)
TrypLE cell dissociation enzyme (Invitrogen)
nhpES cell medium (see recipe)
1 μg/ml ethidium bromide working solution (see recipe)
10 μg/ml KaryoMAX Colcemid solution (Invitrogen)
Hypotonic solution: 0.075 M KCl, 37°C
Fixative: 1:3 (v/v) acetic acid/methanol
0.025% trypsin in DPBS (prepare from 0.5% trypsin stock, see recipe)
2% (v/v) fetal bovine serum (Invitrogen, cat. no. 16000-044) in DPBS
Giemsa stain solution: KaryoMAX Giemsa Stain (Invitrogen) diluted to 6% in Gurr's buffer, pH 6.8 (see below)
Gurr's buffer, pH 6.8: dissolve one Gurr's buffer tablet in 1 liter distilled H_2O

15-ml conical centrifuge tubes
Inverted microscope
Fine glass needle for dissecting ESC colonies: pull a Pasteur pipet as thinly as possible while heating over Bunsen burner, such that a pair of needles with pointed sealed ends (mandatory) are produced, and bend according to preference for optimal access to the wells; alternatively, use commercially available stem cell knives (Swemed cat. no. 25111-109M; *http://www.swemed.com*)
Centrifuge
Glass microscope slides
Beaker of hot water for adjusting humidity/temperature conditions
Slide warmer
Coplin jars
Cytovision Workstation and Genus software (Applied Imaging) *or* bright-field microscope with green interference filter and digital camera, with digital image processing software (e.g., Adobe Photoshop)

Collect cells

1. Remove medium from three wells of a 6-well culture plate of log-phase nhpES cells.
2. Rinse wells with 37°C DPBS, discard, and add 1 ml of 37°C TrypLE to enzymatically loosen/dissociate cells (ES cells will round up in 1 to 2 min; observe with inverted microscope). Add 2 ml nhpES cell medium to inactivate TrypLE.

   MEFs will not dissociate during the first 1 to 2 min; therefore minimizing the time in TrypLE is important in reducing the MEF contamination in the collected ES cells.

3. Working in the original well, tease rounded-up ESC colonies into a near single-cell suspension using a fine glass needle.
4. Add sufficient 1 μg/ml ethidium bromide solution to the well (still containing the TrypLE) for a final concentration of 12 ng/ml. Incubate 40 min at 37°C.

Arrest mitosis

5. Add the microtubule-inhibiting compound Colcemid to this suspension to a final concentration of 120 ng/ml. Incubate 20 min at 37°C.
6. Collect the cell suspension in a 15-ml conical tube and centrifuge for 8 min at 800 × *g*, room temperature.
7. Remove supernatant, then add 1 ml 37°C DPBS to the cell pellet and centrifuge 8 min at 800 × *g*, room temperature.

8. Discard the supernatant and resuspend the pellet in 1 ml of 37°C TrypLE.
9. After 1 min, add 2 to 3 ml nhpES cell medium to the tube to inactivate the TrypLE and repeat centrifugation.
10. Discard supernatant and resuspend pellet thoroughly in as small a quantity of the residual supernatant as possible.

Swell the cells

11. Add 5 ml of 37°C hypotonic solution (0.075 M KCl). Incubate at 37°C for 20 min.
12. Add ~10 drops of fixative (1:3 v/v acetic acid/methanol) to the suspension, gently invert twice to mix, and incubate 5 min at room temperature to prefix the cells.
13. Centrifuge 8 min at 800 × *g*, room temperature. Discard supernatant and resuspend pellet in remaining fluid.

Fix the cells

14. Add 5 ml of fixative slowly to the suspension of fragile prefixed cells while gently tapping the tube.
15. Incubate cells at room temperature for 30 min to fix, then centrifuge 8 min at 800 × *g*, room temperature. Discard supernatant and resuspend pellet in remaining fluid.
16. Repeat steps 14 and 15 twice more.

At this point the fixed cells can be stored at −20°C for several weeks in fixative at ~10,000 cells/ml before proceeding if necessary.

Prepare the slides

17. Remove supernatant from final pellet and resuspend at a concentration of ~10,000 cells/ml in fixative.
18. Using an automatic pipettor with a 20-µl pipet tip, place 10 to 20 µl of cell suspension on a glass slide and examine at 10× magnification for quality of cell preparation, noting number of cells in mitosis and quality of chromosome spread (i.e., if chromosomes are well separated or if numerous chromosomes are lying on top of one another, hindering isolation for karyotyping).
19. Adjust the quality of the slide preparation and fine tune by adjusting humidity and/or temperature factors using a beaker of hot water and/or a slide warmer to optimize quality and spreading of chromosomes.

Individual conditions will vary and investigators will need to determine the optimum conditions in their own laboratories. Further discussion of optimizing chromosome spreads may be found in Bayani and Squire (2004).

Perform GTG banding on chromosomes

20. Age prepared slides on a 75°C slide warmer for 1 to 2 hr, then cool to room temperature and immerse in freshly prepared 0.025% trypsin solution for 25 sec. At end of this time period, immediate immerse in 2% FBS/DPBS for 10 sec.
21. Rinse slides twice in DPBS, then immerse in Giemsa stain solution for 2 to 3 min. Rinse twice in Gurr's buffer and finally rinse in deionized water.
22. Allow slide to air dry.
23. Analyze chromosome spreads using Applied Imaging Cytovision and Genus software according to the manufacturers instructions. Alternatively, image chromosome spreads using a 100× oil objective on a high-quality research microscope with green interference filter, and photograph, preferably using a digital camera.

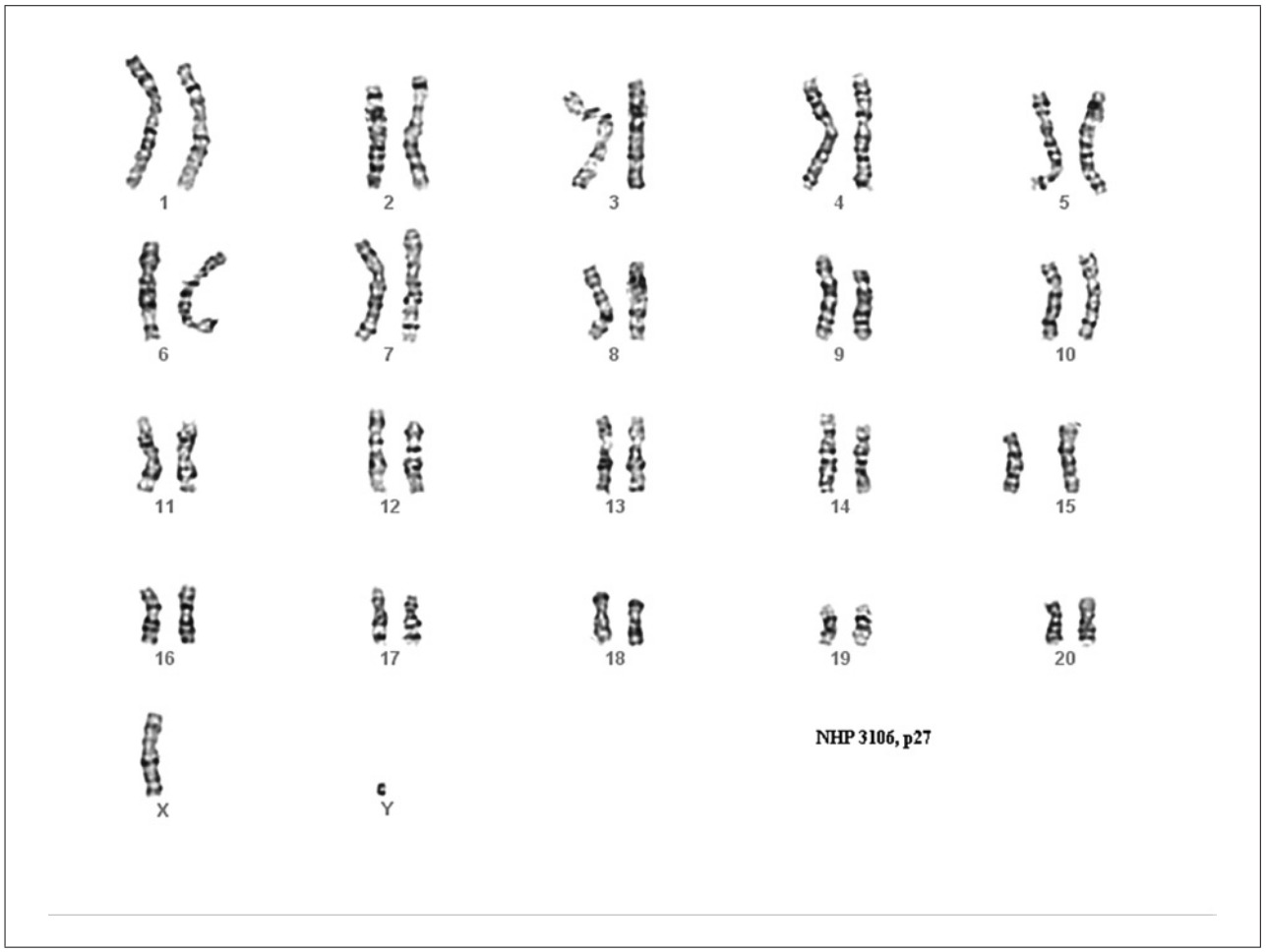

Figure 1A.1.3 G-banded karyotype of a male nhpES cell line. Rhesus monkey cells have a normal karyotype of 20 autosomes and 2 sex chromosomes. The Y chromosome is particularly difficult to observe, as it is very small in this species.

Digital image processing software such as Adobe Photoshop can then isolate individual chromosomes. In this manner a simple chromosome count can be easily completed. For further analysis of correct chromosome type and number see below.

24. Arrange chromosomes in matching pairs according to accepted classifications.

Chromosome designation of the rhesus macaque (Macaca mulatta; Fig. 1A.1.3) is in accordance with the Macaca mulatta chromosome classification proposed by Pearson et al. (1979).

A routine mitotic cell count is 20 metaphases, analyzing chromosomes band-by-band in three cells, two to three photos, and two to three karyotypes. (ACMG, 1999).

SUPPORT PROTOCOL 4

TERATOMA FORMATION IN NOD-SCID MICE

Teratoma formation in immunocompromised mice is a classic pluripotency test and the most stringent measure of pluripotency short of contribution to chimera formation. Chimera formation is unethical using human ES cells (at least into human embryos) and not routinely practical using nhpES cells and NHP embryos.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must conform to governmental regulations for the care and use of laboratory animals.

Materials

5–50 × 10^5 exponentially growing, high-quality ES cells; typically three wells of a 6-well plate (see Basic Protocol; if possible, use cells that have been recently karyotyped; see Support Protocol 3)
Normal saline (0.9% w/v NaCl), sterile
Immunocompromised mice (e.g., NOD-SCID; The Jackson Laboratory), 7 weeks old
Anesthetic solution: 20 mg/ml ketamine/0.5 mg/ml acepromazine in normal saline
10% (v/v) formalin (formaldehyde concentration, 3.7% v/v) in DPBS (Invitrogen, cat. no. 14190-144)
70%, 90%, 95%, and 100% ethanol
Paraffin wax
Hematoxylin
Eosin
Acid rinse: combine 500 ml distilled H_2O and 1 ml glacial acetic acid
Ammonia rinse: combine 480 ml distilled H_2O and 1 ml ammonium hydroxide

1-ml syringe and 25-G needle
Scalpels and scissors
Peloris tissue processor (Vision BioSystems, *http://www.vision-bio.com/*; optional)
Embedding blocks
Microtome
Microscope slides

1. Harvest stem cells by manual passaging (Basic Protocol), centrifuge 5 min at 200 × *g*, room temperature, remove supernatant, and resuspend cells in ~400 μl of normal saline. Load stem cell suspension into 1-ml syringe and attach 25-G needle.

2. Prepare mice by i.p. injection of 100 μl anesthetic solution using a 1-ml syringe and 25-G needle.

   This will not completely anesthetize the mouse, but serves the purpose of relaxing the testis from the abdomen.

3. Inject 100 μl of stem cell solution into the testis of each mouse and return to cage.

   Alternatively cells can be injected subcutaneously in the hind quarters. On an anecdotal basis, it is believed that injection into the testis requires fewer cells for teratoma formation, but this has not been rigorously tested.

4. Monitor tumor formation daily until the tumor is palpable, typically at 12 to 16 weeks post-injection.

5. Euthanize mice by CO_2 asphyxiation and dissect out tumors.

6. Place tumor in 20 ml of 10% formalin in PBS and leave for 48 to 72 hr at room temperature.

   Large tumors (>5 mm) should be pierced with a scalpel or scissors to allow penetration of formaldehyde into deeper tissues. Tumors should be fixed for several days to ensure adequate fixation.

7. After fixation, cut the teratomas into smaller pieces, 3 to 5 mm in diameter, and return to 10% formalin for 8 to 12 hr of further fixation. Process using a Peloris processor for dehydration and embedding or process manually as in the subsequent steps.

8. Dehydrate tissue by immersing successively for 45 min each in 70%, 90%, and 95% ethanol, then three times, each time for 45 min, in 100% ethanol. Next, immerse three time, each time for 45 min, in xylene to clear the samples, then three time, each time for 45 min in paraffin wax (melted at 56° to 62°C) to infiltrate the samples with paraffin. Finally, place samples into blocks and immerse in paraffin for sectioning.

Table 1A.1.1 Hematoxylin and Eosin Staining Protocol

Step	Reagent	Time
1	Xylene	1 min
2	Xylene	1 min
3	Xylene	2 min
4	100% ethanol	30 sec
5	100% ethanol	30 sec
6	95% ethanol	25 sec
7	95% ethanol	25 sec
8	Water	20 sec
9	Hematoxylin	10 min
10	Water	10 sec
11	Water	6 min
12	Acid rinse[a]	6 sec
13	Water	20 sec
14	Ammonia rinse[b]	30 sec
15	Water	8 min
16	Eosin	3 min
17	95% ethanol	10 sec
18	95% ethanol	10 sec
19	100% ethanol	10 sec
20	100% ethanol	10 sec
21	Xylene	1 min
22	Xylene	1 min
23	Xylene	1 min
24	Xylene	1 min

[a] 500 ml distilled H_2O plus 1 ml glacial acetic acid.
[b] 480 ml distilled H_2O plus 1 ml ammonium hydroxide.

9. Cut 0.4-μm sections using a microtome and place on slides. Stain with hematoxylin and eosin using the steps and timing shown in Table 1A.1.1.

It is best to collaborate with a trained pathologist/histologist to analyze the stained sections. Teratomas can be disorienting when first examined. If this is not possible the investigator should consult a reputable pathology text (Rosai, 2004)

REAGENTS AND SOLUTIONS

For culture recipes and steps, use sterile tissue culture–grade water. For other purposes, use deionized, distilled water or equivalent in recipes and protocol steps unless otherwise indicated. For suppliers, see ***SUPPLIERS APPENDIX****.*

Ethidium bromide working solution, 1 μg/ml

Stock solution: Prepare 10 μg/ml ethidium bromide (Sigma) in Hanks' balanced salt solution without calcium and magnesium (Invitrogen). Store up to 3 months at 4°C.

Working solution: Add 10 ml of 10 μg/ml ethidium bromide stock solution to 90 ml sterile distilled water for a working concentration of 1 μg/ml.

MEF medium

Dulbecco's Modified Eagle Medium, high-glucose formulation (Invitrogen) supplemented with:
10% fetal bovine serum (FBS; Invitrogen), heat inactivated
1× Pen/Strep (add from 100× stock; Invitrogen)
1× L-glutamine (add from 100× stock; Invitrogen)
1× nonessential amino acids (add from 100× stock; Invitrogen)
Filter sterilize using 0.22-μm filter
Store up to 1 week at 4°C

nhpES cell medium

80% Knockout DMEM (Invitrogen) supplemented with:
20% (v/v) Knockout Serum Replacement (Invitrogen)
1× Pen/Strep (add from 100× stock; Invitrogen)
1× L-glutamine (add from 100× stock; Invitrogen)
1× nonessential amino acids (add from 100× stock; Invitrogen)
12 ng/ml basic fibroblast growth factor (bFGF; Invitrogen)
10 ng/ml Activin A (Sigma)
10 ng/ml human leukemia inhibitory factor (hLIF; Chemicon)
Filter sterilize using 0.22-μm filter
Store up to 1 week at 4°C

TALP-HEPES medium

Stock solution:
114 mM NaCl
3.2 mM KCl
2 mM $NaHCO_3$
0.4 mM NaH_2PO_4
10 mM sodium lactate (add as 60% syrup)
2 mM $CaCl_2$
0.5 mM $MgCl_2$
10 mM HEPES
100 IU/ml penicillin
1 mg/100 ml phenol red
Filter sterilize using 0.22-μm filter
Store up to 1 month at 4°C
Working solution:
On day of the experiment add:
3 mg/ml BSA Fraction V (Sigma)
50 μg/ml gentamicin
60 ng/ml sodium pyruvate
Filter sterilize using 0.22-μm filter

Trypsin, 0.5% stock and 0.025% working solutions

Stock solution (0.5% trypsin): Dilute 2.5% trypsin (Invitrogen) 1:5 in Dulbecco's phosphate-buffered saline (DPBS; Invitrogen, cat. no. 14190-144). Store up to 6 months at −20°C.

Working solution: (0.025% trypsin): Just before use, dilute 0.5% trypsin stock to 0.025% with DPBS.

Background Information

Mouse embryonic stem cells have primarily been used for the generation of improved animal models (knockouts, knockins), and in this fashion have truly transformed biomedical research. Human embryonic stem cells have the potential to similarly transform medicine by generation of cells with the potential for therapy. They also serve as a model cell for studying very early differentiation events in human embryonic and fetal development. Ethical concerns preclude the in-depth examination of the pluripotency of human embryonic stem cells in chimeras, either with animal embryos or human embryos.

Nonhuman primate embryonic stem cells have the potential to cross the divide between these two species and answer pluripotency questions that cannot be asked using human ES cells. If embryonic stem cells from monkeys can contribute to chimeric offspring like murine embryonic stem cells, this would allow for the development of monkey models for disease that more faithfully represent human disease. Though unlikely to completely replace mouse models due to cost and other constraints, a monkey model for aging and cognitive diseases such as Alzheimer's would be invaluable.

Monkey embryonic stem cells are also the perfect cells to use for preclinical testing of any potential therapies using human embryonic stem cells. Work on the differentiation of nhpES cells is progressing, with successful differentiation reported into neural cells (Calhoun et al., 2003; Kuo et al., 2003; Nakayama et al., 2003; Li et al., 2005), hematopoietic cells (Umeda et al., 2004, 2006), and pigmented retinal epithelium (Haruta et al., 2004). Cells differentiated into neurons have been transplanted into monkey brains (Sanchez-Pernaute et al., 2005; Takagi et al., 2005) with long-term survival, including transfer into a monkey model of Parkinson's disease with early but promising results (Takagi et al., 2005).

Monkey ES cells have been shown to contribute to chimeric embryos (Takada et al., 2002; Mitalipov et al., 2006) but no contribution has been shown in fetuses or offspring to date. It is well known in the mouse embryonic stem cell field that ES cells can maintain pluripotent markers but fail to contribute to chimeric tissues or the germ line. Therefore, it may be necessary to screen dozens of nhpES cell lines before one is found capable of this task.

The derivation of nonhuman primate ES cells has continued successfully but sporadically since the first isolation (Thomson et al., 1995). nhpES cells have been isolated from in vivo–derived embryos (Thomson et al., 1995, 1996) and in vitro embryos including those derived by intracytoplasmic sperm injection (ICSI; Suemori et al., 2001; Mitalipov et al., 2006; Navara et al., 2007). They have even been derived from parthenogenetic embryos (Cibelli et al., 2002). Derivations include three different nonhuman primate species, rhesus monkey (*Macaca mulatta*; Thomson et al., 1995; Mitalipov et al., 2006; Navara et al., 2007), cynomolgus monkey (*Macaca fascicularis*; Suemori et al., 2001; Cibelli et al., 2002), and marmoset (*Callithrix jacchus*; Thomson et al., 1996; Sasaki et al., 2005).

Critical Parameters and Troubleshooting

Before attempting to isolate nhpES cells, investigators should develop the techniques for passaging existing human or monkey embryonic stem cells. Many of the steps require an understanding of the pluripotent phenotype for selection of the highest-quality cells. It would be unfortunate to incur the time and expense of generating NHP embryos and attempting to isolate stem cells, only to lose them as a result of failure to recognize the cells in culture or errors in passaging or preparing mouse embryonic feeder cells.

The nhpES cell medium described in this unit (see recipe) was developed based on published reports that Activin A (Vallier et al., 2005) and increased levels of bFGF (Xu et al., 2005; Levenstein et al., 2006) are helpful in maintaining pluripotency. Additionally, although leukemia inhibitory factor has been shown to be extraneous for pluripotency, most derivation media include this component. The nhpES medium described in this unit has been successfully used in the authors' laboratory, but it is rather costly. Other derivation media have been described (Thomson et al., 1995; Suemori et al., 2001; Sasaki et al., 2005; Mitalipov et al., 2006) for nhpES cells, and investigators may want to look into these if costs warrant.

Embryo quality most likely plays a large role in the success of stem cell derivation. In the authors' research, it has been found that

the embryos that develop fastest in vitro also yield the highest success rate for embryonic stem cell derivation (Navara et al., 2007). This correlates with a long-standing belief by reproductive biologists that the highest-quality embryos also develop the fastest. The authors have isolated stem cells from later-developing embryos, but at rates one-half of that obtained with the more rapidly developing embryos. While it has been shown to be possible to isolate ES cell lines even from embryos believed to have arrested (Zhang et al., 2006), beginning investigators will want to ensure that they are starting with only the best embryos. The authors retain all early cultures, even those from which passaging has been performed, for an additional 2 weeks after passaging to ensure that all potential stem cells have been harvested.

Once cell lines are established, they should be frozen early and often. As soon as the cells exist in multiple cultures, they should be cryopreserved. This is a necessary step for safeguarding against contamination, aneuploidy (see below), or other culture errors.

Perhaps the biggest risk in the culture of embryonic stem cells, particularly for investigators just beginning to culture these cells, is the risk of cells becoming aneuploid in culture. Embryonic stem cells should be tested every 6 months for proper and stable karyotype, and should also be checked when growth conditions change, e.g., in cases where there is faster growth or less differentiation than expected.

In order to ensure the highest-quality immunocytochemistry, cells should be fixed in 37°C formaldehyde as soon as possible after removal from the incubator (within 1 or 2 min). It is best to process the staining all at once, instead of stopping at any given step, and slides should be examined as soon after staining as possible. Commercial antibodies may change over time such that the antibody purchased 6 months ago is not the same antibody purchased today. This can be due to a change in the lot of antibody or a complete reworking of the antibody from the vendor. If an antibody stops working, it will be necessary to test various fixations and antibody dilutions to reoptimize the labeling conditions.

Karyotyping of any cell type requires some adjustments to the system, and this is especially true of embryonic stem cells. Several factors can reduce optimal chromosome spreading and banding, and this in turn can inhibit proper interpretation. If not enough mitotic figures are observed, the concentration and incubation time of Colcemid treatment can be increased. Conversely, if very short chromosomes result, this is generally a sign of too much Colcemid or too long an incubation. Chromosomes can also be lengthened by increasing the ethidium bromide concentration or incubation time.

Fine tuning the slide preparation conditions by modifying the humidity or temperature or by varying the exposure time to hypotonic solution can increase the quality of chromosome spreads. Poor banding is usually a result of over- or under-trypsinization. When adjusting the conditions, trypsin exposure time should be varied by 2-sec intervals. Bands that are not distinct, with diffuse chromosomes, mean that trypsin time should be decreased; conversely, metaphase chromosomes with few light bands indicate that increased time with trypsin is needed.

When interpreting the karyotype, random chromosome loss should not be a concern unless three cells are detected with the same hypodiploidy. If a single hyperploid or aneuploid cell is observed, 20 more cells should be counted. If another identical karyotype is found, it is likely a clone. A repeat karyotype should be performed on the cells to monitor clonal propagation in culture. Aneuploid cells very often have exaggerated pluripotency characteristics, and are thus likely to be selected by manual passaging, making it possible for them to quickly overrun the colony. If this happens, return to an earlier passage from the freezer and throw out the cultures displaying aneuploidy. Alternatively, if no earlier passages exist, single cells can be isolated using a cell sorter, and clones grown from these single cells can be analyzed for pluripotency and proper karyotype. This procedure is incredibly inefficient, but could be used to save a precious cell line.

If teratomas fail to form, the number of cells injected can be increased. This may be an effect of viability after harvesting, and this can be tested using a simple live/dead stain such as trypan blue. Cells for teratoma formation should be of the same high quality as those used for other pluripotency assays. Resist the temptation to use already differentiating cells with the justification that they are going to differentiate anyway.

Anticipated Results

Investigators with a successful history maintaining or propagating existing human or nonhuman primate ES cells should be able to successfully isolate embryonic stem cell lines from 25% to 50% of fully expanded

blastocysts. Faithful attention to manual passaging of only the highest-quality cells should allow for greater than 75% of early established lines to be propagated to stability. Cells with the proper morphology (closely packed cells with a high nucleus:cytoplasm ratio and prominent nucleoli) will display most, if not all, of the described markers for pluripotency and will acquire the others in culture.

Time Considerations

The process of immunosurgery requires ~2 hr. At a time point 48 hr prior to the day of immunosurgery, 6-well plates containing feeder cells should be prepared, and 2 hr before the immunosurgery, the feeder cell medium should be replaced with nhpES cell medium. Attachment of the isolated ICM takes between 24 and 72 hr. Investigators can wait longer, but the success rate of derivation of an ES line from embryos that take longer than 72 hr to attach approaches zero.

It is ~2 weeks from the time of immunosurgery until the derived ES cells are ready for passaging. After this point, they should be passaged every 5 to 7 days.

Immunocytochemical staining takes ~4 hr, not including the overnight incubation for Oct-4 and Nanog antibodies.

RT-PCR analysis of pluripotency can be completed in 6 to 8 hr on a single day, or can be split overnight so that the first day includes RNA isolation, requiring about 45 min, and the next day requires 2 to 3 hr for generating cDNA, performing PCR, and analyzing by gel electrophoresis.

Karyotyping requires 6 to 8 hr on the first day for harvesting the ES cells, fixing them, and preparing glass slides. The next two steps can be completed in 1 day or split over 2 days for convenience. G-banding of the prepared slides requires ~4 hr; allow at least another 4 hr for analysis of the prepared slides, depending on how many slides have been prepared and the familiarity of laboratory personnel with cytogenetic analysis.

Preparing the cells for teratoma formation requires ~1 hr, and injection into an immunocompromised mouse requires another hour. Teratomas require at least 8 weeks to develop, and generally require more than 12 weeks to develop in vitro. Investigators should not try to speed this process by injecting a larger number of cells. The teratoma will become large more quickly but the individual cell types will not have enough time to differentiate; it is difficult to interpret poorly differentiated teratomas.

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Derivation of hESC from Intact Blastocysts

Dusko Ilic,[2] Olga Genbacev,[2] and Ana Krtolica[1, 3]

[1]StemLifeLine Inc., San Carlos, California
[2]University of California, San Francisco, California
[3]Lawrence Berkeley National Laboratory, Berkeley, California

ABSTRACT

This unit describes protocols for culturing human embryos and deriving human embryonic stem cells from the intact blastocyst. Description of the culturing begins with methods for obtaining human blastocysts using pronuclear or cleavage stage embryos left over after in vitro fertilization. Then there is a description of methods that can be used to derive human embryonic stem cell lines from the blastocyst without trophectoderm removal. Also included is a discussion of the critical steps and parameters such as zona pellucida removal, embryo quality assessment, feeder selection, when and how to transfer early embryonic outgrowths. In addition, there are protocols for embryo thawing, seeding of feeder cells, gelatin coating of plates, cleavage and blastocyst stage embryo grading, preparation and storage of reagents and solutions. Finally, there is a discussion of alternative derivation approaches as well as the timeline for the procedures. *Curr. Protoc. Stem Cell Biol.* 1:1A.2.1-1A.2.18. © 2007 by John Wiley & Sons, Inc.

Keywords: human embryonic stem cells (hESC) • inner cell mass (ICM) • trophectoderm (TE) • zona pellucida removal • feeders

INTRODUCTION

Like mouse embryonic stem cells, human embryonic stem cells (hESC) are derived from the inner cell mass (ICM) of pre-implantation embryos and can give rise to cells from all three germ layers (pluripotency). If properly maintained, they can be grown in culture virtually indefinitely while retaining their pluripotency and unlimited self-renewal capacity. It is these characteristics that make hESC ideal candidates for drug testing and future cell replacement therapies. Because hESC share these characteristics with the early embryo cells from which they originate, they can also serve as good models for studies of early human development. This is an understudied area of research because of the limited availability of the relevant tissue material as well as a variety of ethical issues related to its use.

This unit describes protocols related to the derivation of pluripotent embryonic stem cells from human embryos left over after in vitro fertilization (IVF). The Basic Protocol describes a method for deriving embryonic stem cells from the intact zona pellucida–free blastocyst. The authors have used this method (previously described in Genbacev et al., 2005) to derive more than ten hESC lines. Support Protocol 1 describes culturing of embryos from either pronuclear (day 1 single-cell) or cleavage (day 3 8-cell) stage to blastocyst stage followed by zona pellucida removal by acid hydrolysis using Tyrode's solution (Support Protocol 2) or by enzymatic digestion with pronase (Support Protocol 3). In addition, protocols are provided for embryo thawing (Support Protocol 4) and seeding of feeder cells (Support Protocol 5).

NOTE: All procedures described in this unit, including preparation of reagents and solutions, should be performed under sterile culture conditions in either Class II biological

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safety cabinets or laminar flow hoods. For handling embryos, a dissecting microscope should be placed within a laminar flow hood, and a face mask should be worn to prevent contamination.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

NOTE: The described protocols usually require ethics approval from the appropriate institutional review board or equivalent entity. Typically, only embryos donated for research with consent from IVF patients can be used. Regulations may vary depending on geographic area, so inquire locally before initiating this type of research.

BASIC PROTOCOL

HUMAN EMBRYONIC STEM CELL (hESC) DERIVATION

Zona pellucida–free blastocysts are cultured on feeder layers in the presence of human recombinant basic fibroblast growth factor (bFGF) to allow the outgrowth of hESCs.

Materials

KSR embryo culture medium supplemented with 25 ng/ml bFGF (see recipe)
Zona pellucida–free blastocyst-stage embryos (Support Protocols 2 and 3)

26-G needle, sterile
The Stripper micropipettor (MidAtlantic Diagnostics MXL3-STR) and 600-μm polycarbonate tips (MidAtlantic Diagnostics MXL3-600)
1.8-ml cryovials

Additional reagents and equipment for preparing feeder cells in 4- or 6-well tissue culture plates (Support Protocol 5)

Prepare culture plates

1. Prepare feeder cells in 4-well tissue culture plates (Support Protocol 5) 1 to 3 days before plating the zona pellucida–free blastocyst-stage embryo.

   Alternatively, 6-well plates may be used.

   Production of feeder plates should be scheduled to provide freshly plated feeder cells for transfers (see step 7). It is always better to have more wells with freshly plated feeder cells than required for embryos and transfers; plating may sometimes yield wells where feeder cells are not uniformly distributed, and these wells should not be used.

2. One to twelve hours before plating blastocysts, replace the fibroblast medium with KSR embryo culture medium supplemented with 25 ng/ml bFGF (0.5 ml/well for 4-well plates and 3.5 ml/well for 6-well plates).

Establish inner cell mass growth

3. Place the zona pellucida–free blastocyst-stage embryos in the wells of the 4-well plates prepared in step 2 (one embryo per well) and incubate at 37°C in 5% CO_2.

   Because each embryo has different genetic material, each must be plated in a separate well.

   The zona pellucida–free blastocyst-stage embryo should attach to feeder cell layer within 48 hr after plating (Fig. 1A.2.1).

4. Replace the KSR embryo culture medium supplemented with 25 ng/ml bFGF every second day. Observe for growth up to 1 month.

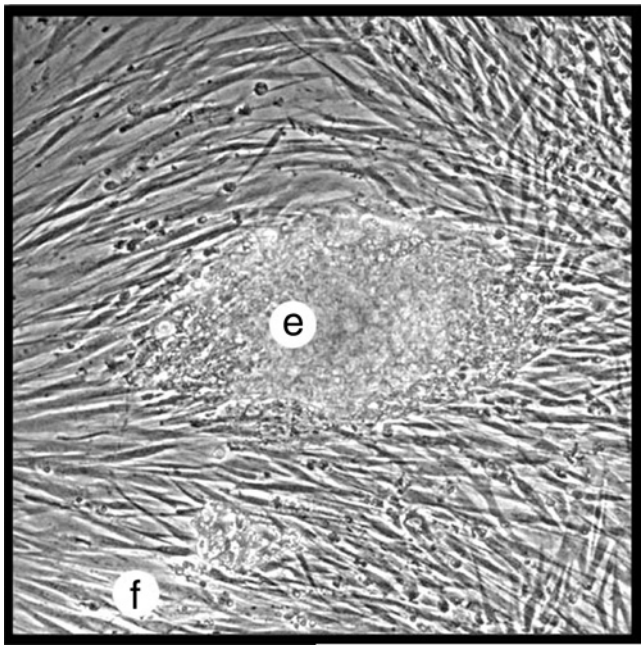

Figure 1A.2.1 Zona pellucida–free blastocyst-stage embryo (e) attached to the feeder cell layer (f).

Trophectoderm cells will form the first outgrowth. Extensive secretion from trophectoderm outgrowth sometimes denudes the area of feeder cells. In such cases, trophectoderm outgrowth should be disaggregated with a sterile needle, usually 6 or 7 days after plating (Fig. 1A.2.2). Because the medium does not support the growth of trophectoderm, it dies off within 10 to 14 days after plating of the embryo.

5. Once ICM outgrowth is observed (~15 to 24 days; Fig. 1A.2.3), replace the medium and dissect the outgrowth into smaller pieces using a sterile needle.

   Movement of the medium in the well while transferring the dish back into the incubator separates the dissected pieces and moves them away from the original outgrowth.

   ICM outgrowth is usually distinguishable 15 to 24 days after plating zona pellucida–free blastocysts on feeder cell layer. At that time, the initial trophectoderm outgrowth will die off.

   Although by definition feeder cells should not be able to proliferate, in some cases a few cells might escape mitotic inactivation with mitomycin C or irradiation (see Support Protocol 5) and can proliferate and fill the well with feeder cells after prolonged culture. Growth of feeder cells will quickly deplete culture medium of the growth factors and nutrients; if feeder cells continue to grow, the medium should be replaced on a daily basis. However, if the growth of feeder cells is prominent, a higher dose of irradiation or mitomycin C should be used for their mitotic inactivation (see Support Protocol 5).

6. Continue to replace KSR embryo culture medium supplemented with 25 ng/ml bFGF every second day and check for growth. Dissect outgrowth again, if present. Leave the clumps in the same well until feeders start detaching from the edges of the well or the well is filled with colonies (see Fig. 1A.2.4).

   The time it takes to reach the point where the hESC are expanded into new wells depends on how fast the hESC divide; all hESC do not proliferate at the same rate.

   The timing for transfer and/or expansion of colonies varies. For example, if there is one slowly growing colony in one well, when the colony is large enough to be dissected it would be best to transfer pieces of it into a new well with fresh feeders. If a colony is still small and feeders start to deteriorate, the colony is transferred to new feeders without

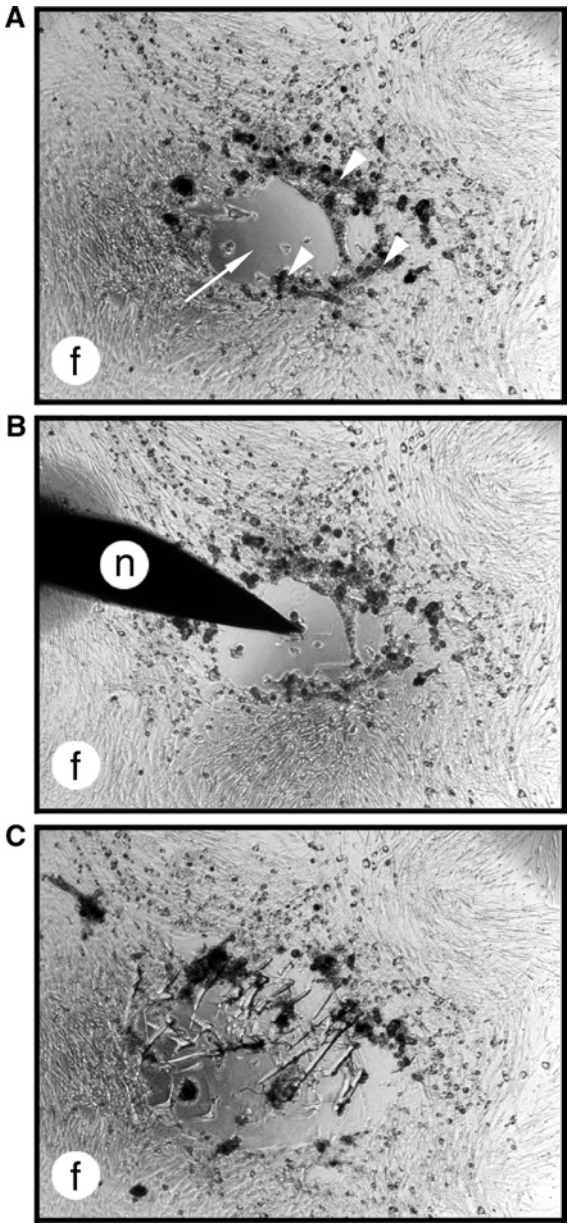

Figure 1A.2.2 Dissection of trophectoderm outgrowth from the attached embryo. (**A**) Initial trophectoderm outgrowth (arrowheads). Arrow points to areas denuded of feeder cell layer (f) due to proteolytic activity of trophectoderm cells. (**B**) Disaggregation of the initial trophectoderm outgrowth with a needle (n). (**C**) Appearance of the area after dissection.

splitting. On the other hand, if there is one fast-growing colony in one well, the colony might be dissected once or twice and the pieces left in the same well until the feeders start to deteriorate.

The viability of the feeder cells can also determine when the hESC colonies are transferred. Detachment of the feeder cells indicates that the cells have aged and that their value as growth-supporting cells has decreased. Some feeders can support hESC for 4 weeks before they deteriorate; others last only 2 weeks.

7. At that time transfer the colonies from each well of the 4-well tissue culture plate into a feeder-containing well of a 6-well tissue culture plate.

The larger surface area in the 6-well plate allows growth of more colonies. Alternatively, 4-well tissue culture plates can also be used for propagating colonies.

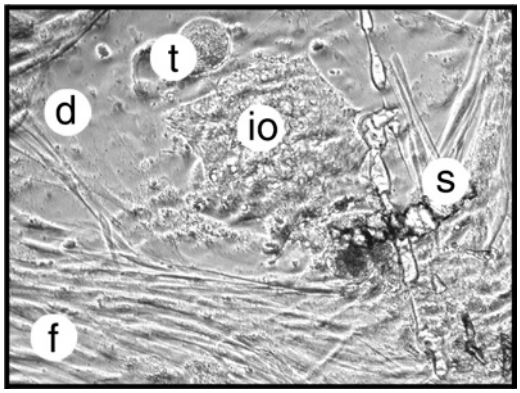

Figure 1A.2.3 Initial ICM outgrowth (io). Visible are the denuded area due to extensive proteolytic secretion of trophectoderm cells (d), feeder cell layer (f), needle scratches remaining from disaggregation of the trophectoderm (s), and dead trophectoderm cell (t).

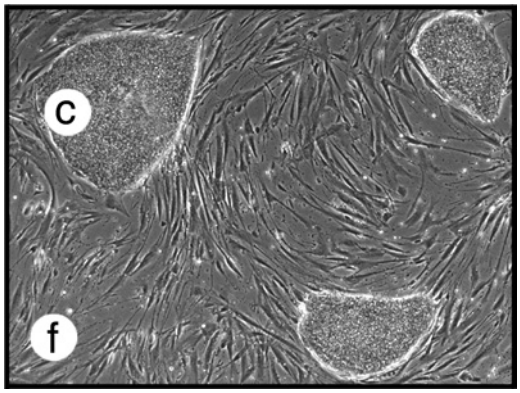

Figure 1A.2.4 Embryonic stem cell colonies (c) on feeder cell layer (f).

During transfer of hESC colonies from one well to another, adjacent feeder cells will be transferred, too. Because there is only small number of such cells and they do not proliferate they will not interfere with further growth and culture of hESC colonies.

Never combine colonies from different embryos in one well because each embryo has its own unique genetic material.

8. Repeat dissection of the colonies until there are at least two wells of the 6-well tissue culture plate with 20 colonies per well.

   The hESC colonies should be propagated as described (also see UNIT 1C.1) until their number is sufficient for freezing (20 to 50 colonies/cryovial).

9. Place cells from at least one well of the 6-well tissue culture plate into one cryovial (minimum of 20 colonies per vial) for freezing (see Phelan, 2006).

10. Continue to expand cells from the other wells for additional frozen cultures and for quality control.

    Do not discard the well from which the original colonies were dissected for at least a week because new colonies may emerge. Whenever possible, dissect only a part of the colony leaving the other part intact, until a sufficient number of wells with colonies is established (three to four wells of the 6-well plate).

    When large areas in the wells lack feeder cells or feeder cells look unhealthy and start detaching and dying, dispose of the plate. Usually, if feeder cells are of good quality, they

can serve their purpose up to 1 month. It is strongly recommended that fresh feeder cells are always plated for each passage of hESC into a new well.

A number of parameters can be evaluated for quality control, depending on the investigator and research being performed, e.g., morphology, proliferation rate, expression and localization of hESC markers, karyotype, telomerase activity, and the ability to differentiate into three germ layers.

SUPPORT PROTOCOL 1

IN VITRO DEVELOPMENT OF BLASTOCYSTS

The blastocyst is the first stage of the human embryo at which two unquestionably distinct cell populations exist: an outer cell layer or trophectoderm and a compact inner cell population called the inner cell mass (ICM). Outgrowth of the ICM cells in culture gives rise to embryonic stem cells. During the cleavage and morula stages of embryo development, differentiation into trophectoderm and ICM is still uncertain. Culturing to the blastocyst stage helps eliminate developmentally arrested embryos and increases chances for successful hESC derivation.

Materials

Appropriate cell culture medium: G-1 v3 Plus medium (Vitrolife) for the 1- to 8-cell stage (day 1 pronuclear to day 3 cleavage); G-2 v3 Plus blastocyst medium (Vitrolife) for the 8-cell (day 3 cleavage) to blastocyst (day 5 or 6) stage

Oil for embryo culture (sterile light mineral oil; Irvine Scientific)

Pronuclear or cleaving embryos from IVF, fresh or frozen (see Support Protocol 4 for thawing directions)

6-cm tissue culture–treated plastic dish (e.g., Falcon 3046)

The Stripper micropipettor (MidAtlantic Diagnostics MXL3-STR) with 135-µm and 600-µm polycarbonate tips (MidAtlantic Diagnostics MXL3-135 and MXL3-600)

1. Place six to seven 30- to 35-µl droplets of the appropriate cell culture medium in a 6-cm tissue culture dish and cover with 5 ml of oil for embryo culture (Fig. 1A.2.5).

   The number of droplets depends on how many embryos will be thawed. To be on the safe side, it is always good to place more drops than necessary.

   Oil for embryo culture is a sterile light mineral oil and is intended for use as an overlay when culturing cells in reduced volumes of medium to prevent evaporation and insulate the medium from changes in osmolarity and pH.

2. Equilibrate medium droplets by preincubating 1 to 3 hr at 37°C in a 5% CO_2 incubator.

3. Attach a 135-µm tip to The Stripper micropipettor (Fig. 1A.2.6) and moisten with cell culture medium as follows:

   a. Carefully attach a sterile Stripper tip to the stainless steel plunger by loosening the knurled collet and depressing the finger pad until the plunger protrudes 0.5 to 1.0 cm past the collet.

   b. Slip on the new tip and push it firmly along the plunger until it stops against the O rings at the tip of the barrel.

   c. Tighten the collet.

   d. Rinse the tip by depressing the plunger until the finger pad contacts the spring housing; immerse the tip into a drop of medium, and slowly release the plunger. Expel the medium by depressing the plunger as before.

   e. To expel any residual medium in the tip, push the finger pad until it enters the spring housing.

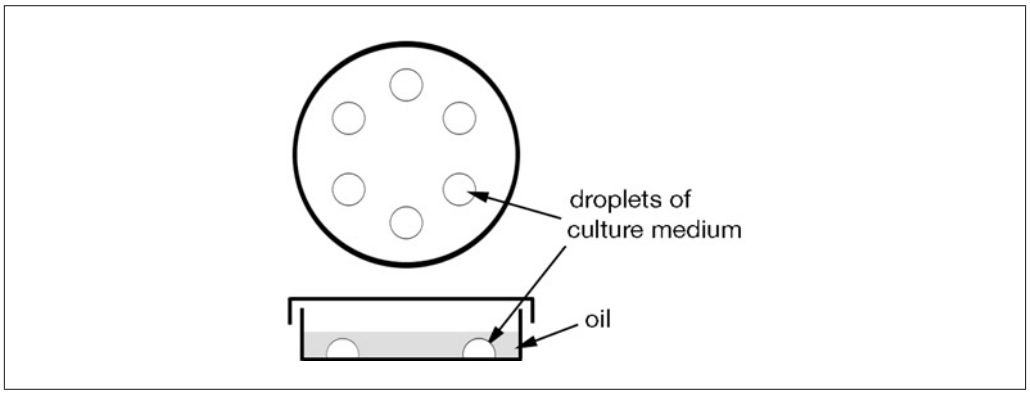

Figure 1A.2.5 Schematic drawing of a dish containing drops of embryo culture medium covered with oil. Label each drop clearly on bottom of the dish.

Figure 1A.2.6 The Stripper micropipettor with tip used for manipulating embryonic cells.

f. Repeat this process a few times to ensure the polycarbonate tip is sufficiently moistened.

The Stripper micropipettor is a precision instrument designed to manipulate gametes or embryos with a minimal amount of fluid transfer. Once the tip has been rinsed, the embryos can be manipulated.

Make sure that the bore of the tip is appropriate for the diameter of the embryo by placing the tip next to the embryo and ascertaining that the inner diameter of the tip will not cause major distortion of the embryo as it is pipetted in and out of the tip. Practice, using discarded mouse, bovine, or hamster eggs/embryos, is recommended.

4. Using the moistened pipettor tip, transfer one to four embryos from the same donor into each drop of the 37°C equilibrated embryo culture medium under oil.

Both fresh and frozen embryos can be used to obtain blastocysts. For thawing frozen embryos see Support Protocol 4.

5. Examine each embryo under the microscope (100×) and assign a grade (see Fig. 1A.2.7).

The embryos with better grades (1 or 2) are more likely to develop into blastocysts. Also, low oxygen tension (5% O_2) and low illumination (20 lux from the ceiling and 100 lux from the microscope) throughout embryo manipulation may improve the blastulation rate (Noda et al., 1994). Special low-oxygen cell incubators are available from various manufacturers.

6. Place the dish at 37°C in 5% CO_2 and transfer embryos every 24 to 36 hr into fresh droplets of the embryo culture medium under oil (prepared as described in steps 1 and 2). When embryos start to expand in size, transfer them with a 600-μm tip instead of the 135-μm tip.

7. When the embryos reach the blastocyst stage, proceed with zona pellucida removal (Support Protocol 2 or 3).

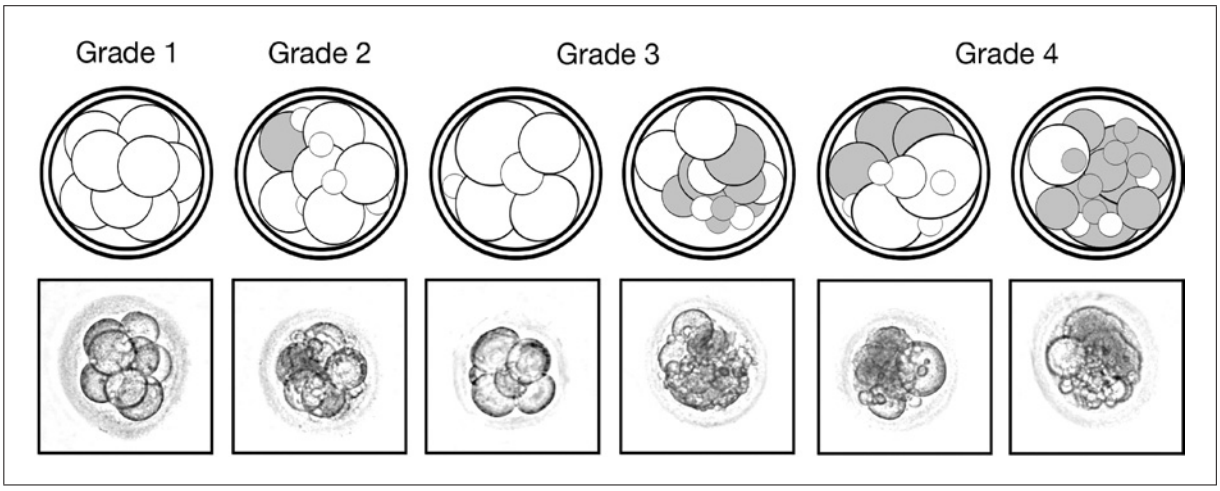

Figure 1A.2.7 Grading criteria for embryos at the cleavage stage (day 3 embryos). Grade 1: Equal size blastomeres without any cell fragmentation. Grade 2: Equal size blastomeres with some cell fragmentation. Grade 3: Unequal size blastomeres with no or little cell fragmentation or equal size blastomeres with moderate cell fragmentation. Grade 4. Unequal size blastomeres with moderate fragmentation or massive cell fragmentation regardless of blastomere size. Gray shading indicates nonviable cells.

SUPPORT PROTOCOL 2

REMOVAL OF THE ZONA PELLUCIDA WITH ACIDIFIED TYRODE'S SOLUTION

The zona pellucida is a protective extracellular glycoprotein matrix layer surrounding oocytes and pre-implantation embryos. As the embryo grows, the zona pellucida becomes thinner, and prior to implantation into the uterine wall, the embryo hatches out of the zona pellucida completely. Assisted hatching (in vitro removal of zona pellucida) can be accomplished in several different ways. This protocol describes removal of the zona pellucida with acidified Tyrode's solution. Removal using pronase treatment is detailed in Support Protocol 3.

Materials

KSR embryo culture medium with and without 25 ng/ml bFGF (see recipe)
Acidified Tyrode's solution (Irvine Scientific)
Embryos in culture (Support Protocol 1)
4-well tissue culture plate with feeder cells (Support Protocol 5)
G-2 v3 Plus blastocyst medium (Vitrolife)

6-cm tissue culture dish with cell culture–treated surface (e.g., Falcon 3046)
The Stripper micropipettor (MidAtlantic Diagnostics MXL3-STR) with 600-μm (MidAtlantic Diagnostics MXL3-600) and other appropriate size tips
Microscope with camera

1. Place six separate 50-μl drops of KSR embryo culture medium on a cell culture–treated surface of a sterile 6-cm tissue culture dish.
2. Place two 50-μl drops of acidified Tyrode's solution in the same dish; mark the drops of acidified Tyrode's solution to avoid error.

   CAUTION: *Acidified Tyrode's solution has a pH of 2.1 to 2.5. Use appropriate precautions in handling it.*

   One dish with the drops of KSR medium and Tyrode's solution should be prepared for each embryo to be treated.
3. Remove the embryo from the culture drop under oil using The Stripper micropipettor with an appropriate size tip and transfer it into a drop of KSR embryo culture medium.

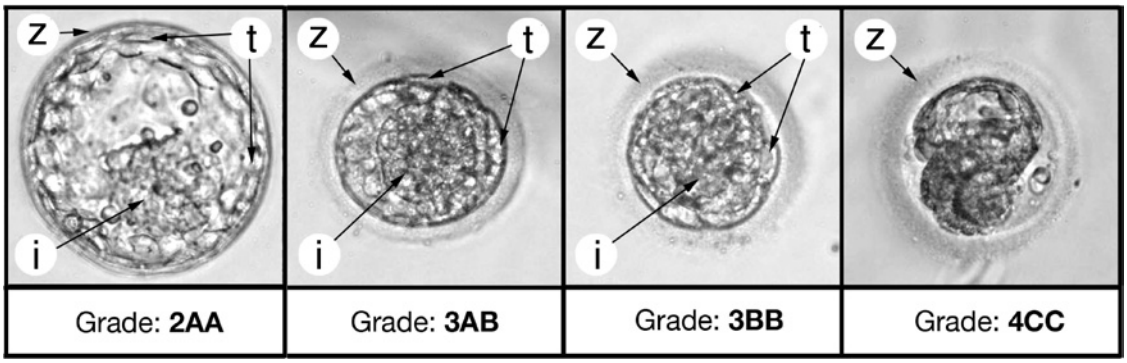

Figure 1A.2.8 Grading examples for embryos at the blastocyst stage. Blastocyst-stage embryo score is a number based on the morphology, size of the inner cell mass (i), and the viability of cells as judged under the microscope on the indicated days after in vitro fertilization, according to the following rules: 1 = fully expanded or hatching on day 5; 2 = fully expanded or hatching on day 6 or moderate expansion on day 5; 3 = moderate expansion on day 6 or early cavitation on day 5; 4 = early cavitation day 6 or morula on day 5 or 6. Add to the number score (1 to 4) two alphabetic scores: the first one to grade inner cell mass (i) and the second one to grade trophectoderm (t) according to the following rules: A = large inner cell mass or continuous trophectoderm with good cell-cell adhesion; B = medium inner cell mass or areas in trophectoderm with poor cell-cell adhesion; C = no visible inner cell mass or sparse granular trophectoderm cells. Featured examples: 2AA, fully expanded blastocyst on day 6 with a large inner cell mass and continuous trophectoderm; 3AB, moderately expanded blastocyst on day 6 with a large inner cell mass and discontinuous trophectoderm; 3BB, moderately expanded blastocyst on day 6 with a poor inner cell mass and discontinuous trophectoderm; 4CC, moderately expanded blastocyst on day 6 with no visible inner cell mass or distinguishable trophectoderm. z, zona pellucida.

4. Examine the blastocyst-stage embryo under the microscope, record an image, and assign a grade (see Fig. 1A.2.8).

   Do not treat embryos that have initiated hatching (Fig. 1A.2.9) with acidified Tyrode's solution. Instead, transfer them onto feeders in G-2 v3 Plus medium and place in the cell incubator. Replace the G-2 v3 Plus medium with KSR embryo culture medium supplemented with 25 ng/ml bFGF once the embryo has completely hatched and detached from the zona pellucida (from 2 to 12 hr).

5. Transfer the embryo into the first drop of acidified Tyrode's solution for a brief rinse, and then transfer to the second drop of acidified Tyrode's solution. Watch carefully for the dissolution of the zona pellucida (5 to 30 sec).

6. As soon as the zona pellucida is dissolved, quickly rinse the embryo by pipetting it up and down in the first drop of KSR embryo culture medium using The Stripper micropipettor with a 600-μm tip.

7. Transfer the embryo into the next drop and repeat the procedure until embryo reaches the sixth drop. Examine the embryo to ensure that the zona pellucida was completely removed (Fig. 1A.2.10).

8. Place the zona pellucida–free embryo into a well of 4-well tissue culture plate with feeder cells in 0.5 ml KSR embryo culture medium supplemented with 25 ng/ml bFGF.

   Place only one embryo into each well. Because each embryo has its own unique genetic material, it is crucial not to mix them.

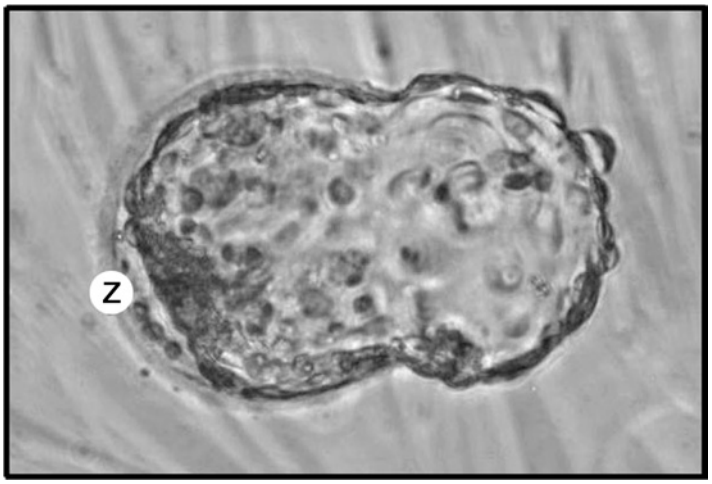

Figure 1A.2.9 Hatching blastocyst-stage embryo. z, zona pellucida. Note the break in the zona pellucida on the right side of the blastocyst. The zona pellucida–free half of the blastocyst protrudes through the hole in the zona pellucida while the other half (on the left) is still surrounded by it.

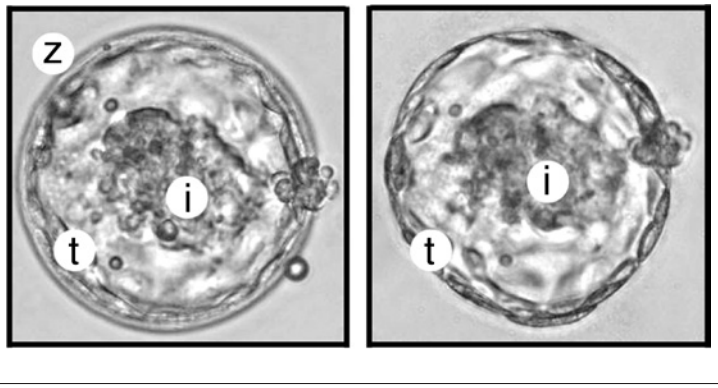

Figure 1A.2.10 Zona pellucida removal. Blastocyst-stage embryo before (left) and after (right) zona pellucida removal with acidified Tyrode's solution. Labels: i, inner cell mass; t, trophectoderm; z, zona pellucida. Change in embryo shape is a sign that the zona pellucida is dissolved.

SUPPORT PROTOCOL 3

REMOVAL OF THE ZONA PELLUCIDA WITH PRONASE

Zona pellucida removal with acidified Tyrode's solution is a rapid process, and it is quite easy for the unskilled experimenter to irreparably damage the embryo. Therefore, some experimenters use the pronase method to remove the zona pellucida, a more time-consuming process that decreases the likelihood of the inadvertent embryo damage. While use of acidified Tyrode's solution is preferred in the cases when hESC may have potential therapeutic use, because it eliminates the use of animal-derived enzyme (pronase), pronase treatment is in other aspects equivalent to acid hydrolysis with Tyrode's solution.

Materials

KSR embryo culture medium with and without 25 ng/ml bFGF (see recipe)
0.5% (w/v) pronase E (Sigma) in KSR embryo culture medium (see recipe)
Embryos in culture (Support Protocol 1)
4-well tissue culture plate with feeder cells (Support Protocol 5)

6-cm tissue culture dishes with cell culture–treated surface
The Stripper micropipettor (MidAtlantic Diagnostics MXL3-STR) and 600-µm tips

1. Place six separate 50-µl drops of KSR embryo culture medium on a cell culture–treated surface of a sterile 6-cm tissue culture dish (for washing the embryo after pronase treatment).
2. Place two 50-ml drops of 0.5% pronase in the same dish; mark pronase drops to avoid error.
3. Remove embryo from the culture drop under oil using The Stripper micropipettor with a 600-µm tip and transfer into a drop of KSR embryo culture medium.
4. Examine the blastocyst-stage embryo under the microscope and assign a grade (see Fig. 1A.2.8).

   Do not treat embryos that have initiated hatching with pronase. Instead, transfer them onto feeders in the blastocyst medium and replace the medium with KSR embryo culture medium supplemented with bFGF once the embryo has completely hatched and detached from the zona pellucida.

5. Transfer the embryo into the first drop of pronase for a brief rinse, and then transfer to the second drop of pronase. Transfer dish into incubator and incubate 3 min at 37°C.
6. Remove the dish from the incubator and examine the embryo for presence of the zona pellucida. If the zona pellucida is still present, incubate the dish again ~1 min at 37°C. Repeat as many times as necessary.
7. As soon as the zona pellucida is dissolved, quickly transfer the embryo to the first drop of KSR embryo culture medium.
8. Transfer the embryo to the next drop and repeat until the sixth drop. Examine the embryo to ensure that the zona pellucida was completely removed.
9. Place the zona pellucida–free embryo into the well of a 4-well tissue culture plate with feeder cells in 0.5 ml KSR embryo culture medium supplemented with 25 ng/ml bFGF.

   Place only one embryo into each well. Because each embryo has its own unique genetic material, it is crucial not to mix them.

THAWING EMBRYOS

SUPPORT PROTOCOL 4

Embryo cryopreservation is a relatively new technique. The first pregnancy from a frozen and thawed human embryo was reported in 1983, and a birth from this source occurred the following year. Of ~100,000 cases of assisted reproductive technology in the United States in 2000, ~16% of the cases used frozen and thawed embryos. In 2000, live birth rates per thaw cycle were 18.3% versus 26.6% from the fresh embryo transfer. Theoretically, if there are no temperature variations, the embryos can be frozen indefinitely and still be successfully recovered. Embryos are gradually cooled from the body temperature to −196°C in the presence of cryoprotectants (e.g., propanediol) that prevent damage from intracellular ice formation and interact with membranes during their transition from a pliable to a rigid state. Thawing, which means bringing frozen embryos to room temperature, is a quick process, taking less than 2 min. However, the most critical aspect of the process is a slow step-wise exchange of cryoprotectant fluids with culture medium. Once the thawing is completed, the embryo is assessed for cryodamage. If there is no blastomere loss during cryopreservation, cryopreserved embryos are equivalent to fresh embryos. However, some healthy embryos may not survive the stress of freezing and thawing without partial cellular damage and blastomere lysis.

Materials

Embryos frozen in straws under liquid nitrogen (from IVF center)
Embryo Thaw Media Kit containing solutions T1, T2, and T3 (Irvine Scientific)
100 mg/ml human serum albumin solution (HSA; Irvine Scientific)
Modified human tubal fluid medium (mHTF; Irvine Scientific)

6-cm tissue culture dish (e.g., Falcon, 3046)
The Stripper micropipettor (MidAtlantic Diagnostics MXL3-STR) and appropriate size tips

Prepare solutions

1. Verify, using the accompanying documentation, that the straw removed from the liquid nitrogen storage tank contains embryos at the desired stage of development.

   In vitro fertilization clinics usually freeze embryos at the cleavage stage (day 3), although some may also freeze them at the single-cell, pronuclear stage (day 1) or at the blastocyst stage (day 5 or 6). Thaw media kits are not the same for cleavage- and blastocyst-stage embryos.

2. Bring solutions T1, T2, and T3 from the Embryo Thaw Media Kit to room temperature.

3. Add 12 µl of 100 mg/ml stock solution HSA to 1 ml mHTF. Bring to room temperature. Prepare a second 1-ml aliquot and warm to 37°C.

   Do not use any bottle of HSA which shows evidence of particulate matter, cloudiness, or is not clear pale yellow in color. To avoid problems with contamination, discard any excess medium or HSA stock that remains after the procedure is completed.

Set up thaw plates

4. Put 50 µl of solution T1 into a 6-cm tissue culture dish, and mark the drop as number 1.

   Embryo thaw solution T1 is a 1.0 M propanediol solution containing 0.2 M sucrose in mHTF supplemented with 12 mg/ml HSA. During the thawing procedure, the cryoprotectant propanediol is removed, and the embryos are rehydrated. Because of its high molecular weight, sucrose does not pass through the plasma membrane, and therefore it is included in the thawing solution to aid in the removal of cryoprotectant via osmosis.

   Several embryos may be placed into each drop of thawing solution, but to ensure that there is no potential for cross-contamination; only embryos from the same donor should be placed together.

   The arrangement of the drops of the different solutions on the same or different plates depends on how many embryos are being thawed. More than three drops in one dish might be too close and easily mixed.

5. Put 50 µl solution T2 into the 6-cm tissue culture dish, and mark the drop as number 2.

   Embryo thaw solution T2 is a 0.5 M propanediol solution containing 0.2 M sucrose in mHTF supplemented with 12 mg/ml HSA.

6. Put 50 µl solution T3 into the 6-cm tissue culture dish, and mark the drop as number 3.

   Embryo thaw solution T3 is a 0.2 M sucrose in mHTF supplemented with 12 mg/ml HSA.

7. Put 50 µl of HSA/mHTF medium into a separate 6-cm tissue culture dish, and mark the drop as number 4.

8. Fill a 50-ml test tube with sterile water heated to 30°C to act as a water bath.

Thaw embryo

9. Remove the straw containing frozen embryos from the liquid N_2 storage. Hold straw in the air for 30 to 40 sec, then immerse in the 30°C water bath for 40 to 60 sec to thaw.

   While identifying the correct straws, keep them in the liquid nitrogen to prevent temperature increase.

10. Remove the plastic top of the straw. Hold the straw at an angle against a sterile tissue culture dish and push content out, drop by drop.
11. Using the Stripper micropipettor with an appropriate size tip transfer the embryo(s) to drop number 1 with solution T1 and leave 5 min at room temperature.
12. Transfer the embryo(s) to drop number 2 (solution T2) and incubate 5 min at room temperature.
13. Transfer the embryo(s) to drop number 3 (solution T3) and incubate 10 min at room temperature.
14. Transfer the embryo(s) to drop number 4 (mHTF/HSA medium) and incubate 10 min at room temperature.
15. Put 50 µl of prewarmed HSA/mHTF medium into a separate 6-cm tissue culture dish, and mark the drop as number 5. Transfer the embryo(s) to drop number 5 (HSA/HTF medium) prewarmed to 37°C and incubate 10 min at 37°C.
16. Proceed with embryo culture as described in Support Protocol 1.

PLATING OF FEEDER CELLS

SUPPORT PROTOCOL 5

Human embryonic stem cells were originally derived on feeder layers of mitotically inactivated mouse embryonic fibroblasts (Thomson et al., 1998). The incorporation of nonhuman sialic *N*-glycolylneuraminic acid (Neu5Gc) from nonhuman feeder layers and medium by hESC leads to an immune response mediated by natural anti-Neu5Gc antibodies present in most humans (Martin et al., 2005); in cases when there is potential for therapeutic uses of the hESC, it is advantageous to replace mouse embryonic fibroblasts as feeder cells with feeder cells of human origin or, ideally, with a feeder-layer-free culture environment (Ilic, 2006). Among human feeder cells that support not only growth but also derivation of hESC lines, human foreskin (Amit et al., 2003; Hovatta et al., 2003) and placental fibroblasts (Genbacev et al., 2005) are the most easily accessible.

Materials

0.5% (w/v) gelatin (see recipe)
Phosphate-buffered saline (PBS), calcium and magnesium free (Gibco/Invitrogen)
Fibroblasts: irradiated and frozen mouse or human cells (see Conner, 2000; Nagy, 2003)
Fibroblast feeder medium (see recipe), prewarmed to 37°C
15-ml centrifuge tube, sterile
6-well, tissue culture–treated plates (e.g., Corning) *or*
4-well, tissue culture–treated plated (e.g., Nunc)
Additional reagents and equipment for counting cells (Phelan, 2006)

Prepare gelatin-coated plates

1. Add 0.5% gelatin to the tissue culture plates (0.5 ml/well of 4-well plate or 2 ml/well of 6-well plate) and incubate at least 2 hr at 37°C. Swirl to wet the entire surface of the wells.

2. Aspirate the gelatin and either use plates immediately or fill wells with PBS and leave in the 37°C incubator until use (maximum 3 days).

Thaw and plate irradiated fibroblasts

3. Thaw a cryovial of irradiated fibroblasts at 37°C and transfer contents into a sterile, 15-ml centrifuge tube containing 9 ml prewarmed fibroblast feeder medium.
4. Centrifuge the cells 5 min at 700 × *g*, room temperature.
5. Remove the supernatant and resuspend the cell pellet in fresh fibroblast medium.
6. Count the resuspended cells (see Phelan, 2006) and adjust the cell number according to the plating plan (see step 7 annotation) with additional fibroblast medium.
7. Plate the cells in a volume of fibroblast feeder medium and at cell density adjusted to the surface area of the cell culture plate used (to give 70% to 80% confluency within 3 days).

   The optimal number of cells should be determined for each lot and type of irradiated cells. When determining the number of cells to be plated, use 1.5 × 10^4 cells/cm^2 as a starting point. For example, plate 2 – 4 × 10^4 cells in 0.5 ml fibroblast culture medium per well of a 4-well tissue culture plate. Ideally, feeders will be 70% to 80% confluent at the time of embryo plating and not longer than 3 days in culture. However, thawed and plated irradiated fibroblasts may be used as feeders up to 1 week after plating. They are kept in the cell incubator until used.

   Irradiated fibroblasts are mitotically inactivated, which means that they can only complete a cell division cycle initiated prior to the irradiation, but cannot divide any further. However, in some cases, a few cells might escape mitotic inactivation with mitomycin C or irradiation and proliferate to fill up the well with feeder cells after prolonged culture.

   Some feeders can support hESC for 4 weeks before they deteriorate, while others are only good for about 2 weeks. How often feeders should be prepared must be determined by the investigators for each type and preparation of feeders used in their laboratories.

8. Change the medium once, 1 day after plating.

REAGENTS AND SOLUTIONS

For culture recipes and steps, use sterile tissue culture–grade water. For other purposes, use deionized, distilled water or equivalent in recipes and protocol steps. For suppliers, see ***SUPPLIERS APPENDIX****.*

Fibroblast feeder medium

360 ml Dulbecco's modified Eagle medium (DMEM), high glucose (Gibco/Invitrogen)
90 ml medium 199 (Gibco/Invitrogen)
50 ml heat-inactivated fetal bovine serum (Hyclone): prepared by dividing into 50-ml aliquots and storing up to 1 year at −20°C
Sterilize by passing through a 0.22-μm 1 cellulose acetate, low-protein-binding filter (Corning) and store up to 1 month at 4°C.

Gelatin, 0.5% (w/v)

50 ml 2 % (w/v) gelatin, Type B (Sigma)
150 ml H_2O
Sterilize by passing through a 0.22-μm low-protein-binding filter (Corning), divide into 10-ml aliquots, and store up to 1 year at −20°C.

Thawed 0.5% gelatin can be stored up to 1 week at 4°C.

Human recombinant basic fibroblast growth factor (bFGF) stock, 10 µg/ml

100 µg human recombinant basic fibroblast growth factor (bFGF; R&D), four 25-µg vials
10 ml diluted human serum albumin solution: prepared by diluting 20 µl 100 mg/ml human serum albumin solution (Irvine Scientific) with 10 ml calcium- and magnesium-free PBS (Gibco/Invitrogen)

Make up a 10 µg/ml solution of bFGF by dissolving four 25-µg vials of bFGF in a total of 10 ml diluted human Serum Albumin (HSA)/PBS in the original vials. Pool the solutions and sterilize by passing through a 0.2-µm surfactant-free cellulose acetate syringe filter (e.g., Corning 431219) (prefiltered with HSA solution diluted 1/10 in PBS). Divide into 1-ml aliquots and store up to 1 month at −20°C or up to 1 year at −80°C. Upon thawing, record the thawing date on the tube and store thawed aliquots up to 1 month at 4°C.

Do not use any bottle of HSA that shows evidence of particulate matter or cloudiness or is not clear pale yellow in color. To avoid problems with contamination, discard any excess medium or HSA stock that remains after the procedure is completed.

KSR embryo culture medium, with and without 25 ng/ml bFGF

400 ml Knockout Dulbecco's modified Eagle medium (e.g., Gibco/Invitrogen)
100 ml Knockout Serum Replacement (Gibco/Invitrogen)
5 ml 200 mM L-glutamine (Gibco/Invitrogen)
5 ml 10 mM modified Eagle medium nonessential amino acids solution, 100× stock (Gibco/Invitrogen)
1 ml 0.1 mM 2-mercaptoethanol
Sterilize by passing through a 0.22-µm cellulose acetate, low-protein-binding filter unit (Corning). Store up to 1 month at 4°C.

When required, add human recombinant bFGF (see recipe) to an aliquot of KSR embryo culture medium in a sterile tube to a final concentration of 25 ng/ml. Store up to 24 hours at 4°C.

2-Mercapoethanol stock, 0.1 mM

Combine 53 µl 99% 2-mercapoethanol (Sigma) with water to a final volume of 15 ml. Sterilize by passing through a 0.2-µm regenerated cellulose syringe filter (Corning), and divide into 1.5-ml aliquots. Store up to 6 months at –20°C.

COMMENTARY

Background Information

Embryonic stem cells (ESC) originate from the pre-implantation mammalian embryo. As it travels down the oviduct, a fertilized oocyte (or zygote) divides to generate a 16- and 32-cell morula (Johnson and McConnell, 2004). With subsequent cell divisions, a blastocoel cavity forms in the center of the morula and embryonic cells differentiate into two morphologically distinct populations within the blastocyst: an outer layer of cells comprising the trophectoderm, which will form placenta, and the inner cell mass (ICM) that will give rise to the fetus.

The cells from the ICM give rise to ESC in culture. However, the pluripotent cell population that exists for a short time within ICM of the developing blastocyst is most likely not identical to the derived ESC. During derivation, ESC undergo epigenetic changes to adjust to cell culture conditions and therefore acquire certain characteristics which separate them from the embryonic cells from which they originate (see Krtolica and Genbacev, 2007). However, hESCs share with embryonic ICM cells a pluripotent capacity and capability of self-renewal (Amit et al., 2000; Draper and Fox, 2003).

During ESC differentiation in culture, as well as embryonic differentiation in vivo, heterochromatin formation selectively suppresses gene expression, resulting in a loss of pluripotent capacity (Rasmussen, 2003). It is interesting to note that while the differentiation

potential of the ICM cells in vivo is not equivalent to a totipotent zygote—they do not form placenta and some other extraembryonic tissues—ESC in culture may have somewhat extended differentiation capacity and can give rise to trophectoderm-like cells (Xu et al., 2002).

Unlike the majority of somatic cells which undergo telomere shortening with each cell division and as a result have finite life span that ends with senescent arrest (Krtolica and Campisi, 2002), ESC express telomerase, a reverse transcriptase that adds telomeric DNA to chromosome ends thus preventing telomere shortening and growth arrest (Verfaillie et al., 2002; Carpenter et al., 2003). In this way, ESC maintain their telomere length at 8 to 12 kb and are capable of unlimited self-renewal (Verfaillie et al., 2002). When grown in culture, they exhibit a virtually indefinite replicative lifespan—some ESC lines have been propagated for years without any signs of slowing down.

Although reported derivation rates vary significantly between the investigators, there does not appear to be consistent difference in the efficiency of derivation between those who use the isolated ICM and those who start with the intact blastocysts. However, using intact blastocysts provides some advantages:

It eliminates technically challenging step of ICM isolation which requires either micromanipulator for the mechanical/laser dissection or immunosurgery.

It abrogates the exposure of the embryos to animal-derived complement that is used to destroy trophectoderm cells during immunosurgery, a most common procedure for the isolation of the ICM. This may be advantageous in case derived ESC are intended for clinical use.

It avoids risk of damaging the ICM during removal of trophectoderm.

It enables use of underdeveloped blastocysts in which ICM may not be clearly visible.

That said, some groups reported high efficiency of ESC derivation using isolated ICM, and there is no question that both methods can yield ESC of similar characteristics.

Critical Parameters

All tissue culture must be performed in Class II biological safety cabinets or laminar airflow workstations. All reagents and media must be sterilized (except for presterilized embryo media) by passing through 0.22-μm filters and should be discarded after their expiration date. Embryo transfer and removal of the zona pellucida should be performed in the shortest possible time to reduce stress and exposure to nonoptimal culture conditions.

Even if all procedures are performed correctly, the embryo may not give rise to hESC. The success of hESC derivation ultimately depends on two parameters: quality of the embryos and quality of the feeder cells. In the authors' experience, embryos with larger and well defined inner cell masses are more likely to give a rise to an hESC line. It is essential that feeders are freshly plated (1 to 3 days before use) and at the right density. It is also recommended that feeder cells used for derivation be from the passages/population doublings within the first 30% to 50% of their lifespan (i.e., between passages 7 and 12 if split 1:2 for human placental fibroblasts, passages 4 to 5 for mouse embryo fibroblasts, and $<$18 passages for foreskin fibroblasts). However, earlier passages may yield more efficient derivation); earlier passages are better.

Troubleshooting

To reduce technical problems, staff involved in hESC derivation should be experienced in handling cleavage- and blastocyst-stage embryos. If not, it is recommended that they practice first on mouse, bovine, or hamster eggs/embryos.

If removal of the zona pellucida is a challenge, one can wait for spontaneous hatching. In this case, the expanded blastocyst-stage embryo should be transferred onto feeders in the blastocyst medium. The medium should be replaced with KSR medium supplemented with bFGF once the embryo has completely hatched and detached from the zona pellucida. Because spontaneous hatching may or may not occur, and it happens only in the fully expanded high-grade blastocyst-stage embryos, relying only on hatched blastocysts would markedly reduce number of embryos available for hESC derivation.

In case grade 1 and 2 embryos with A or B inner cell mass consistently fail to give rise to an ESC line, it is recommended that the feeders used should be replaced by earlier passage and/or different feeder cell line.

If the growth of feeder cells is prominent in ESC cultures, a higher dose of irradiation or mitomycin C should be used for their mitotic inactivation.

Anticipated Results

Good quality cleavage stage (day 3) embryos (grades 1 and 2) are likely to develop into blastocysts with an efficiency of 40% to 50%. For lower grade embryos the efficiency of reaching the blastocyst stage is significantly lower. It is anticipated that between 20% and 50% of blastocysts will be able to give rise to embryonic stem cell colonies. The efficiency of derivation depends in large part on the quality of the blastocyst and the number and quality of inner cell mass cells in particular. Typically, blastocysts with the inner cell mass grade A or B are more efficient in yielding the ESC line. Some of the derived ESC lines may develop abnormal karyotypes, and it is therefore recommended that the initial freezing of ESC be performed as early as possible. The karyotype of each newly derived ESC line should be analyzed by G-banding after initial freezing and every 5 to 10 passages thereafter.

Time Considerations

Thawing embryos

The procedure takes approximately 2 hr, including media preparation.

Culturing embryos

Time needed depends on the number of drops and number of embryos. Preparation of embryo culture medium in droplets and covering with oil requires 1 to 2 min per one 6-cm tissue culture dish. Drops need to be pre-incubated for at least 1 hr prior to their use. Transfer of embryos from one drop to another is fairly quick, and it should take less than a minute per drop. After thawing, it takes 2 days for pronuclear-stage (day 1) embryos to reach cleavage stage. It takes 2 to 3 days for cleavage-stage (day 3) embryos to develop into blastocysts.

Removal of the zona pellucida

The process of zona pellucida removal will depend on the number of embryos, competence of the operator, and technique used. Approximately 10 min should be sufficient to remove the zona pellucida from one embryo using acidified Tyrode's solution, including assigning the grade. Using pronase instead of acidified Tyrode's solution will prolong zona pellucida removal by 3 to 5 min.

hESC derivation

Preparation of the KSR embryo culture medium takes about 30 min including thawing of premade frozen aliquots of various constituents. Preparation of bFGF and 2-mercaptoethanol stock and dividing in aliquots takes about 30 min for each. Placing embryos after zona pellucida removal onto feeders should not take more than few minutes. Attachment of embryos onto feeders takes between 24 and 48 hr. The derivation process from the moment embryos are placed onto feeders until the first visible embryonic stem cell colony outgrowth arises takes usually between 2 and 4 weeks. It takes additional 4 to 12 weeks to expand cells enough to be able to freeze them.

Plating feeder cells

To prepare fibroblast growing medium and gelatin stock takes about 30 min for each. Gelatin coating of tissue culture plates and thawing and plating of irradiated fibroblasts takes about 3 hr or more, depending on the number of plates being coated.

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SECTION 1C
Culture and Maintenance of Undifferentiated Embryonic Stem Cells

INTRODUCTION

All somatic and germ cells are derived from the inner cell mass (ICM) of blastocyst stage embryos. While the ICM is only a transient structure in vivo, stable in vitro cultures of pluripotential embryonic stem cells (ESCs) can be derived from it. How can ESCs be maintained in the undifferentiated state? Ever since the first derivation of ESCs from mice in 1981 it was clear that soluble factors are important for the expansion of ESCs. The *differentiation inhibitory activity* (DIA) critical for mouse ESCs was later shown to be *leukemia inhibitory factor* (LIF). Recently, the *bone morphogenetic protein* (BMP) pathway has emerged as a second major signaling pathway in mouse ESCs. Standard mouse ESC culture conditions consist of media containing LIF and fetal bovine serum (FBS; known to contain BMP-like activities) and use of gelatin-coated dishes or mouse embryonic fibroblasts (MEFs) as feeder cells.

With the recent derivation of primate and human ESCs it quickly became apparent that their optimal culture conditions differ significantly from those established for mouse ESCs: LIF is dispensable, and the BMP pathway should be inhibited rather than stimulated. Furthermore, addition of *basic fibroblast growth factor* (bFGF or FGF-2) and stimulation of the *transforming growth factor beta* pathway are critical. Accordingly, most laboratories do not use FCS in hESC media. Instead, a commercially available serum replacement, *knockout serum replacement* (KOSR; Invitrogen) containing albumin, insulin, transferrin, and other agents found in serum; is commonly used. Despite these differences, MEFs have also proven useful as feeder cells.

There are several reasons for the development of chemically defined ESC growth conditions. (1) Conventional media exhibit significant lot-dependent variability that require extensive testing of undefined components such as FBS or KOSR and may compromise the reproducibility of results. (2) The ideal medium would only include components necessary for optimal cell growth and function, thereby simplifying the analysis of molecular mechanisms that regulate ESCs. (3) Exposure to undefined xeno-products (e.g., FBS, KOSR, or MEFs) poses significant risks of molecular or microbiological contamination that may limit the clinical utility of human ESCs and their derivatives. Chemically defined media that allow derivation and long-term expansion of mouse and human ESCs have recently been developed.

An important difference between mouse and human ESC culture is that mouse ESCs can easily be passaged as single cells while human ESCs generally should be passaged as clumps. The reason for the more laborious and less efficient "clump passaging" of human ESCs is that long-term use of single-cell passaging techniques often leads to the emergence of karyotypically abnormal cells. However, techniques such as genetic manipulation, subcloning, and cell sorting are therefore significantly more challenging with clump-passaged human ESCs. Recovery of cryo-preserved human ESC clumps is also less efficient than that of frozen mouse ESCs. While the conventional method of slow freezing in the presence of DMSO yields acceptable results for at least short-term storage of human ESC clumps, the more cumbersome vitrification technique is the

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current method of choice when both long-term storage and the highest rate of recovery are essential.

In *UNIT 1C.1*, protocols for the expansion of karyotypically normal human ESCs are provided. The unit begins with a method for mechanically passaging human ESCs; this is followed by a method for enzymatic short-term bulk expansion.

Thorsten M. Schlaeger

Expansion of Human Embryonic Stem Cells In Vitro

Magdaline Costa,[1] Koula Sourris,[1] Tanya Hatzistavrou,[1] Andrew G. Elefanty,[1] and Edouard G. Stanley[1]

[1]Monash University, Clayton, Victoria

ABSTRACT

This unit describes a protocol for the large-scale expansion of karyotypically normal human embryonic stem cells (hESCs). hESCs can be maintained indefinitely as dense colonies that are mechanically cut into pieces, which are subsequently transferred to fresh organ culture dishes seeded with primary mouse embryonic fibroblasts (MEFs). hESCs can also be enzymatically passaged (bulk culture); however, over time, this style of culturing may lead to the acquisition of chromosomal abnormalities. Nevertheless, enzymatic passaging can be used for short periods (up to 25 passages) without the appearance of cells with abnormal karyotypes. *Curr. Protoc. Stem Cell Biol.* 1:1C.1.1-1C.1.7. © 2007 by John Wiley & Sons, Inc.

Keywords: human embryonic stem cells (hESCs) • mechanical passaging • enzymatic passaging • hESC expansion

INTRODUCTION

This unit describes a protocol for the large-scale expansion of karyotypically normal human embryonic stem cells (hESCs). hESCs can be maintained indefinitely as dense colonies that are mechanically cut into pieces, which are subsequently transferred to fresh organ culture dishes seeded with primary mouse embryonic fibroblasts (MEFs; Thomson et al., 1998; Reubinoff et al., 2000). hESCs can also be enzymatically passaged (bulk culture; Amit et al., 2000); however, over time, this style of culturing may lead to the acquisition of chromosomal abnormalities (Draper et al., 2004). Nevertheless, enzymatic passaging can be used for short periods (up to 25 passages) without the appearance of cells with an abnormal karyotype.

This unit begins with a method for the propagation of hESCs in organ culture, followed by a protocol for large-scale expansion of hESCs that can be used subsequently for experiments.

NOTE: The following procedures are performed in a Class II biological hazard flow Hood or a laminar-flow hood.

NOTE: All solutions and equipment coming into contact with live cells must be sterile, and proper aseptic technique should be used accordingly.

NOTE: All incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

BASIC PROTOCOL 1

MAINTENANCE OF hESC CULTURES BY MECHANICAL PASSAGING (ORGAN CULTURE)

This protocol is used for the long-term maintenance of hESCs. During organ culture, the cells are nonenzymatically passaged and can be continuously cultured for over 2 years without the acquisition of an abnormal karyotype. Cells are maintained in organ culture

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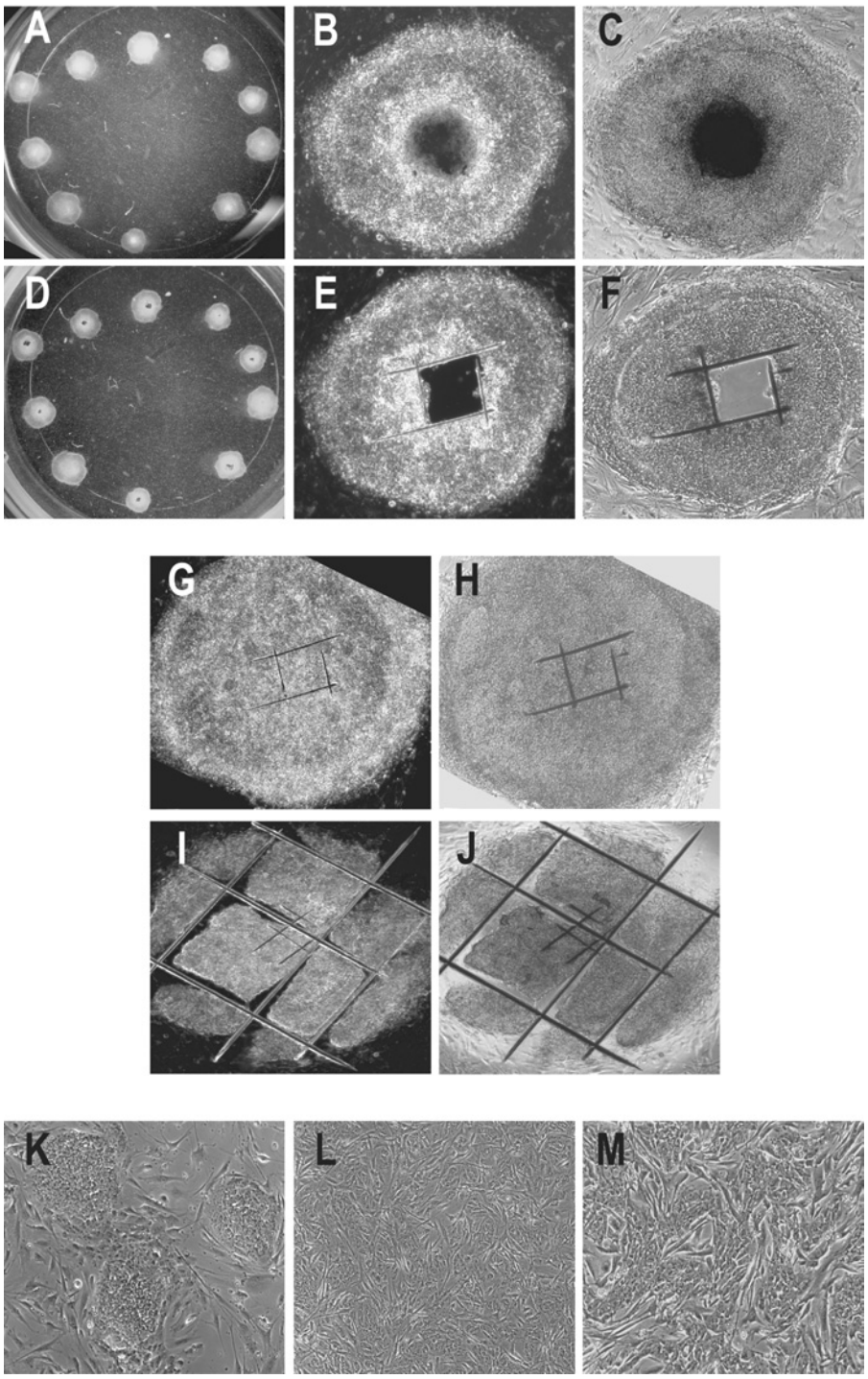

Figure 1C.1.1 Photomicrographs of hESCs during various stages of culture. (**A**) Overview of day 5 hESC colonies grown on MEFs. (**B**) and (**C**) 50× magnification of a single colony under phase-contrast and bright-field visualization, respectively. The colony has a dense raised area. (**D**) Overview of day 5 hESC colonies grown on MEFs with the central raised areas eliminated. (**E**) and (**F**) 50× magnification of a single colony under phase contrast and bright field, respectively with the central raised area eliminated. (**G**) and (**H**) 50× magnification of the same colony shown in (E) and (F) 2 days later (day 7) under phase contrast and bright field, respectively . (**I**) and (**J**) 50× magnification of a day 7 colony under phase contrast and bright field, respectively, sliced into a grid motif prior to dislodgement and transfer to new organ culture dishes. (**K**) 50× magnification of hESCs in bulk culture passage 1. (**L**) 50× magnification of hESC in bulk culture passage 2 after treatment with TrypLE Select. (**M**) 50× magnification of hESCs in bulk culture on feeders at reduced density on day of application.

dishes as dense colonies and passaged once every 7 days based on published methods (Thomson et al., 1998; Reubinoff et al., 2000) with modifications as described.

Materials

hESCs, starting from macroscopic colonies (~1-mm diameter) grown on MEFs (*UNIT 1A.2*)
Mitotically inactivated (irradiation- or mitomycin C–treated) mouse embryonic fibroblasts (MEFs; Nagy et al., 2003; Conner, 2000)
hESC medium (see recipe)

Stereomicroscope
26-G × ½-in. (0.45 × 13–mm) needles
1-ml syringe
60 × 15–mm center-well organ culture dishes

Additional reagents and equipment for hESCs grown on feeder cells (*UNIT 1A.2*) and mitotically inactivated mouse embryonic fibroblasts (Conner, 2000)

Maintain hESCs in organ culture dishes

1. At a time point 2 days before transfer, excise differentiated and/or raised regions within each colony using a 26-G needle attached to a 1-ml syringe (Fig. 1C.1.1, A through F).

   These procedures are performed under a stereomicroscope.

2. On the day before transfer, plate mitotically inactivated MEFs (Conner, 2000) onto the center well of gelatinized organ culture dishes at a density of $6 \times 10^4/cm^2$ in 1 ml MEF medium.

3. On the day of transfer, day 7, using a 26-G needle, cut each colony into a grid motif containing approximately eight pieces per colony and dislodge with the same 26-G needle (Fig. 1C.1.1, G through J).

4. Replace medium in organ culture dishes (prepared in step 2) with 1 ml hESC medium and transfer up to ten pieces into the center well.

5. Change the medium to fresh hESC medium daily.

   Using this method a single organ culture plate containing 10 colonies should yield ~18 organ culture plates after 3 weeks of passaging—a sufficient number to enter the enzymatic passaging protocol described below.

EXPANSION OF hESC IN BULK CULTURE

BASIC PROTOCOL 2

This protocol is used for the large-scale expansion of hESCs. During bulk culture, the cells are enzymatically passaged, up to 25 times, without the acquisition of an abnormal karyotype.

Materials

hESCs in organ culture dishes (Basic Protocol 1)
hESC medium (see recipe)
Trypsin (see recipe) *or* TrypLE Select (Invitrogen)
Phosphate-buffered saline without $CaCl_2$, without $MgCl_2$(CMF-PBS)
Soybean Trypsin Inhibitor (Invitrogen), optional

26-G × ½-in. (0.45 × 13–mm) needles
1-ml syringe
200-µl Gilson pipet (optional)
75-cm^2 flask containing preseeded mitotically inactivated MEFs at a density of $4 \times 10^4/cm^2$, $2 \times 10^4/cm^2$, and $1 \times 10^4/cm^2$ (Connor, 2000)

150-cm^2 tissue culture flask with vented cap
Stereomicroscope

Additional reagents and equipment for mitotically inactivated mouse embryonic fibroblasts (Connor, 2000) growing hESCs in organ culture dishes (Basic Protocol 1), cell counting (Phelan, 2006), and electroporation (Costa et al., 2007)

Expand ES cells in bulk culture

1. Using a 26-G needle attached to a 1-ml syringe, cut colonies from 18 organ culture dishes (~10 colonies/dish) into a grid motif to generate ~25 small pieces per colony.
2. Dislodge the sliced colonies from the dish either with the same needle or with a 200–µl Gilson pipet. Collect these small pieces into a 15-ml centrifuge tube.
3. Centrifuge the pieces 3 min at 480 × *g*, 4°C. Resuspend the entire pellet in 10 ml hESC medium and plate onto a gelatinized 75-cm^2 flask containing irradiated MEFs seeded previously at a density of 4 × 10^4/cm^2 (Fig. 1C.1.1, K).

   This is designated as the first passage in bulk culture. At this stage the cells have not been exposed to any enzyme and are still tightly bound together in small clumps.
4. At a time point 3 days later, wash the hESCs once with 3 ml CMF-PBS.
5. Add 2 ml of trypsin or TrypLE Select and incubate for 5 min at 37°C or until the cells have dislodged from the dishes.

   If trypsin is used as the dissociation agent, then a neutralization step is required such as washing the cells with 10 ml serum-containing medium or adding 1 ml soybean trypsin inhibitor.
6. During these first enzymatic passages, break up hESC colonies by trituration to produce predominantly single cells with some small clumps remaining.
7. After trypsinization, collect the cells by centrifuging 3 min at 480 × *g*, 4°C.
8. Resuspend the cells in 10 ml hESC medium and transfer to a fresh gelatinized 75-cm^2 flask preseeded the day before with MEFs at a density of 2 × 10^4/cm^2 (Fig. 1C.1.1L).

   There is usually extensive cell death associated with this first enzymatic passage (50%). The amount of cell death decreases with subsequent passages.
9. Passage the cells enzymatically biweekly (refer to steps 4 to 8), to ensure that the colonies remain small.

   This generally allows the cultures to be expanded ~1:2 on each passage. For the first time passaging to 150-cm^2 flasks, transfer the contents of a 75-cm^2 flask to one 150-cm^2 flask (1:2 passage)

   By this stage the cell numbers increase rapidly and by passage 4 to 5 there are enough cells for applications such as electroporation, transfection, or differentiation.

Day before application

10. Enzymatically passage cells (refer to steps 4 to 8) and transfer the cells into gelatinized 150-cm^2 flasks containing irradiated MEFs seeded at a density of 1 × 10^4/cm^2.

    A lower MEF density is required for enzymatically passaged hESCs than for those in organ culture. This may be related, in part, to the fact that the MEFs are replaced more often as a consequence of more frequent passaging. As a rule, on the day of application, aim for a semiconfluent flask of cells, i.e., ~8 × 10^6 hESC/150 cm^2.

Day of application

11. In the morning, change the medium on the cells to fresh hESC medium (Fig. 1C.1.1M).

12. Harvest cells using 2 ml trypsin or TrypLE Select (steps 4 to 5) after first washing with CMF-PBS.

    If trypsin is used as the dissociation agent, then a neutralization step is required such as washing the cells with 10 ml serum-containing medium or adding 1 ml soybean trypsin inhibitor.

13. Perform cell count (Phelan, 2006) and subtract total feeder number from the count.

    For example, an area of 150-cm^2 will contain ~1.5 × 10^6 feeders in the total cell count.

14. Use the cells for electroporation (Costa et al., 2007), transformation, or differentiation.

REAGENTS AND SOLUTIONS

For culture recipes and steps, use sterile tissue–grade water. For other purposes, use deionized, distilled water or equivalent in recipes and protocol steps. For suppliers, see ***SUPPLIERS APPENDIX****.*

Gelatin 0.1% (w/v)

Dissolve 0.5 g of gelatin (from porcine skin) in 500 ml distilled water and autoclave. Store at room temperature indefinitely.

Gelatinized flasks/plates

Prior to addition of MEFs, coat all plates and flasks with enough 0.1% (w/v) gelatin solution (see recipe) to cover the surface. Remove gelatin after 5 min.

hESC medium

DMEM/F12 (Invitrogen) containing:
20% (v/v) Knockout Serum Replacement (Invitrogen)
10 mM non-essential amino acids
2 mM L-glutamine
1× penicillin/streptomycin (add from 200× stock, e.g., Invitrogen)
50 mM 2-mercaptoethanol
10 ng/ml bFGF (Amit et al., 2000; see recipe)
Store up to 1 week at 4°C

MEF medium

DMEM containing:
10% (v/v) heat-inactivated fetal bovine serum (FBS)
2 mM L-glutamine
1× penicillin/streptomycin (add from 200× stock, e.g., Invitrogen)
Store up to 4 weeks at 4°C

Recombinant human basic fibroblast growth factor (bFGF)

Resuspend lyophilized bFGF (PeproTech) to a final concentration of 10 μg/ml in CMF-PBS containing 0.1% (w/v) BSA and 1 mM DTT. Store at −80°C according to manufacturer's instructions.

Trypsin, 0.125% (w/v)

Trypsin/EDTA (0.25% trypsin EDTA; Invitrogen, no. 25200-056) supplemented with:
2% (v/v) chicken serum (Hunter Antisera, no. 110)
Dilute 1:2 with CMF-PBS
Store for 4 weeks at 4°C

COMMENTARY

Background Information

ESCs are pluripotent cells that are isolated from the inner cell mass of the blastocyst-stage embryo and can be cultured indefinitely in vitro (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998). Their ability to differentiate into multiple cell types (Evans and Kaufman, 1981; Nagy et al., 1993; Thomson et al., 1998; Reubinoff et al., 2000) makes them a suitable substrate for studies involving drug discovery (McNeish, 2004), human development, and cell therapies (Menendez et al., 2005).

hESCs are often cultured as dense colonies which are mechanically cut into small pieces and transferred from one organ culture dish to another (Thomson et al., 1998; Reubinoff et al., 2000). This method is preferred for long-term maintenance of hESCs as it reduces the level and frequency of stress associated with passaging and reduces the incidence of karyotypic abnormalities. Collagenase Type IV is commonly used for the large-scale passaging of hESCs (Amit et al., 2000); however, because this enzyme usually yields cell clumps, precise cell numbers can only be estimated making this method unsuitable for situations where precise cell numbers or single cells are required. Replacing Collagenase Type IV with either trypsin or TrypLE Select enables hESCs to be passaged as a single-cell suspension. All enzymatic passaging methods eventually select for cells which are adapted to such methods. Over time, such adaptations may include the acquisition of chromosomal aberrations (Draper et al., 2004) that provide a selective advantage to cells grown under these conditions. For this reason it is recommended that enzymatic passaging only be used transiently for the generation of large cell numbers required for experiments rather than as a method for routine long-term hESC maintenance. Uses for enzymatically passaged cells include electroporation of $\sim 1 \times 10^7$ cells, typical spin EB (embryoid bodies) method for differentiation of 3×10^5 hESCs per 96-well plate with 3000 cells per well. Most experiments would use 10 to 30 plates.

Critical Parameters and Troubleshooting

hESCs are cultured using two different techniques, each with different requirements. The two techniques are listed below.

Organ culture: the feeder density influences the thickness of the hESC colony. If the feeder density is too high then the colonies will be very thick and will tend to tear when being cut for passaging. If the feeder density is sparse, the colonies will be thin and the cut pieces will fray when being manually dislodged from the dish during passaging.

Bulk culture: The time it takes to achieve confluence from one passage to the next is influenced by the number and size of colonies present in the initiating culture. Although the hESCs are eventually passaged as a single-cell suspension they reform colonies on the dish as the cells proliferate. The longer these colonies are left to regrow between passages, the harder it is to dissociate them, which in turn leads to higher levels of cell death once passaged. Passaging the cells biweekly regardless of the number of colonies per flask prevents the colonies from becoming too large and difficult to dissociate.

Anticipated Results

This protocol generates large numbers of karyotypically normal hESCs, suitable for numerous applications such as electroporation and differentiation. After 5 bulk (enzymatic) passages, expect $4 \times 150\text{-cm}^2$ flasks each containing $\sim 8 \times 10^6$ hESCs each ($\sim 3.2 \times 10^7$ total).

Time Considerations

hESC colonies from one organ culture dish containing 10 colonies should be able to be distributed among six new dishes. It should take 2 weeks to go from 1 dish to 18. During the third week the colonies on the 18 dishes are mechanically transferred to a single 75-cm^2 flask (passage 1) which is subsequently passaged 3 days later (passage 2) using either trypsin or TrypLE select. By passage 4 (week 4),

there should be sufficient cells to generate two confluent 150-cm^2 flasks. At this stage, cells which are to be used for experiments are passaged onto flasks seeded with MEFS at a reduced density (Passage 5). Alternatively, cells can be enzymatically passaged 20 to 25 times without the appearance of chromosomal abnormalities. Under such circumstances, excess cells generated at each passage can be fed into other applications.

Acknowledgement

We thank Elizabeth Ng for her valuable contribution to the development of the organ culture protocol described in this article.

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Germ Layer Induction in ESC—Following the Vertebrate Roadmap

Jim Smith,[1] Fiona Wardle,[1] Matt Loose,[2] Ed Stanley,[3] and Roger Patient[4]

[1]Wellcome Trust/Cancer Research UK Gurdon Institute, University of Cambridge, Cambridge, United Kingdom
[2]Institute of Genetics, University of Nottingham, Nottingham, United Kingdom
[3]Monash Immunology and Stem Cell Laboratories, Monash University, Clayton, Victoria, Australia
[4]Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, United Kingdom

ABSTRACT

Controlled differentiation of pluripotential cells takes place routinely and with great success in developing vertebrate embryos. It therefore makes sense to take note of how this is achieved and use this knowledge to control the differentiation of embryonic stem cells (ESCs). An added advantage is that the differentiated cells resulting from this process in embryos have proven functionality and longevity. This unit reviews what is known about the embryonic signals that drive differentiation in one of the most informative of the vertebrate animal models of development, the amphibian *Xenopus laevis*. It summarizes their identities and the extent to which their activities are dose-dependent. The unit details what is known about the transcription factor responses to these signals, describing the networks of interactions that they generate. It then discusses the target genes of these transcription factors, the effectors of the differentiated state. Finally, how these same developmental programs operate during germ layer formation in the context of ESC differentiation is summarized. *Curr. Protoc. Stem Cell Biol.* 1:1D.1.1-1D.1.22. © 2007 by John Wiley & Sons, Inc.

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LESSONS FROM FROGS (AND FISH)

Inductive Interactions in the Early Embryo

The most important mechanism by which cell fate is determined in the developing vertebrate embryo involves inductive interactions, in which one group of cells makes a signal that is received, and acted upon, by adjacent tissue. Interactions of this sort are most easily studied in embryos that are accessible to the experimenter, including those of the chick, zebrafish, and frog, and among these species, most insights have come from the frog, *Xenopus laevis*. In this introductory unit the authors summarize these results, discuss how they might apply to other species, including mammalian embryos, and then consider to what extent they might allow the experimenter to manipulate the differentiation of embryonic stem cells (ESC). The unit focuses on early developmental decisions, in which cells become committed first to endoderm, mesoderm, or ectoderm, and then to particular regions of these germ layers, such as dorsal versus ventral mesoderm or neural versus non-neural ectoderm. This is done because it is necessary that cultured ESC successfully negotiate these early decisions before they follow pathways leading to the formation of specialized cell types such as pancreas, heart, or liver.

The first step: Dorsalization of the embryo

Most of the understanding on inductive interactions during early vertebrate development derives from work on the amphibian species, *Xenopus laevis*. *Xenopus* offers many advantages to the developmental biologist: the embryo is large, and therefore easy to inject,

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dissect, and manipulate; it is available in large numbers, and therefore suitable for biochemical approaches; it has, at early stages, a reliable fate map, so that one can reliably interpret experiments that are designed to alter cell fates; and its cells are laden with yolk, so that they can survive in a very simple salts solution in the absence of poorly characterized components such as fetal bovine serum. Together, these advantages have made *Xenopus* the organism nonpareil for the analysis of inductive interactions.

When it is laid, the *Xenopus* egg is a sphere about 1.4-mm in diameter. It has a heavily pigmented animal hemisphere, which comes to lie uppermost in the water, and a paler, yolkier, vegetal half that forms the southern hemisphere. At this early stage, and, as far as is known, in contrast to the mammalian egg, some RNAs are differentially localized within this large cell. Two such RNAs are of particular note. One encodes VegT (also known as Brat, Xombi, and Antipodean), a member of the T-box family of transcription factors (Lustig et al., 1996; Stennard et al., 1996; Zhang and King, 1996; Horb and Thomsen, 1997), and the other encodes Vg1, a member of the transforming growth factor type β (TGF-β) family (Weeks and Melton, 1987). More of these will be discussed later in the unit. For now, suffice it to say that these RNAs move to their vegetal positions during oogenesis, and that their position in this region of the embryo is crucial for normal development.

Immediately after being laid the egg appears, and is, radially symmetrical about the animal-vegetal axis. Polarity is imposed upon the egg at the time of fertilization, when the position of sperm entry defines the side of the egg that will eventually form posterior and ventral structures (Vincent and Gerhart, 1987). The mechanism by which this occurs is not well understood, but it is associated with the rotation, of ∼30°, of a shell of cortical cytoplasm, just beneath the egg plasma membrane (Vincent and Gerhart, 1987). The position of sperm entry defines the orientation of this rotation, and the significance of the rotation for the specification of the anterior/dorsal to posterior/ventral axis is demonstrated by experiments in which the rotation is inhibited. For example, this can be done by irradiating the vegetal hemisphere of the newly fertilized embryo with UV light (Scharf and Gerhart, 1980; Holwill et al., 1987). This prevents rotation and the embryo develops without a head or axial structures. All that is formed is a mass of ventral tissue that includes, for example, large amounts of blood (Cooke and Smith, 1987). The loss of anterior tissue is not due to nonspecific effects of UV irradiation, because rotation can be restored by tipping the fertilized egg to one side, and this completely rescues the embryo, such that it forms a perfectly normal tadpole (Scharf and Gerhart, 1980).

It is not known how rotation specifies dorsal and anterior regions of the embryo, although it is clear that signaling by members of the Wnt pathway is involved. Thus, ectopic expression of members of the Wnt family in the early *Xenopus* embryo causes the formation of an additional head (McMahon and Moon, 1989; Smith and Harland, 1991; Sokol et al., 1991), and inhibition or stimulation of Wnt signaling causes, respectively, the loss or enhancement of anterior/dorsal structures (Heasman et al., 1994; Kofron et al., 2001). Most significantly, depletion of maternal Wnt11 mRNA causes embryos to develop without a head or anterior structures, a phenotype that can be rescued by the re-introduction of exogenous Wnt11 mRNA (Tao et al., 2005). Rotation of the cortical layer of cytoplasm is now thought to reposition maternal Wnt11 mRNA from its original vegetal location to a position closer to the equator of the embryo, where it is free to diffuse into deeper cytoplasm. This allows Wnt11 protein to accumulate in dorsal vegetal cells and to activate the canonical Wnt signal transduction pathway in this region of the embryo. It is also possible that cortical rotation causes the dorsal enrichment of Wnt co-receptors, or components of the Wnt signal transduction pathway, in a mechanism that would further enhance the level of Wnt signaling in this region of the embryo (Dominguez and Green, 2000).

One of the consequences of Wnt signaling in the dorso-vegetal region of the embryo is the activation of the *Siamois* gene, which is involved in the formation of dorsal and axial tissues (Lemaire et al., 1995; Fan and Sokol, 1997; Engleka and Kessler, 2001).

The second step: Mesoderm induction

As is remarked above, there is no evidence yet for localized RNAs or other determinants in the mammalian egg, and indeed the extent to which the early mammalian embryo is patterned at preblastocyst stages remains controversial (Hiiragi et al., 2006; Zernicka-Goetz, 2006). However, at later stages, similarities between amphibian and amniote development are more obvious, and the analysis of mesoderm induction in *Xenopus* has indeed

informed studies of mouse development and of ESC differentiation (Gadue et al., 2006).

The mesoderm of the amphibian embryo derives from the equatorial region of the embryo. Pioneering experiments by Nieuwkoop showed that this germ layer (and part of the endoderm) forms in this position as the result of an inductive interaction in which cells of the vegetal hemisphere (which inherit, for example, Vg1 RNA and protein from the vegetal region of the fertilized egg) act on overlying cells (Nieuwkoop, 1969; Sudarwati and Nieuwkoop, 1971). Nieuwkoop demonstrated this interaction by dissecting cells from the animal pole region of the embryo (which normally become ectoderm), and juxtaposing them with vegetal cells (which normally become endoderm). Individually neither cell population would form mesoderm, but the combination of cells produced large amounts of mesodermal tissue such as muscle, and lineage labeling experiments revealed that the mesoderm derived from the ectodermal tissue, indicating that the inducing signal derives from the endoderm.

Mesoderm inducing factors

Work in the late 1980s and early 1990s revealed that two classes of signaling molecules have the ability to mimic the effect of the vegetal cells. These include members of the fibroblast growth factor (FGF; Slack et al., 1987; Kimelman et al., 1988) and transforming growth factor type β (TGF-β) families (Albano et al., 1990; Asashima et al., 1990; Smith et al., 1990; Thomsen et al., 1990). The most powerful inducers proved to be members of the TGF-β family, and especially activin and the *Xenopus* nodal-related (Xnr) genes (Jones et al., 1995; Joseph and Melton, 1997; Takahashi et al., 2000), and since this time two other related proteins have been characterized as mesoderm-inducing factors: Vg1, whose transcripts are expressed in the vegetal region of the oocyte and egg (Weeks and Melton, 1987; Dale et al., 1993; Thomsen and Melton, 1993; Birsoy et al., 2006), and derrière, which, like activin and the nodal-related genes, is expressed zygotically (Sun et al., 1999a).

When applied to isolated animal pole regions, all of these inducing factors, including members of both the FGF and TGF-β families, can cause the activation of mesoderm- and endoderm-specific genes and the differentiation of mesodermal cell types. One difference between the two families of signaling molecules is that FGF family members induce predominantly ventral and posterior cell types, and fail to induce the expression of genes that are expressed in prospective anterior and dorsal tissues, such as *goosecoid* (Green et al., 1990). In contrast, the members of the TGF-β family tend to induce a wide spectrum of tissues, from ventral and posterior to dorsal and anterior, and they do this in a concentration-dependent manner, with lower concentrations inducing posterior/ventral tissues and high doses inducing anterior and dorsal structures (Green and Smith, 1990; Green et al., 1992; Green, 1994; Gurdon et al., 1994, 1995, 1996). These dose-dependent effects of the TGF-β family are discussed below.

The FGF and the TGF-β families employ different signal transduction pathways, the former inducing mesoderm through the MAP kinase pathway (Gotoh et al., 1995; LaBonne et al., 1995; Umbhauer et al., 1995) and the latter through the Smad family (Hill, 2001). Limited knowledge of the transcriptional regulation of known FGF and TGF-β target genes (Watabe et al., 1995; Howell and Hill, 1997; Latinkic et al., 1997; Howell et al., 1999; Germain et al., 2000; Lerchner et al., 2000) provides a molecular basis for the difference in the inducing activities of the two classes of signaling molecules. Most significant, however, is the conclusion confirmed by one of the first experiments to employ transgenesis in *Xenopus* species that the role of FGF signaling is to maintain mesodermal identity rather than to induce it (Kroll and Amaya, 1996).

Concentration-dependent effects of inducing factors

It is interesting that members of the TGF-β family are able to induce different types of mesoderm, and to activate the expression of different mesoderm- and endoderm-specific genes, at different concentrations. Unfortunately, rather little is known about the mechanism through which this occurs. Work by Gurdon and colleagues has shown that 100 molecules of activin bound to a single animal pole cell are sufficient to induce expression of the pan-mesodermal gene *brachyury*, while 300 molecules are required to extinguish *brachyury* and to activate *goosecoid* (Dyson and Gurdon, 1998). It is also known that protein synthesis is required for the downregulation of *brachyury* expression that occurs at high doses of activin, suggesting that these high doses of a TGF-β family member induce a repressor of *brachyury* (Papin and Smith, 2000). One candidate for such a repressor is Goosecoid itself, and indeed mutation of a Goosecoid binding site in a 381-base

pair *brachyury* promoter fragment prevents the down-regulation of a *brachyury* reporter construct that occurs at high activin concentrations (Latinkic et al., 1997). However, it is unlikely that Goosecoid is the only repressor of *brachyury* that is induced at high levels of activin, because inhibition of Goosecoid activity does not affect the down-regulation of endogenous *brachyury* that occurs at high activin concentrations (Papin and Smith, 2000). This phenomenon requires further investigation, because, as is discussed below, it is likely that pattern formation in the developing embryo occurs in response to gradients of TGF-β family members, and attempts to direct ESC differentiation must take account of the concentrations of the inducing factors that are used.

The most direct evidence that gradients of inducing factors activate different genes in different regions of the embryo, and specify different cell types, comes from experiments in which the functions of the genes are inhibited. Developmental biologists know of eight TGF-β family members that are expressed in the embryo and that have mesoderm-inducing activity: Vg1, activin, Xnr1, Xnr2, Xnr4, Xnr5, Xnr6, and derrière (see references above). It has not yet proved possible to inhibit the action of all of these factors individually, but antisense and dominant-negative approaches have demonstrated that the maternally expressed gene *Vg1* (Birsoy et al., 2006), and the zygotically activated *activin* (Piepenburg et al., 2004) and *derrière* (Sun et al., 1999a) are all required for proper mesoderm formation. Of the nodal-related genes, only *Xnr1* has been studied individually (Toyoizumi et al., 2005), and it proves to be required for proper specification of the left-right axis of the embryo. However, simultaneous inhibition of all the *Xenopus* nodal-related genes by increasing expression of a truncated form of *Cerberus*, termed *Cerberus-short*, causes the progressive loss of dorsal and then ventral gene expression, consistent with the idea that gradients of these TGF-β family members specify cell types in the developing embryo (Agius et al., 2000).

It is not yet clear how such gradients are established or how the inducing factors traverse fields of responding cells, although the higher expression of the nodal-related genes at the dorsal side of the embryo is thought to derive from the enhanced VegT, Vg1, and Wnt signaling in this region of the embryo (Agius et al., 2000), and it seems likely that inducing factors travel in the extracellular milieu rather than through an intracellular route such as transcytosis (Williams et al., 2004).

Refining the pattern: Inhibiting BMP signaling

Different concentrations of TGF-β family members, such as activin and the nodal-related genes, can establish differences between the prospective anterior/dorsal and posterior/ventral regions of the late blastula and early gastrula regions of the embryo. These differences, however, are rather crude. Dissection of tissue from different regions along this axis of the embryo reveals that there are only two well-defined domains, a smaller one on the dorsal side of the embryo, where the dorsal lip of the blastopore will appear, and a larger one that comprises the rest of the equatorial region of the embryo (Dale et al., 1985). Culture of the former region reveals that this is specified to form notochord and muscle, while the latter region forms predominantly blood. Significantly, these results stand in contrast to the fate map of the embryo at this stage, which shows that most of the muscle of the embryo derives from the larger, ventral, region of the prospective mesoderm (Dale and Slack, 1987).

How can these observations be reconciled? Further grafting experiments show that the region of the embryo that will form the dorsal lip of the blastopore is the source of signals that can dorsalize adjacent ventral mesoderm (Smith and Slack, 1983). Indeed, if this region, known as Spemann's organizer, is grafted to the ventral region of a host embryo, the host develops a secondary axis on its ventral side (Spemann, 1938). Interactions between Spemann's organizer and the rest of the embryo refine the spatial patterns of gene expression and cell differentiation along the anterior/dorsal to posterior/ventral axis.

Identification of the signals produced by Spemann's organizer that are responsible for dorsalization came from several types of experiments. Expression cloning identified the gene *noggin* (Smith and Harland, 1992), which was capable of rescuing embryos that had been ventralized by treatment with UV light (see above). An in situ hybridization screen showed that *chordin* is expressed in the dorsal region of the embryo, with subsequent experiments revealing that it too had dorsalizing activity (Sasai et al., 1994); and analysis of *follistatin*, whose gene product was already known to inhibit activin signaling, showed that this gene is also expressed dorsally and is capable of dorsalizing ventral mesoderm (Hemmati-Brivanlou et al., 1994). All of these factors have in common the fact that they can

inhibit signaling by bone morphogenetic proteins (BMPs), factors which previously had been shown to have powerful ventralizing activity in the *Xenopus* embryo (Dale et al., 1992; Jones et al., 1992), and subsequent work demonstrated that these inhibitors can set up a gradient in the embryo, high in the anterior/dorsal region and low in posterior/ventral tissues (Jones and Smith, 1998). BMP family members such as BMP4 are expressed in a widespread fashion throughout the embryo (with the exception of the dorsal region; Hemmati-Brivanlou and Thomsen, 1995), so that the gradient of BMP inhibitors establishes a reverse gradient of BMP activity. It is this gradient that creates positional information along the anterior/dorsal to posterior/ventral axis of the embryo.

Like Vg1, activin, the Xnr proteins, and derrière, BMPs are members of the TGF-β family, but they differ because they signal through different cell surface receptors and different members of the Smad family. Activin and the other mesoderm-inducing factors signal through Smad2 or Smad3, which form heteromeric complexes with Smad4; BMPs signal through Smad1, Smad5, and Smad8, which also form complexes with Smad4 (Hill, 2001; Schier, 2003).

The organizer produces many inhibitors

The discovery of noggin, chordin, and follistatin as factors that pattern the anterior/dorsal to posterior/ventral axis of the embryo by inhibiting BMP signaling was quickly followed by the identification of other inhibitors of extracellular signaling molecules. These include Frzb-1 (Leyns et al., 1997), a secreted protein containing a domain similar to the putative Wnt-binding region of the frizzled family of transmembrane receptors, and Dickkopf-1 (Dkk1; Glinka et al., 1998), a ligand for the Wnt co-receptor LRP6. Both of these molecules inhibit signaling by Wnt family members, and have the effect of inhibiting the ventralizing effects of proteins such as Wnt8, which is expressed throughout the lateral and posterior/ventral regions of the early gastrula-staged embryo. They therefore establish an effective reverse gradient of Wnt signaling, further refining positional information along the dorso-ventral axis of the embryo.

Perhaps the most remarkable inhibitor produced by Spemann's organizer, however, is Cerberus, which inhibits the activities of BMP, Wnt, and nodal-related signaling (Bouwmeester et al., 1996; Glinka et al., 1997; Piccolo et al., 1999). Ectopic expression of Cerberus in the *Xenopus* embryo causes the formation of an extra head (Bouwmeester et al., 1996), indicating, remarkably, that formation of the head does not require special or novel inducing activities but rather the inhibition of several signaling pathways.

Later inductive interactions and implications for embryonic stem cells

This description of the induction and patterning of the mesoderm has emphasized (1) the importance of intercellular signaling by secreted polypeptide growth factors, (2) the fact that these molecules can exert concentration-dependent effects, and (3) the observation that effective gradients of such growth factors can be established by reverse gradients of secreted inhibitors. These principles also apply to later stages of embryonic development. For example, neural induction by Spemann's organizer in the gastrula-stage embryo is largely a consequence of the inhibition of BMP signaling in the prospective ectoderm (Harland, 1994), together with contributions from FGF and probably other signaling families (Launay et al., 1996; Sasai et al., 1996; Linker and Stern, 2004). At later stages and in other tissues, other families of signaling molecules also play a role, including hedgehog and notch signaling. A thorough knowledge of these signaling events will be invaluable in coming to understand the ways in which one can direct and influence ESC differentiation.

Integration of Intracellular Responses to Embryonic Induction

The distinct combinations and levels of embryonic signals experienced by different cells in the embryo differentially induce the expression or activities of transcription factors (TFs); and the particular combination of TFs active in a given cell determines its phenotype. The responses of TFs to embryonic signals together with interactions between TFs constitute a genetic regulatory network (GRN), describing the state of a given cell at a particular time (Loose and Patient, 2004; Koide et al., 2005). To understand germ layer induction, it is necessary to fully understand the construction, dynamics, and stability of these networks as differentiation proceeds.

Here, because of the volume of data available, the authors concentrate on mesendoderm induction in *Xenopus*, but much of what has been found in *Xenopus* is the same or very similar in zebrafish, making it very likely that the fundamental mechanisms discussed here

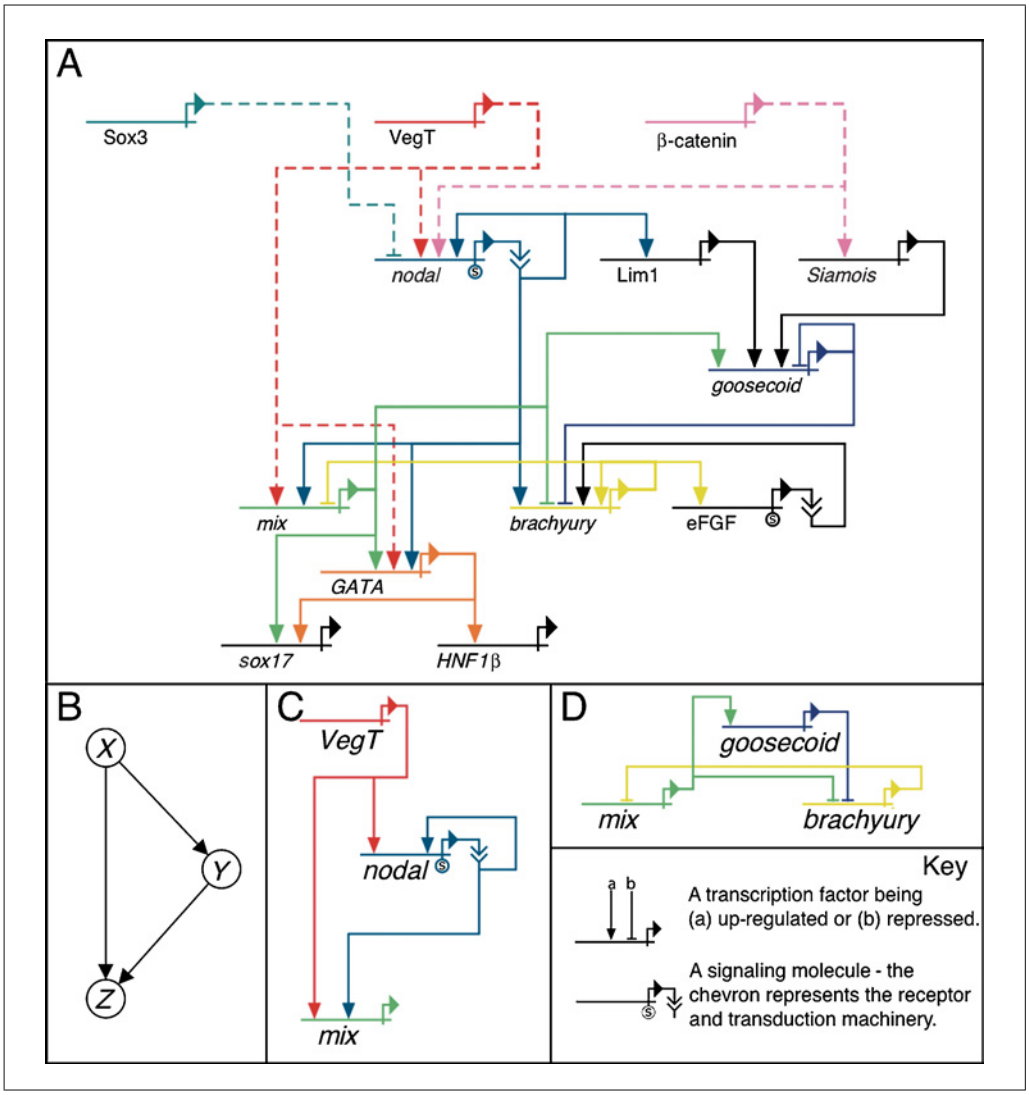

Figure 1D.1.1 Legend at right.

serve as a model for vertebrate germ layer formation generally. In vertebrates, the three primary germ layers, ectoderm, mesoderm, and endoderm, are classically regarded as the first signs of differentiation in the embryo proper, with differentiation to extra-embryonic tissues taking place concomitantly. However, a number of observations suggest that the initial separations may not be as clean as previously thought (Rodaway and Patient, 2001; Wardle and Smith, 2004). In addition, subdivisions within the mesoderm become apparent in some cases before mesodermal and endodermal fates have been distinguished. Thus, skeletal muscle and notochord progenitors become distinguishable from endodermal precursors before blood and cardiovascular progenitors. Observations of this nature have led to the use of the term mesendoderm, and comparisons with more primitive organisms indicate that the distinction may in fact be quite ancient.

In order to begin to appreciate the implications of the *Xenopus* network for our understanding of ESC programming, the authors have built a simplified version of this network, which takes account of reduced gene numbers in mammals and illustrates the key principles (Fig. 1D.1.1A). GRNs are composed of many smaller networks, termed motifs (for a review see Lee et al., 2002 or Babu et al., 2004). These motifs occur more often than would be expected in a random network with the same degree of connectivity as the network under study. Such motifs are easiest to identify computationally, and the program mFinder has been used to identify motifs in a recent update of the *Xenopus* mesendoderm network used (Milo et al., 2002; Loose and Patient, 2004). The complete network is scale-free, that is, all genes do not display equal connectivity. Some of the TFs studied to date display substantially more connectivity than others making them potentially master regulators. The following

will highlight some of these and the motifs found in the *Xenopus* GRN, and will try to draw out the lessons to be learned for ESC differentiation.

Feed forward loops

The most striking thing about mesendoderm formation in *Xenopus* is the extent to which the maternal T-box TF, VegT, dominates proceedings (Zhang et al., 1998; Xanthos et al., 2001; Taverner et al., 2005; Heasman, 2006). Of 54 genes currently in the network, 26 are regulated directly by VegT. A clue to its function in the network emerges when considering which network motifs feature VegT. VegT features heavily in a common motif, the feed forward loop (see Fig. 1D.1.1B). Feed forward loops consist of three genes, *X*, *Y*, and *Z*, where *X* regulates *Y* and *Z*, and *Y* regulates *Z* (Milo et al., 2002). Studies of the properties of feed forward loops reveal two likely functions: either accelerated or delayed expression of the target *Z*, depending on the nature of the interactions between the three genes (activating or repressing) and the way in which the *X* and *Y* genes interact at *Z* (both or either required for expression; see Mangan et al., 2003). Of the 63 such feed forward motifs identified in the *Xenopus* network, 41 are initiated by VegT. Furthermore, the majority of these 41 motifs are of the form that would be expected to accelerate the onset of expression of the target. The targets of the feed forward motifs in the *Xenopus* GRN include both mesodermal and endodermal TFs, suggesting that cells in which maternal VegT is expressed have the potential to become both mesoderm and endoderm, i.e., a molecular demonstration of their mesendodermal potential. Of the remaining 22 feed forward loops identified in the updated *Xenopus* mesendodermal network, 13 are initiated by nodal-related signals, 5 by β-catenin, driven maternally by Wnt11 signaling (Tao et al., 2005), and 4 by Mix family TFs or GATA factors.

Thus, in the *Xenopus* embryo, the localization of maternal determinants is a crucial aspect of mesendoderm formation. The significance of this for programming ESCs remains to be seen. For example, even though zebrafish express a maternal T-box TF, its activities have not yet been demonstrated to be equivalent to VegT in *Xenopus* (Bjornson et al., 2005), so it is possible that this is a derived characteristic of *Xenopus*. Alternatively, the loss of such a wide range of activities might be a derived characteristic of zebrafish, in which case, one should be looking for the distribution of T-box transcription factors in ESCs and determining the extent to which they

Figure 1D.1.1 (*at left*) (**A**) A reduced network representing the key interactions occurring within the *Xenopus* mesendoderm network at the start of gastrulation (approximately stage 10.5; Loose and Patient, 2004). In order to reduce the complexity of the network, the authors have combined the interactions of key gene family members into one representative gene. Thus, the *nodal* family members (and derrière) are combined into one *nodal* gene, the *mix* family members into one *mix* and the *GATA* genes into one *GATA* gene. For the *nodal* and *mix* families, this reduction reflects the fact that there is only one *mix* and one *nodal* homolog in mouse and human. In contrast, the reduction of the *GATA* family into one representative reflects the difficulty of distinguishing the targets of individual GATA factors at this time, although recent work has begun to make some headway (Afouda et al., 2005). The interactions shown in the diagram represent the combined input and output of all family members in the original network. No explicit conflicts were found within families (i.e., one family member positively regulating, another negatively), although for some interactions, individual members have been shown to regulate a subset of the targets of the whole family. In these cases the authors have assumed that the ability to regulate a given target has been lost by the individual family member as opposed to having been gained by several family members. In order to clearly represent the topology of the network, genes that do not feed back into the network have not been shown. The exception to this is the two targets of the *GATA* family, *sox17* and *HNF1*β. At later time-points these genes will feed back into the network. *sox17* and *HNF1*β also represent endoderm within the network. Dashed lines illustrate interactions that are initiated by a maternal message, although later, zygotic expression will take over. Examples of feed forward loops (B) can be traced in this network, for example *VegT* to *nodal* to *mix* (illustrated in C) and *mix* to *GATA* to *sox17*. (**B**) Illustration of a feed forward motif. Gene *X* activates both *Y* and *Z*, gene *Y* activates *Z*. A feed forward loop can have any combination of activation and repression, resulting in 8 different sub-classes of feed forward loop. (**C**) A sample feed forward loop involving *VegT*, *nodal*, and *mix*. Note that *nodal* is able to autoregulate and can therefore maintain *mix* expression in the absence of *VegT*. (**D**) This motif is extracted from panel A and shows the antagonism between *mix* and *brachyury*. In addition, g*oosecoid* is activated by *mix* and in turn repressed *brachyury* expression.

correlate with mesendoderm differentiation. In the absence of a maternal T-box TF, expression of a T-box TF could be induced by FGF as seen for Brachyury and FGF4 (eFGF) in the *Xenopus* mesendoderm network (Isaacs et al., 1994; Casey et al., 1998; see below). Thus, in the absence of a maternal T-box transcription factor with the full range of activities in ESCs, FGF signaling may play a greater role in establishing the mesoderm and the endoderm. As discussed below, nodal-related signaling is often downstream of VegT and the nodal-related family members share many of the targets of VegT. Therefore, another possible substitute for a maternal T-box determinant could be nodal signaling itself. Finally, in the case of maternal β-catenin, whose nuclear localization is driven by maternal Wnt11, the evidence suggests that this pathway is intact in ESCs (Lindsley et al., 2006).

Autoregulation

An important consideration when driving a cell along a specific differentiation pathway is how the cell will maintain the new pathway once the driving stimulus has been removed. In addition to the example of FGF and Brachyury mentioned above, the key initial driver of the *Xenopus* mesendoderm network, VegT, drives expression of the nodals, of which Xnr1, 2, and 4 can positively autoregulate (Osada et al., 2000; Cha et al., 2006). Of the 63 feed-forward motifs identified thus far, 27 involve a nodal family member in the Y position of the motif (see Fig. 1D.1.1B,C). Thus, the initial activating push that is provided by VegT is gradually replaced by nodal signaling, and this signal has the ability to maintain its expression and the pathway remains active as VegT is depleted. Thus, autoregulation is another key motif in genetic regulatory networks contributing to cell memory and forward momentum. Buffering of positive autoregulation is provided by negative regulators, such as antivin/lefty, itself a target of nodal signaling (Cha et al., 2006).

Cross-antagonism

An important concept in differentiation, in addition to activation of specific programs, is the shutting down of alternative pathways. A control motif unique to cells that have to make lineage decisions is cross-antagonism. In the most complete examples, this motif has two master regulators each trying to drive different programs, with each regulator positively autoregulating its own expression, providing a driving force for that particular program. In addition to this, each factor antagonizes the other's activities and so inactivates the alternative pathway. Examples of this can be found in lineage decisions in the blood. Here, GATA-1 drives the erythroid/megakaryocyte program and the Ets factor, Pu.1, drives other myeloid outcomes (Nerlov et al., 2000; Zhang et al., 2000; Swiers et al., 2006). This switch can be modified by altering the levels of the master regulators (Galloway et al., 2005; Rhodes et al., 2005).

The closest motif with characteristics of cross-antagonism in the *Xenopus* mesendoderm network involves Mix.1 and Brachyury. These TFs repress each other's expression and likely in part underpin the choice between endoderm and mesoderm from the mesendodermal layer (Lemaire et al., 1998). Although there is no evidence for direct autoregulation of Mix.1 or Brachyury, the autoregulatory loop between Brachyury and FGF4 provides this function. The apparent absence of autoregulation on the Mix.1/endoderm pathway may be compensated for by the role of Goosecoid, which throws the switch towards Mix.1/endoderm, because it represses Brachyury and is itself downstream of Mix.1 (see Fig. 1D.1.1D; Latinkic et al., 1997; Germain et al., 2000). This relationship between Goosecoid and Brachyury likely also underpins the anteroposterior patterning of the mesoderm, with continued Brachyury expression being restricted to the posterior mesoderm by Goosecoid repression. Mix.1 favors endoderm differentiation by driving expression of GATA factors and Sox17. Both factors drive expression of endodermal genes throughout the future endoderm in *Xenopus* and zebrafish, and, later in the anterior of the embryo. Sox17 also regulates Goosecoid positively, thereby reinforcing the endodermal lineage decision there (Latinkic and Smith, 1999; Sinner et al., 2004, 2006; see below).

Both Mix.1 and Brachyury are downstream of the same nodal-related signaling factors and are initially found co-expressing in the same cells (Lemaire et al., 1998), suggesting that cells are initially programmed with the potential to differentiate into either germ layer and subsequent downstream interactions select one germ layer or another. As already discussed, the choice of germ layer is determined by the combination or concentration of the inducing signals. Thus, individual cells within the mesendoderm may have indistinguishable molecular phenotypes reflecting the broad potential of these cells.

Network states

Microarray based profiling of gene expression in embryos is revealing new interactions important for endoderm and mesoderm specification, and these will need to be incorporated into the GRN (Dickinson et al., 2006; Sinner et al., 2006). However, it is already clear that GRNs are four dimensional, with levels of individual transcription factors varying in both space and time between, and possibly also within, fields of cells. In vivo the network of an individual differentiating cell will pass through various intermediate states and there may be a limited number of ways of constructing the stable network responsible for the phenotype of a specific cell type. A better understanding of these phenomena in developing embryos will likely enable enhanced control over ESC differentiation.

Direct Target Genes

The activity of early regulatory transcription factors must be translated into downstream responses, such as the cell movements of gastrulation and the onset of cell differentiation. This is achieved through regulating the expression of downstream targets, which may themselves be regulatory factors or signals giving rise to GRNs as discussed above, or may be direct effectors of cell movement or differentiation. Below the authors discuss some direct targets and the roles they play in mediating the activities of a few of these early regulatory transcription factors in mesendoderm.

Mesoderm

Direct targets of Brachyury

Brachyury is expressed throughout the mesoderm of early vertebrate embryos as an immediate response to mesoderm induction, and it is required for many aspects of mesoderm formation. Inhibition of Brachyury function and mutations in the gene reveal that its orthologs in mouse, frog, and zebrafish are required during gastrulation for normal morphogenetic cell movements, for notochord and posterior mesoderm formation, and for the establishment of left-right asymmetry (Smith, 2001; Amack and Yost, 2004). Moreover, ectopic activation of *Xenopus* Brachyury in *Xenopus* ectodermal explants (animal caps) is sufficient to induce ventral and lateral mesodermal cell fates in those cells (Cunliffe and Smith, 1992). Double mutants uncover additional roles for Brachyury that are not evident in the single mutant due to redundancy with other factors. For instance double mutants in *ntl* (no tail, the zebrafish Brachyury homolog) and *spt* (*tbx16*) or zygotic *oep* reveal a role for Brachyury in trunk somite and blood formation in zebrafish (Schier et al., 1997; Amacher et al., 2002).

Brachyury exerts its effects on mesodermal cells both through regulating cell fate, as shown by down regulation of target genes when Brachyury activity is decreased or absent, and through regulating cell movements during gastrulation, because cells do not move to their correct positions in *brachyury* mutant embryos. Research over the last decade or so has also identified a handful of direct targets of Brachyury in *Xenopus*, including Xwnt11, Vent2b, and FGF4 that mediate both cell fate choices and cell movement (Casey et al., 1998; Tada et al., 1998; Tada and Smith, 2000; Messenger et al., 2005). For instance, Wnt11, acting through the noncanonical Wnt pathway, regulates the convergent extension movements of gastrulation in both *Xenopus* and zebrafish (Heisenberg et al., 2000; Tada and Smith, 2000). Vent2b, on the other hand, is a transcriptional repressor that plays a role in BMP-mediated specification of ventral and paraxial mesoderm, at least in part through its ability to repress *goosecoid* expression (Ladher et al., 1996; Onichtchouk et al., 1996; Schmidt et al., 1996; Trindade et al., 1999; Melby et al., 2000). Brachyury binds the promoter of Xvent2b in vivo, and probably does so in combination with Smad1, a transducer of BMP signals, in ventral lateral mesoderm (Messenger et al., 2005).

FGFs are involved both in regulating gene expression and cell movement. Inhibition of FGF signaling causes loss of posterior somites and notochord, and defects in gastrulation movements in *Xenopus* and zebrafish (Amaya et al., 1993; Griffin et al., 1995). Similarly, FGF signaling is required for normal mesoderm formation in mouse embryos (Deng et al., 1994; Yamaguchi et al., 1994). Several different FGFs are expressed in the mesoderm of *Xenopus* (*fgf3*, *fgf4* [*efgf*], and *fgf8b*) and zebrafish (*fgf8*, *fgf17*β, and *fgf24*) and act in combination to regulate gene activity which patterns and maintains mesoderm (Furthauer et al., 1997; Fisher et al., 2002; Draper et al., 2003; Fletcher et al., 2006b). For instance, FGF4 and Brachyury are involved in a positive autoregulatory feedback loop (Isaacs et al., 1994; Casey et al., 1998), and both FGF4 and FGF8b regulate MyoD expression (Fisher et al., 2002; Fletcher et al., 2006b). Inhibition of FGF signaling also causes defects in cell movement during gastrulation, and

evidence in chick embryos suggests that different FGFs can act as chemoattractants or chemorepellents to ensure that migrating mesodermal cells arrive at their correct destination during gastrulation (Yang et al., 2002).

Interestingly, target genes of Brachyury that directly mediate notochord differentiation have yet to be isolated. Furthermore, as mentioned above, during the early stages of mesendoderm formation, Brachyury is expressed in cells that will eventually become endoderm, although expression resolves into mesodermal cells as development proceeds (Rodaway et al., 1999; Wardle and Smith, 2004). Similarly Brachyury-expressing cells in embryoid bodies have the potential to go on and form both mesoderm and endodermal lineages (Fehling et al., 2003; Kubo et al., 2004). Given this, it is possible that Brachyury also regulates the expression of early endodermal genes, although targets are yet to be identified.

Endoderm

Direct targets of Sox17

In *Xenopus*, *sox17* alleles (there are three) are required in combination for normal endoderm formation, while in zebrafish, Sox17 and the related factor, Sox32/Casanova, both play a role in endoderm formation, with Casanova acting upstream of *sox17*. Inhibition of all Sox17 activity in *Xenopus* leads to downregulation of endodermal markers, abnormal gut formation, and inhibition of gastrulation movements (Clements and Woodland, 2000). Similarly, mouse embryos null for *sox17* have defects in gut formation (Kanai-Azuma et al., 2002).

Direct targets of Sox17 in the gastrula stage *Xenopus* embryo include *endodermin*, *HNF1*β, *foxa1*, and *foxa2* which are expressed in the endoderm of early embryos (Clements and Woodland, 2003; Ahmed et al., 2004; Sinner et al., 2004). *foxa2* is also expressed in the floorplate of the neural tube, and mutants in zebrafish *foxa2* (*monorail*) show a defect in floor plate differentiation (Norton et al., 2005). On the other hand, inhibition of *Xenopus* Foxa2 in ventral tissues using an engrailed repressor construct causes expression of dorsoanterior mesendodermal markers and some decrease in anterior structures, while ectopic expression of *foxa2* in *Xenopus* inhibits the expression of mesoendodermal markers and causes severe defects in gastrulation (Suri et al., 2004). The difference in phenotypes in *Xenopus* and zebrafish may be due to the engrailed repressor construct inhibiting additional fox-related factors, the *monorail* mutation not being a complete null, and/or other factors taking over the role of *foxa2* in mutant zebrafish. Inhibiting *HNF1*β activity in *Xenopus* using an engrailed repressor construct inhibits mesoderm induction by vegetal explants and causes defects in mesoderm formation if localized to the marginal zone, while inhibition or augmentation of *HNF1*β activity in *Xenopus*, using mutated human alleles, leads to defects in pronephros formation (Vignali et al., 2000; Wild et al., 2000). Hence, although these downstream targets play some role in aspects of mesendoderm formation, it is not clear from these experiments which aspects of Sox17 activity they are involved in, and it is evident that more targets remain to be isolated.

Targets of GATA factors

In *Xenopus* and zebrafish GATA 4 to 6 mediate endoderm formation. Ectopic expression of GATA 4, 5, or 6 in *Xenopus* animal caps induces endodermal markers, such as *sox17*β and *HNF1*β, while knock down of GATA 5 and 6 activity in the whole embryo using antisense morpholinos leads to defects in gut morphology (Weber et al., 2000; Afouda et al., 2005). In zebrafish, mutations in *gata5* (*faust*) lead to a decrease in early endodermal markers and subsequent defects in gut morphogenesis (Reiter et al., 1999, 2001).

Little is known about direct targets of GATA factors, although experiments in which protein synthesis was inhibited suggest that GATA6, and to some extent GATA5, is able to directly activate expression of *sox17*β and *HNF1*β in *Xenopus* (Afouda et al., 2005). GATA factors are also involved in migration of the leading edge mesendoderm across the blastocoel roof during gastrulation (Fletcher et al., 2006a), although the direct targets that mediate this activity are not known.

Identification of direct targets

Clearly, in order to better understand how the actions of regulatory transcription factors induced in response to mesendoderm-inducing signals are translated into cell movements and the onset of cell differentiation, it is necessary to have a better understanding of the direct targets that mediate those processes, and particularly those targets that directly affect those processes.

One method of identifying direct targets is by a candidate approach. For instance, subtractive screens or, more recently, microarray experiments can be used to identify genes

whose expression is altered in response to activation or inhibition of a transcription factor (Saka et al., 2000; Taverner et al., 2005). Using protein synthesis inhibitors, such as cycloheximide, one can then identify whether these genes are directly regulated transcriptional targets of that factor (Taverner et al., 2005).

Another, more high-throughput method of identifying directly bound targets of a particular transcription factor, or other DNA binding protein, in embryos or ESCs is chromatin immunoprecipitation combined with genomic microarrays (Taverner et al., 2004; Boyer et al., 2005; Wardle et al., 2006). In this method, often known as ChIP-chip or location analysis, cells or embryos are treated with formaldehyde to cross-link the proteins to DNA. The DNA is then fragmented (ranging in size from ~0.2 to 2 kb) and antibodies to the factor of interest are used to immunoprecipitate it and identify the DNA bound to it in vivo. The isolated DNA is then amplified, labeled, and hybridized to a microarray containing genomic promoter sequences. Additional methods to identify genomic sequences directly bound by a factor that have been used in other systems such as *Drosophila* embryos, *C. elegans*, or mammalian cell lines include Dam-ID (van Steensel et al., 2001; Greil et al., 2006) and ChIP-cloning (Weinmann et al., 2001; Oh et al., 2006) or ChIP coupled with pair-end ditag sequencing (Wei et al., 2006). Cloning and sequencing the isolated fragments has the advantage of identifying all genomic regions that are associated with the factor, whereas currently genomic microarrays for vertebrate organisms contain only a subset of the genome such as sequences around the gene promoter, although this is likely to change as new technologies allow larger whole genomes to be studied.

These powerful direct genomic binding approaches will eventually lead to the identification of all the direct targets of each of the regulatory TFs involved in germ layer formation. These data will enhance the GRNs described above and allow for a more complete understanding of the control circuits of differentiation. They will in addition eventually identify the end game of each differentiation pathway with a complete readout of which regulatory TFs drive each of the terminal differentiation products. Together with the information on the embryonic signals that control the activities of these regulatory TFs, the ability to control differentiation will be substantively improved.

GERM LAYER INDUCTION DURING EMBRYONIC STEM CELL DIFFERENTIATION

Developmental Potential of ESCs

ESCs are pluripotent immortal cells derived from the inner cell mass of the preimplantation mammalian blastocyst (Evans and Kaufman, 1981; Martin, 1981). The clearest demonstration that ESCs are capable of generating all of the cell types found in the adult comes from experiments in which normal fertile mice composed entirely of ESC-derived cells were generated by aggregating clusters of ESCs with tetraploid embryos (Nagy et al., 1993). In these experiments, instructive signals from the tetraploid embryo–derived extraembryonic tissues initiated a cascade of developmental programs within the ESC aggregates, culminating in the formation of a complete animal. Similarly, the application of exogenous instructive signals to aggregates of ESCs in vitro also initiates a cascade of differentiation resulting in the generation of cells representing multiple tissue types.

Developmental Congruence and Directed Differentiation

The factors governing establishment of the embryonic germ layers, ectoderm, mesoderm, and endoderm, have been determined from embryological studies, predominantly using model systems such as zebrafish and *Xenopus* (Kimelman and Griffin, 2000; Loose and Patient, 2004; Tam et al., 2006). These factors form part of an evolutionarily conserved genetic regulatory network that coordinates gene expression, cell movement, and differentiation. Within these networks, specific genes mark or regulate sequential embryonic stages as cells pass through a series of progressive developmental restrictions culminating in their irreversible commitment to the germ layers, ectoderm, endoderm, and mesoderm. These same steps can be observed during the early phases of ESC differentiation, with cells sequentially expressing genes representing inner cell mass, epiblast, primitive streak, mesoderm, and endoderm (Hirst et al., 2006). Likewise, the commitment of ESC differentiation to a particular developmental program can be precipitated by the same extracellular factors found to be important for the execution of corresponding programs within the embryo (see Fig. 1D.1.2). This correspondence between what happens in the embryo and what happens during ESC differentiation in vitro

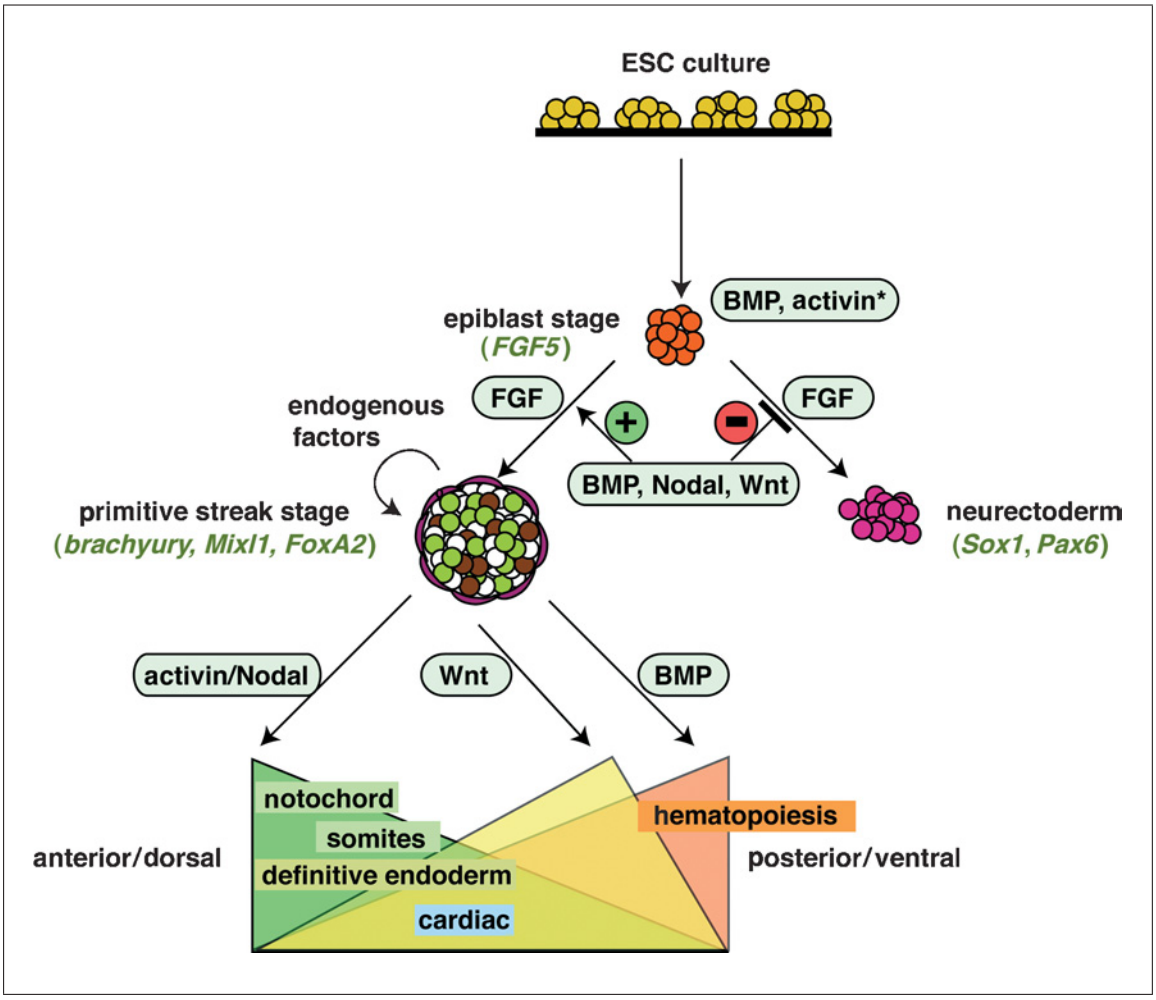

Figure 1D.1.2 Schematic representation of key steps and factors involved in the specification of the germ layers from differentiating ESCs. BMP and Activin-like signals are required for expansion of cell numbers as cells traverse the in vitro equivalent of an epiblast stage (Mishina et al., 1995; Song et al., 1999), marked by expression of *FGF5*. In the absence of BMP, Wnt, or Nodal signals, epiblast cells adopt a default neurectodermal fate marked by expression of genes including *Sox1* or *Pax6*. In the presence of BMP, Nodal, or Wnt signals, cells commit to the in vitro equivalent of primitive streak formation, marked by expression of genes such as *brachyury*, *Mixl1*, and *FoxA2*. FGF signals may play a role in both the neurectodermal and mesendodermal differentiation pathways (Dell'Era et al., 2003; Sun et al., 1999b; Ying et al., 2003), although this has not yet been assessed in conditions free from extraneous influences. Once mesendoderm formation is initiated, the continued presence of a robust Activin-like signal promotes the formation of anterior-dorsal mesoderm (notochord, somites, cardiac) and definitive endoderm. Whereas, high levels of BMP activity favor the generation of posterior-ventral hematopoietic mesoderm. The overlapping triangles representing Activin, Wnt, and BMP activity serve as a reminder that all of these signaling pathways must be intact for correct patterning of the emerging mesendoderm. Activin* signifies that the identity of the ligand responsible for the activin-like signal at this stage is unclear.

represents a developmental congruence between the two systems. The existence of developmental congruence allows studies of embryo development in vivo to be used as a reference for understanding and manipulating ESC differentiation in vitro.

A second concept underlying the manipulation of ESC differentiation systems is encapsulated in the phrase "directed differentiation." This term has been used to describe ESC differentiation protocols that give the experimenter more of the cell type they desire and a lower proportion of unwanted cell types. Taken together, the ideas of developmental congruence and directed differentiation provide a conceptual framework for the development of ESC differentiation protocols aimed at producing large numbers of specific cell types.

Neural induction

A critical idea surrounding the process of germ layer formation is the concept of the

default differentiation pathway; the commitment of cells to a particular lineage in the absence of signals to the contrary. This idea had its origins in studies of *Xenopus* development in which it was demonstrated that ectodermal cells deprived of the opportunity for cell-cell interactions adopted a neural fate (Grunz and Tacke, 1989; Green, 1994). Subsequent work demonstrated that a blockade of BMP signaling or of the activity of Brachyury, a critical target gene of BMP/Nodal/Wnt signaling, was sufficient to permit neural differentiation of naive ectoderm (Rao, 1994; Hawley et al., 1995; Grunz, 1996; Hansen et al., 1997). These studies were possible because cells of the *Xenopus* embryo survived in a medium devoid of constituents with inductive activities. Application of this principle applied to ESC differentiation required the development of a medium capable of supporting cell growth and survival without imposing a predetermined differentiation outcome (Johansson and Wiles, 1995; Wiles and Johansson, 1997). Experiments performed using such a chemically defined media (CDM) showed that in the absence of serum, BMPs, or Activin A, differentiating ESCs up-regulated expression of the neural marker Pax6, suggesting cells had embarked on a neural differentiation pathway (Wiles and Johansson, 1997). These and subsequent studies demonstrated that BMP, Wnt, and Nodal signaling are able to block neural differentiation of ESCs, mirroring findings from studies of zebrafish and *Xenopus* development (Finley et al., 1999; Wiles and Johansson, 1999; Kimelman and Griffin, 2000; Ikeda et al., 2005; Watanabe et al., 2005). That neurectoderm formation does indeed represent a default differentiation pathway for ESCs has since been confirmed using serum-free low-density embryoid body (EB) and monolayer culture differentiation systems where cell-cell interactions are minimized (Tropepe et al., 2001; Ying et al., 2003; Smukler et al., 2006).

Although it is unclear what factors in serum are responsible for blocking neural differentiation (and inducing mesendoderm differentiation), the fact that the neural default can be restored by BMP, Wnt, or Nodal antagonists suggests that serum inhibits neural development by inducing secretion of proteins that activate these signaling pathways (Aubert et al., 2002; Gratsch and O'Shea, 2002; Pera et al., 2004; Watanabe et al., 2005). Addition of retinoic acid (RA) during the early phases of ESC differentiation can also promote neural differentiation (Meyer et al., 2004; Okada et al., 2004; Chiba et al., 2005). Again, although the molecular mechanism by which RA exerts this affect has not been determined, it is possible that RA's ability to induce expression of the Wnt antagonist, dkk1, may be important for RA-dependent neural differentiation (Verani et al., 2006). Finally, it is worth noting that once neurectodermal fate has been established, the same pathways whose signaling were blocked in order to achieve neural induction in the first instance are used to pattern the nascent neurectodermal to specific cell fates (Okada et al., 2004; Chiba et al., 2005; Irioka et al., 2005; Kawaguchi et al., 2005; Watanabe et al., 2005; Su et al., 2006). The use of the same signaling pathways to induce and then subsequently pattern cells of the nascent germ layers is a theme common to both neurectoderm and mesendoderm formation.

Mesendoderm induction

Gene targeting studies have demonstrated that the secreted factors BMP4 (Winnier et al., 1995), Nodal (Conlon et al., 1994), Wnt3 (Liu et al., 1999), and FGF8 (Sun et al., 1999b) are required for primitive streak formation in the mouse. Studies using chimeric embryos, in which the expression of these genes is absent from either the embryonic or extraembryonic region, point to distinct roles for these proteins during germ layer formation and patterning. Analysis of chimeras of BMP4 null embryos and wild-type ESCs showed that BMP4 expression within extraembryonic ectoderm is required for the initiation of gastrulation (Fujiwara et al., 2001). Conversely, Nodal expression within the epiblast is required for gastrulation while its expression in extraembryonic endoderm regulates induction and patterning of anterior neural tissues (Varlet et al., 1997). Recent work suggests that BMP4, Nodal, and Wnt3 form a regulatory loop whereby Nodal signals from the epiblast maintain expression of BMP4 in the extraembryonic ectoderm, which in turn induces expression of Wnt3 in proximal epiblast (Ben-Haim et al., 2006). Intriguingly, these studies also showed that Nodal is unable to directly induce expression of either Brachyury or Wnt3, implying Nodal acts indirectly through up-regulation of extraembryonic BMP4, which in turn activates embryonic Wnt3 expression. Wnt3 maintains Nodal expression in the epiblast thus resulting in the establishment of a positive reinforcing feedback loop (Ben-Haim et al., 2006).

Paralleling findings from studies in the embryo, experiments using ESCs differentiated in vitro suggest that BMP, Activin, and Wnt signals are all capable of initiating the

formation of mesendoderm (Johansson and Wiles, 1995; Kubo et al., 2004; Park et al., 2004; D'Amour et al., 2005; Ng et al., 2005; Tada et al., 2005; Yasunaga et al., 2005; Gadue et al., 2006; Lindsley et al., 2006). By and large, findings from ESC differentiation studies can be rationalized in the context of developmental congruence between the in vitro and in vivo systems. For example, a number of reports have now shown that sustained Activin signaling promotes formation of definitive endoderm (DE) from ESCs (Kubo et al., 2004; D'Amour et al., 2005; Tada et al., 2005; Yasunaga et al., 2005; Gadue et al., 2006), consistent with the requirement for Nodal signaling in gut endoderm formation and with lineage tracing experiments showing that DE arises from the anterior primitive streak, a region of robust Nodal expression (Lowe et al., 2001; Lawson and Schoenwolf, 2003; Tam et al., 2003). Similarly, BMP4 promotes the formation of hematopoietic mesoderm (Johansson and Wiles, 1995; Wiles and Johansson, 1997; Li et al., 2001; Park et al., 2004; Ng et al., 2005) and cardiac mesoderm (Honda et al., 2006; Hosseinkhani et al., 2007), the latter in a concentration-dependent manner, reflecting findings from studies in *Xenopus* and zebrafish documenting the role of BMP4 as a morphogen (Dosch et al., 1997; Neave et al., 1997). Finally, it has recently been established that Nodal and Wnt signaling are both required for induction of primitive streak genes during ESC differentiation (Gadue et al., 2006), providing an in vitro correlate of genetic ablation studies in the developing mouse embryo (Conlon et al., 1994; Liu et al., 1999).

CONCLUSIONS

Although the similarities between ESC differentiation and early mammalian development are compelling, one must also bear in mind that there is no a priori reason why cells must behave according to embryological principles. Indeed, the ability to maintain immortal, pluripotent ESCs is a poignant reminder that in vitro cultures can lead to the generation of nonphysiological cell types. Moreover, the outcome desired by the embryo is not the same as that sought by the experimenter. Whereas the aim of embryogenesis is to produce an animal, the aim of directed differentiation protocols is often to generate pure populations of a single cell type. Thus it is possible that many of the considerations that constrain the course of cell differentiation within the embryo may not necessarily apply to ESC differentiation. Nevertheless, because the embryo has already mapped out differentiation pathways for the generation of every known cell type, it would seem prudent to begin with nature's road map. In this regard, the most detailed maps are those generated from the study of frogs and fish, organisms that continue to provide new insights into the molecular mechanisms underlying vertebrate development.

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SECTION 1E
Isolation of Stem Cells from Extraembryonic Tissues

INTRODUCTION

This section focuses on methods for obtaining stem cells from the extraembryonic membranes and, more specifically, the placenta and umbilical cord. Compared to human and nonhuman primate embryos, little is known about the nature of progenitor cells that are harbored within the placenta and its associated extraembryonic structures (e.g., the amnion, the fluid it produces, and the umbilical cord). However, there is a great deal of interest in interrogating this compartment because the component cells, either embryonic or fetal depending on the gestational age of the tissue, could be an important source of stem progenitors. The differentiative capacity of these cells also awaits investigation. For example, we do not know whether primate extraembryonic stem cells have the apparently irreversible lineage restrictions that are imposed during the early stage of mouse development or whether they retain more plasticity, which in turn would greatly expand their utility as both research and clinical tools.

The contributions to this section provide insights into these outstanding questions. At one end of the spectrum, *UNIT 1E.1* describes a method for isolating a subpopulation of placental cells that can be directed toward a hepatocyte fate. This surprising finding suggests possible differences in the molecules basis of embryonic and extraembryonic lineage restriction in mice and humans. *UNIT 1E.2* describes methods for producing stem cells from amniotic fluid and placenta.

In summary, it is very likely that the extraembryonic tissues are an interesting source of many different progenitor populations. Of note is the fact that they are routinely discarded after birth. Thus, compared to cells obtained from the embryo or fetus proper, fewer regulatory issues are involved in studies of cells isolated from the amnion/chorion, making the extraembryonic tissues a source of human progenitors that is routinely and widely available to the research community. Nevertheless, we note that the same institutional approvals and HIPPA regulations that are required for work with other tissues apply here as well.

Susan J. Fisher

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Isolation of Human Placenta-Derived Multipotent Cells and In Vitro Differentiation into Hepatocyte-Like Cells

Hsing-I Huang[1]

[1]Cathay General Hospital, Taipei, Taiwan

ABSTRACT

Several types of progenitor cells can be isolated from various human adult tissues such as bone marrow, adipose tissues, and umbilical cord. Placental tissue collected after labor and delivery can provide a valuable source for adult stem cells. These progenitor cells, termed placenta-derived multipotent cells (PDMCs), are fibroblast-like cells which can attach on the bottom of culture vessels. PDMCs are capable of differentiating into various cells such as adipocytes, osteoblasts, chondrocytes, and neurons. Recently, we showed that PDMCs also possess the ability to differentiate into hepatocyte-like cells. This unit describes the protocols for isolation of PDMCs from human term placental tissue and for setting up in vitro differentiation of PDMCs toward hepatocyte-like cells. These cells not only express the characteristics of human liver cells, but also demonstrate several functions of typical hepatocytes. *Curr. Protoc. Stem Cell Biol.* 1:1E.1.1-1E.1.9. © 2007 by John Wiley & Sons, Inc.

Keywords: placenta • hepatocytes • differentiation • isolation • multipotent progenitors

INTRODUCTION

This unit presents procedures for isolation of placenta-derived multipotent cells (PDMCs; Fig. 1E.1.1) from human placental tissues and a protocol for in vitro differentiation of these cells into hepatic cells. The first protocol (see Basic Protocol 1) presents a method for isolation of the progenitor cells from term placenta. Human term placenta should be kept sterile and processed no later than 24 hr after the delivery. The placental tissue is then minced to small pieces. After treatment with trypsin/EDTA, the freed cells are washed and then seeded on culture vessels. The critical parts of successful isolation include keeping the tissue and cells clear of bacterial or fungal contamination and keeping the tissue cells alive. Once the tissues are dried or fixed in fixative solution, they are not appropriate materials for culture.

This unit also describes a method that allows the induction of differentiation of isolated PDMCs toward hepatocyte-like cells (see Basic Protocol 2). Expanded PDMCs are seeded on poly-L-lysine-coated plates and treated with defined medium. A change in cellular morphology from fibroblast-like to polygonal epithelial-like can be observed within 7 days of treatment. Critical to the success of this protocol are the coating of culture surfaces and the growth factors used to stimulate the differentiation. However, after the differentiation, these cells lose their proliferation capacity; thus, the cell numbers will not increase with continued cultivation.

The protocols in this unit work for human placental tissues but not for mouse placenta. In addition, the procedures should not be used for processing other human fetal tissues such as amniotic membrane. PDMCs cannot be isolated from every placental tissue sample. However, keeping the tissue sterile and carefully handling it can increase the rate of successful PDMC isolation to ~50%.

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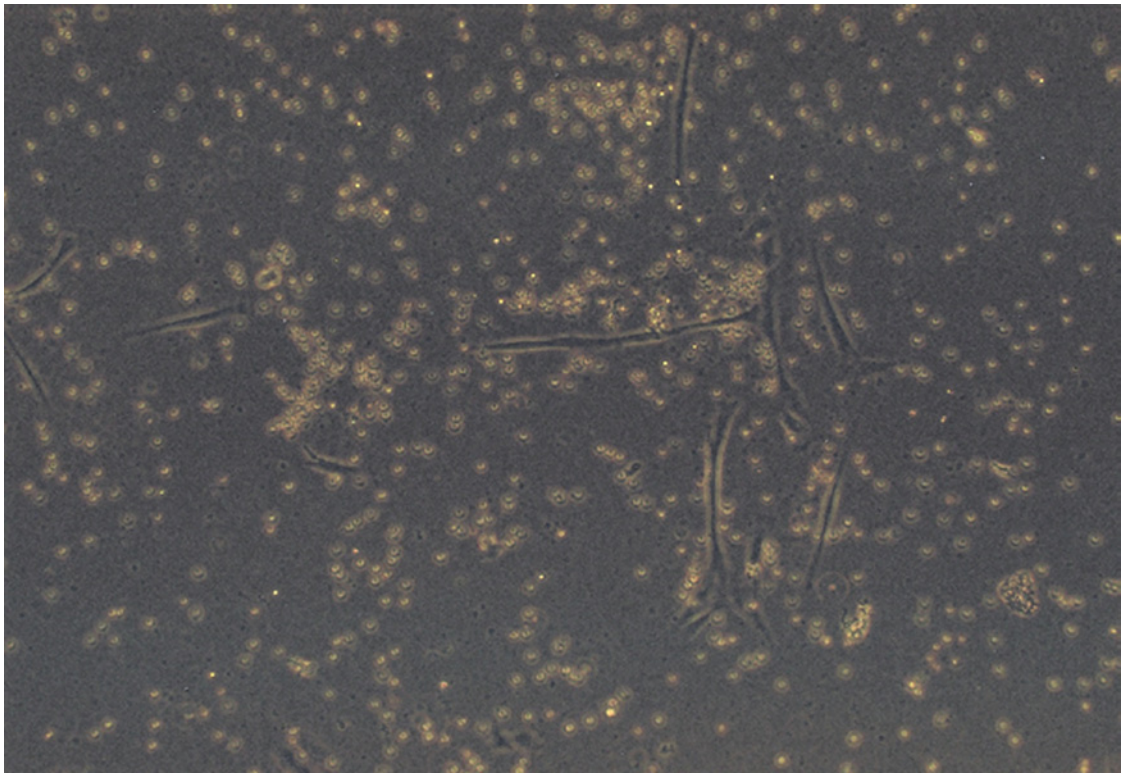

Figure 1E.1.1 Fibroblast-like cells appear on culture vessels 10 days after the first seeding of placental cells. Medium was changed on day 7.

NOTE: Ethics approval required.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All culture incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

BASIC PROTOCOL 1

ISOLATION OF PDMCs FROM HUMAN PLACENTA

This protocol describes a simple method for isolation of PDMCs from human placental tissue. Human placenta contains various cell populations including trophoblasts, epithelial cells, and some blood cells. However, most of these cells are incapable of attachment and proliferation under these culture conditions. After cultivation for 2 weeks, epithelial cells and fibroblast-like cells will appear as colonies. Finally, only the fibroblast-like cells can keep dividing. The epithelial cells will constitute 10% to 20% of the culture; the fibroblasts will not overgrow.

Materials

Donor for term placenta
Expansion medium (see recipe), prewarmed before use
Dulbecco's phosphate-buffered saline without calcium or magnesium (CMF-DPBS; Invitrogen, cat. no. 21600)
70% (v/v) ethanol
Trypsin/EDTA solution: 0.5% (w/v) trypsin/0.5 mM EDTA (Invitrogen, cat. no. 15400)

100-mm culture dishes
15-ml polypropylene centrifuge tubes
Tweezers, sterile
Scissors, sterile
Centrifuge
25-cm^2 tissue culture flasks
Inverted microscope

Additional reagents and equipment for counting cells (Phelan, 2006)

Collect and wash placental tissue

1. Collect placental tissue samples immediately after delivery. Place each sample in 10 to 20 vol sterile cold expansion medium (~1:1 ratio of tissue to medium) and place in a transport container. Keep cold and transport to the laboratory as soon as possible (<24 hr).

   The author uses the part of the placenta near the umbilical cord. Placental tissue is collected from the fetal side of the placenta.

   PDMC will be verified by immunophenotyping of the appropriate markers (see Commentary).

   Term placenta is full of blood (maternal and fetal) that has to be removed by extensive washing in a large volume of CMF-DPBS or culture medium. At least 1000 ml of washing solution is needed per 100 g of tissue (wet weight) to remove trapped blood cells (see step 2).

2. Rinse placental tissue sample eight to ten times, each time with 25 ml CMF-DPBS, to remove trapped blood cells. Rinse the tissue sample briefly with 5 ml 70% ethanol, then twice, each time with 10 ml CMF-DPBS, and put in a 100-mm culture dish.

   This step can be performed repeatedly if necessary.

3. For a 7-g sample, add 7 ml of expansion medium to the culture dish to keep the sample moist.

   Dried-out tissue samples cannot be used for the cell culture experiments because many cells are dead.

4. Remove the amniotic membrane. Use tweezers and scissors to mince the placental tissues into small pieces (<0.2 cm^3).

   In the author's laboratory, PCR analysis to detect SRY (see Commentary) is performed to confirm that placental tissue is of fetal origin.

5. Decant the culture medium.

6. Apply 10 ml CMF-DPBS to wash the minced tissues, and remove the medium.

   This procedure is to remove the medium to improve the effects of trypsin/EDTA digestion. Thus, this step can be repeated several times.

Digest sample

7. For 3 g tissue, add 3 ml of trypsin/EDTA solution to the culture dish so as to completely cover the cells. Incubate 10 min.

   The incubation time should not exceed 30 min because it could harm or the cells or cause cell death

8. Add 7 ml expansion medium to the 100-mm culture dish to stop the trypsin/EDTA reaction.

9. Transfer the entire suspension from the culture dish to a 15-ml centrifuge tube.

10. Centrifuge 5 min at 258 × *g*, room temperature. Decant the supernatant. Resuspend pellet in 10 ml of expansion medium.

Seed cultures

11. Seed the cells on 100-mm tissue culture dishes or 25-cm^2 tissue culture flasks (poly-L-lysine treatment not needed). Begin incubation.

12. At day 2 and day 3, check by microscopy for signs of bacterial or fungal contamination.

    If contamination occurs, do not open the caps; dispose of the infected culture vessels immediately.

Expand cultures

13. After incubation for 7 days, check for the appearance of attached cells on the culture surfaces using an inverted microscope. Remove unattached cells and old medium by aspirating the medium, and replace with 10 ml fresh expansion medium. From this point on, change the medium every 7 days.

    Sometimes colonies can be seen at this time point.

14. After another 7 days, check for the appearance of attached cells on the culture surfaces using an inverted microscope.

    At this time, several cell colonies could be observed. More attached cells should be seen than at step 13.

15. Subculture the cells as follows when they reach 90% confluency:

    a. Remove culture medium and wash attached cells with CMF-DPBS.
    b. Add 3 ml trypsin/EDTA solution and incubate 5 min at 37°C.
    c. Add 10 ml expansion medium to inactivate trypsin.
    d. Transfer cell suspension to a 15-ml conical centrifuge tube.
    e. Centrifuge 5 min at 258 × *g*, room temperature. Remove supernatant.
    f. Resuspend cells in fresh culture medium and replate in fresh culture dishes.

BASIC PROTOCOL 2

HEPATIC DIFFERENTIATION OF PDMCS IN VITRO

This protocol describes the steps for setting up a differentiation experiment. PDMCs can be induced to differentiate into hepatocyte-like cells, which express liver cell–specific markers and show some bioactivities restricted to hepatocytes.

Materials

0.01% (w/v) poly-L-lysine solution (mol. wt. 70,000 to 150,000; Sigma-Aldrich), filter sterilized
Dulbecco's phosphate-buffered saline without calcium or magnesium (CMF-DPBS; Invitrogen, cat. no. 21600)
Culture of PDMCs, 80% to 90% confluent (Basic Protocol 1)
Trypsin/EDTA solution: 0.5% trypsin/0.5% mM EDTA (Invitrogen, cat. no. 15400)
Expansion medium (see recipe)
Medium A (see recipe)
Medium B (see recipe)

6-well tissue culture plates or 35-mm tissue culture dishes
15-ml centrifuge tubes
Centrifuge

Additional reagents and equipment for counting viable cell (Phelan, 2006)

Prepare culture vessel

1. Cover the inner surfaces of culture vessels with 0.01% poly-L-lysine solution and incubate 30 min at room temperature.

   There are several reports of experiments using fibronectin as coating material for induction of hepatic differentiation; however, fibronectin does not work with PDMCs.

2. Withdraw coating solution from culture vessels using pipets.
3. Wash the coated surfaces twice, each with 2 ml CMF-DPBS twice.
4. Air dry the culture dish in a sterile tissue culture hood.

Collect PDMCs

5. Harvest PDMCs from culture dish by treating the attached cells with 3 ml of trypsin/EDTA solution for 5 min at 37°C. Decant the trypsin/EDTA solution.
6. Add 7 ml 37°C expansion medium to the culture dish to stop the activity of trypsin. Collect PDMCs by pipetting culture medium over the surface of the plate.
7. Transfer the PDMCs and medium to a 15-ml centrifuge tube.
8. Centrifuge 5 min at 179 × *g*, room temperature. Aspirate the supernatant.
9. Wash the harvested PDMCs three times, each time by adding 10 ml expansion medium, centrifuging 5 min at 179 × *g*, room temperature, and removing the supernatant and resuspend the pellet in 10 ml expansion medium.
10. Count the number of viable harvested PDMCs using trypan blue exclusion.
11. Adjust the cell concentration to 1×10^6 cells/ml by adding expansion medium.

Establish differentiation cultures

12. Seed the PDMCs at a concentration of 1.7×10^4 cells/cm^2 on poly-L-lysine-coated culture vessels.

    The seeding concentration is critical for setting up the differentiation experiments.

13. Incubate 18 to 24 hr.
14. After incubation, carefully remove the culture medium from the culture vessels, wash the attached cells with 2 ml CMF-DPBS, then add 2 ml medium A to replace the decanted culture medium.

    The wash procedure should be performed gently to avoid the loss of attached cells. Because PDMCs do not adhere tightly to the dish, rapid movements can cause the detachment of these cells.

15. Incubate another 12 to16 hr.

Induce and monitor differentiation

16. Remove the medium A and wash the PDMCs twice, each time with 2 ml CMF-DPBS.
17. Add 2 ml medium B the dish to induce cell differentiation.
18. Incubate cultures. Observe and check the differentiation of cells frequently.

    The changes of cell morphology from fibroblast-like to epithelial-like can be observed 3 to 5 days after the addition of medium B.

    The author has applied immunohistochemical staining with anti-HepPar-1 (Dako) to identify differentiated cells.

REAGENTS AND SOLUTIONS

For culture recipes and steps, use sterile tissue culture–grade water. For other purposes, use deionized, distilled water or equivalent in recipes and protocol steps. for suppliers, see ***SUPPLIERS APPENDIX****.*

Expansion medium

Dulbecco's Modified Eagle Medium, high-glucose formulation (DMEM-HG) supplemented with:
10% (v/v) fetal bovine serum (FBS)
100 U/ml penicillin
100 μg/ml streptomycin
3.7 g/liter $NaHCO_3$
Store up to 1 month at 4°C

Medium A

Mixture of 60% (v/v) Dulbecco's Modified Eagle Medium, low-glucose formulation (DMEM-LG) and 40% (v/v) MCDB 201 medium (Sigma-Aldrich), supplemented with the following:
1× insulin-transferrin-selenium (ITS; Sigma, cat. no. I3146 or Invitrogen) supplement (add from 100× stock)
4.7 μg/ml linoleic acid
1× (1 mg/ml) bovine serum albumin (BSA, Cohn Fraction V)
10^{-9}M dexamethasone
10^{-4}M L-ascorbic acid 2-phosphate (Sigma)
100 U/ml penicillin
100 μg/ml streptomycin
2% (v/v) fetal bovine serum (FBS)
10 ng/ml recombinant human epidermal growth factor (Invitrogen)
10 ng/ml recombinant human PDGF-BB (R&D Systems)
Store up to 2 weeks at 4°C

Medium B

Mixture of 60% (v/v) Dulbecco's Modified Eagle Medium, low-glucose formulation (DMEM-LG) and 40% (v/v) MCDB 201 medium (Sigma-Aldrich), supplemented with the following:
1× insulin-transferrin-selenium (ITS; Sigma, cat. no. I3146 or Invitrogen) supplement (add from 100× stock)
4.7 μg/ml linoleic acid
1× (1 mg/ml) bovine serum albumin (BSA, Cohn Fraction V)
10^{-9} M dexamethasone
10^{-4} M L-ascorbic acid 2-phosphate (Sigma)
100 U/ml penicillin
100 μg/ml streptomycin
10 ng/ml human fibroblast growth factor 4 (Sigma)
20 ng/ml recombinant human hepatocyte growth factor (Peprotech)
Store up to 2 weeks at 4°C

COMMENTARY

Background Information

Stem cells are known to be capable of differentiating toward various types of somatic cells. However, stem cells isolated from the different origins have varying differentiation potential. Usually, the stem cells isolated from embryonic tissue such as the blastocyst have been considered to have superior differentiation abilities in comparison with stem cells isolated from adult tissues. However, recent research provides evidence that several adult stem cells, the progenitors isolated from adult

tissues, are able to "transdifferentiate" toward cells that belong to other lineages. The transdifferentiation potential of adult stem cells thus opens the door to applying these cells in future medical therapy.

Recently, the author's laboratory and other groups reported that placental tissue could serve as a source for isolation of progenitor cells (In't Anker et al., 2004; Yen et al., 2005). These cells, termed placenta-derived multipotent cells (PDMCs), have fibroblast-like morphologies and multilineage differentiation ability. PDMCs share some properties with bone marrow-derived mesenchymal stem cells (BM-MSCs), including the expression of CD105, CD29, CD44, CD90, SH3, and SH4, and the ability to differentiate into mesodermal lineage cells. However, PDMCs are negative for STRO-1, which is a marker found on BM-MSCs. In addition, PDMCs possess differentiating abilities comparable to BM-MSCs. PDMCs have been demonstrated to be able to differentiate into cells from all three germ layers (Yen et al., 2005; Chien et al., 2006). In comparison with bone marrow, placental tissue has the advantages of being an easily accessible and relatively young tissue. It appears that placenta may serve as an attractive source of progenitor cells.

One of the advantages of these protocols is easy handling; the tissue processed in the author's laboratory is usually no more than 8 cm $\times$ 2 cm $\times$ 1 cm from the area near the umbilical cord, which is smaller than the amount used for some other protocols. The time needed for processing is short when compared to protocols that take several hours for enzymatic digestion. Once PDMCs are isolated, they can be easily propagated and characterized according to their expression of specific immunophenotypes or by means of differentiation assays for the various types of cells. PDMCs can be frozen and preserved in liquid nitrogen. According to the author's experience, thawed PDMCs express the same properties as the cells before they have been frozen including the ability to differentiate into the same types of cells to the same extent.

The liver has long been considered an organ that is capable of repairing itself. Liver stem cells, which are located within the canals of Hering, can differentiate into parenchymal hepatocytes and bile ductular cells (Evarts et al., 1987). Recent findings demonstrate that adult stem cells isolated from non-liver tissues such as bone marrow, umbilical cord blood, and human islet can be induced to differentiate into hepatocyte-like cells (Petersen et al., 1999; Kakinuma et al., 2003; von Mach et al., 2004). Furthermore, some of the differentiated cells not only express hepatocyte-specific markers (CD105, CD29, CD44, CD90, SH3, and SH4) but also possess liver cell bioactivities, including urea production, albumin secretion, and glycogen storage (Schwartz et al., 2002; Lee et al., 2004).

In addition to the similarities to BM-MSCs, PDMCs are positive for alpha fetoprotein (AFP) and c-Met expression (Chien et al., 2006). AFP and c-Met are markers of hepatic stem cells in the early-stage human embryo (Kubota et al., 2002; Suzuki et al., 2004). Based on these characteristics, the author's group tried setting up hepatic differentiation experiments for PDMCs. Using the method described in Basic Protocol 2, results were obtained suggesting that differentiated PDMCs show hepatocyte-like morphology and express liver cell–specific markers as assessed by immunocytochemical staining using anti-hepatocyte antibody (OCH1E5; Dako; Kakinuma et al., 2003). Bioactivity assays also indicate that PDMC-derived hepatocyte-like cells can take up lipoprotein and even store glycogen (Chien et al., 2006). These results are similar to those obtained using BM-MSCs as the progenitors.

Critical Parameters and Troubleshooting

It is essential to test the batch of fetal bovine serum, based on growth assays of PDMC and maintenance of the undifferentiated state, before setting up the experiments. Some batches of serum work well in PDMC culture while other batches could induce the senescence of progenitor cell cultures. However, increasing the percentage of serum in expansion medium seems not to yield any difference in cell growth rate. Medium containing 10% serum is sufficient for propagation.

The freshness of collected placental tissues is another factor that can affect successful isolation of PDMCs. The placenta samples should be processed within 24 hr after delivery. During transport and storage, keep the tissue in 4°C refrigerator, especially if the sample has to wait more than 24 hr to be processed. The tissue should be handled aseptically to minimize the possibility of bacterial or fungal contamination. That is the reason why the author prefers to rinse the tissue with alcohol before processing it; cell toxicity is minimized by keeping ethanol exposure short and

immediately washing with DPBS following the ethanol rinse.

Placental tissues contain various blood cells, including red blood cells and some leukocytes. Some blood cells remain in the cultures; some of these are unattached cells and can easily be removed when the medium is exchanged, while others are attached to the plate bottom. These latter cells attach tightly to the culture surface and are not released by trypsin treatment; therefore, they can be separated from PDMCs.

In some protocols describing the isolation of MSCs, the seeding concentration seems to be an important factor for successful isolation of stem cells. According to the authors' experience, the initial seeding density is not a critical factor in isolation of PDMCs. However, plating at excessively low densities ($<10^5$ cells/cm^2) may yield no colony formation.

For the hepatic differentiation assay, the coating of the culture surfaces makes a difference. The type of coating material plays an important factor in hepatic differentiation of PDMCs. In the author's laboratory, poly-L-lysine has proven effective in induction of the differentiation, while fibronectin has been shown to be ineffective. However, the mechanism by which the coating reagent exerts its effects on cell differentiation is not yet understood.

The other factors that affect differentiation are the growth factors and reagents added to medium B (see Reagents and Solutions), which is used to induce differentiation. The effects that these molecules have on cells might be through cell surface receptors, or they may produce changes in the intracellular concentrations of some specific ions. They may activate or inactivate a specific transduction pathway, thus driving the target cells to convert to specific types of cells. Hepatic growth factor (HGF) and fibroblast growth factor 4 (FGF-4) are effective growth factors in hepatic differentiation of PDMCs as well as bone marrow–derived MSCs. However, some reagents, such as DMSO, an effective inducer for progenitor cells such as adipose stromal cells (Seo et al., 2005), are toxic to PDMCs.

When no cells attach to culture dishes or flasks, there are several possible explanations. First, the placental tissue might not be fresh enough, so the native progenitor cells die when the tissue sample is waiting for processing. Second, the seeding concentration might be too low. Third, the composition of medium might be altered. One of the most critical factors in isolation of PDMCs is the medium. The serum must be checked before use to ensure that it supports the growth of PDMCs.

Several factors can affect the outcome of the differentiation experiment. These factors include the passage of cells used (preferably passage numbers 4 to 15) and the varied differentiation abilities of each clone. Try using more placentas and using cells in their early passages to help obtain good differentiation results.

Anticipated Results

When successfully isolated, colonies containing 10 to 50 fibroblast-like PDMCs will appear on the surface of culture dishes after 2 to 3 weeks of culture. The morphology of these cells is long and thin. They can be easily identified by their appearance when compared to other attached cells, such as the multinucleated trophoblasts and the polygonal epithelioid cells. However, because they are easily propagated and removed by trypsin treatment, a homogeneous population of PDMCs can be attained after subculturing these cells several times (three to four passages). For the hepatic differentiation experiments, a change in cell morphology can be observed after 3 to 5 days treatment with medium B. The expression of some hepatocyte-specific markers can be detected as early as day 7.

Time Considerations

The time required for isolation of PDMCs depends on how many placental tissue samples need to be processed. For one sample, 2 to 3 hr are sufficient for the first day's work (Basic Protocol 1, steps 1 to 11). Medium changes can be completed in 20 min. The greatest time requirement in this protocol is for the chopping of the tissue samples.

The hepatic differentiation experiment takes ~1 hr on day 1 for coating the culture vessels. Counting the cells and seeding them on coated plates, the major task on day 2, can be completed in 1 hr. Medium change for differentiation experiments takes only several minutes. However, the media used for this experiment should be prepared and tested (for sterility and ability to support growth of cells) before the experiment is begun.

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Isolation of Mesenchymal Stem Cells from Amniotic Fluid and Placenta

Shaun A. Steigman[1] and Dario O. Fauza[1]

[1]Department of Surgery, Children's Hospital Boston and Harvard Medical School, Boston, Massachusetts

ABSTRACT

Diverse progenitor cell populations, including mesenchymal, hematopoietic, trophoblastic, and possibly more primitive stem cells can be isolated from the amniotic fluid and the placenta. At least some of the amniotic and placental cells share a common origin, namely the inner cell mass of the morula. Indeed, most types of progenitor cells that can be isolated from these two sources share many characteristics. This unit will focus solely on the mesenchymal stem cells, the most abundant progenitor cell population found therein and, unlike some of the other stem cell types, present all through gestation. Protocols for isolation, expansion, freezing, and thawing of these cells are presented. Preference is given to the simplest methods available for any given procedure. *Curr. Protoc. Stem Cell Biol.* 1:1E.2.1-1E.2.12. © 2007 by John Wiley & Sons, Inc.

Keywords: amniotic fluid • placenta • mesenchymal stem cells • fetus • neonate • amniotic stem cells • stem cells • tissue engineering • fetal tissue engineering

INTRODUCTION

Both the amniotic fluid and the placenta contain a heterogeneous population of progenitor cells, which includes mesenchymal, hematopoietic, trophoblastic, and, perhaps, more primitive stem cells. At least some of the amniotic and placental cells share a common origin, namely the inner cell mass of the morula, which gives rise to the embryo, yolk sac, mesenchymal core of the chorionic villi, chorion, and amnion. Thus not surprisingly, most if not all types of progenitor cells that can be isolated from the amniotic fluid and the placenta share many characteristics.

The cellular profile of the amniotic fluid changes predictably during gestation as the amniotic cavity receives cells from the fetus, and possibly from the placenta as well. The mechanisms responsible for the production and turnover of the amniotic fluid also contribute to the cell types present in the amniotic cavity. In addition to the spectrum of cells generally found in the amniotic fluid, certain fetal pathologic states (e.g., neural tube and body wall defects) may lead to the accessibility of cells not normally found therein.

The multilineage potential of the different amniotic and placental stem cell populations has only recently begun to be explored. The most abundant population in amniotic fluid and nondecidual placenta, and the most extensively studied to date, is the mesenchymal stem cell (MSC), which can be isolated from these sources throughout gestation. Amniotic fluid and placental MSCs have been shown to differentiate at least into adipogenic, chondrogenic, myogenic, and osteogenic lineages. These cells can also give rise to nonmesenchymal cell types under appropriate conditions, raising the question as to whether "mesenchymal" is actually the best term to describe them. It is likely that their full differentiation spectrum remains to be entirely defined. The potential clinical value of amniotic fluid and placental MSCs in regenerative therapies has generated much interest of late. This unit will focus on MSCs only, as well as on the simplest methods available for any given procedure.

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The unit starts with the isolation and expansion of MSCs from either amniotic fluid (Basic Protocol 1) or placental samples (Basic Protocol 2). Cell isolation is achieved through a straightforward combination of natural selection by the culture medium and mechanical separation. Two means of implementing the mechanical separation are presented: multiple wells (Basic Protocols 1 and 2) or coverslips (Alternate Protocols 1 and 2). The unit proceeds to describe the basic methods for handling these MSCs, including passaging (Support Protocol 1) and freezing and thawing (Support Protocol 2), independent of whether they were isolated from placental or amniotic fluid sources.

NOTE: Ethics approval for the described protocol is usually required from the appropriate institutional research office, typically an Institutional Review Board (IRB) for human cells or Institutional Animal Care and Use Committee (IACUC) for animal cells.

NOTE: For all procedures described in this unit, sterile facilities for tissue culture and reagent preparation are required. If nondisposable instruments and containers are to be used, wash-up and sterilization facilities will also be required.

NOTE: Experiments should be performed under sterile conditions in either Class II biological hazard flow hoods or laminar flow horizontal draft hoods. When working with human cells, Class II biological hazard flow hoods are recommended.

NOTE: All incubations are performed in a humidified, 37°C, 5% CO_2 incubator unless otherwise specified.

BASIC PROTOCOL 1

ISOLATION OF MESENCHYMAL STEM CELLS FROM AMNIOTIC FLUID

Human amniotic fluid is typically obtained from diagnostic amniocentesis and occasionally from therapeutic amnioreductions. Conceivably, one could also procure it during delivery. Although the chemical and cellular composition of the amniotic fluid varies with gestational age, MSCs can be reliably isolated from it at any time during pregnancy. A very simple technique is outlined below for isolating amniotic fluid–derived MSCs (also referred to as mesenchymal amniocytes) based on natural selection from the culture medium and on mechanical separation.

Materials

Amniotic fluid
Mesenchymal-20 medium (see recipe)
Dulbecco's phosphate-buffered saline (cation-free; CMF-DPBS; Invitrogen)
0.025% (w/v) trypsin/0.04% (w/v) EDTA (Invitrogen)

15- and 50-ml conical centrifuge tubes (BD Biosciences)
Centrifuge
6-well culture plate precoated with collagen type I (BD Biosciences)
10-cm tissue culture dishes (BD Biosciences)
Inverted microscope

Collect amniotic fluid sample

1. Place 40 ml amniotic fluid into 50-ml conical centrifuge tube.

   A sample size greater than 10 ml is recommended, particularly when initially implementing these protocols, although a minimal sample size of as little as 2 ml is feasible. For these small amounts of amniotic fluid, a 15-ml conical tube should be used instead.

2. Store amniotic fluid up to 48 hr at 4°C until ready to use.

   Regardless of source, the amniotic fluid sample should be processed within 48 hr and preferably within 24 hr of harvest.

Plate cells

3. Centrifuge the amniotic fluid sample 15 min at 500 × *g*, room temperature.
4. Aspirate the supernatant and discard. Resuspend the pellet in 6 ml mesenchymal-20 medium.
5. Plate 1 ml of the cell suspension into each well of a 6-well collagen-coated culture plate and incubate 48 hr.

   Upon inspection, mostly dead cells and cellular debris will be evident; typically, fewer than 2% of the cells therein are viable (see Fig. 1E.2.1).

   Instead of collagen, other options for coating the wells are fibronectin and laminin.
6. After 48 hr, change the medium by aspirating and replacing 1 ml per well.

   Mesenchymal amniocytes will adhere to collagen-coated plates by 48 hr; this step removes the nonadherent cells.
7. Aspirate and replace 1 ml of medium per well every 3 days thereafter.

Select MSCs

8. After 7 to 14 days, inspect individual wells for predominance of amniocytes with the characteristic "mesenchymal" morphology (see Fig. 1E.2.2) and for absence of contamination. Select only these wells for expansion.

   This time period can be fairly variable. Accordingly, daily plate inspections are recommended.

   The typical mesenchymal morphology is that of attached, spindle-shaped cells.

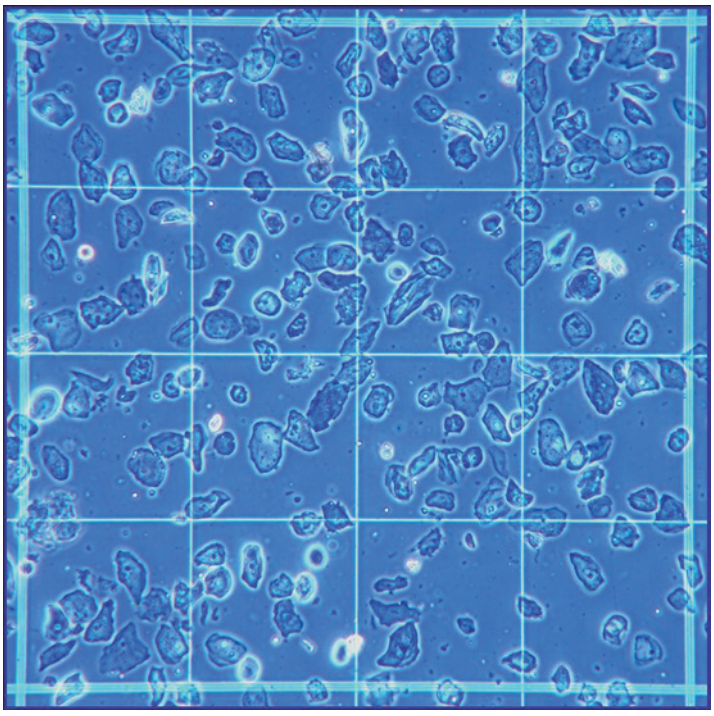

Figure 1E.2.1 Typical aspect of a fresh amniotic fluid sample after centrifugation, under trypan blue exclusion. Most cells are dead (blue cells); fewer than 2% of them are viable (unstained cells). Many cell debris are also present. (Magnification 100×)

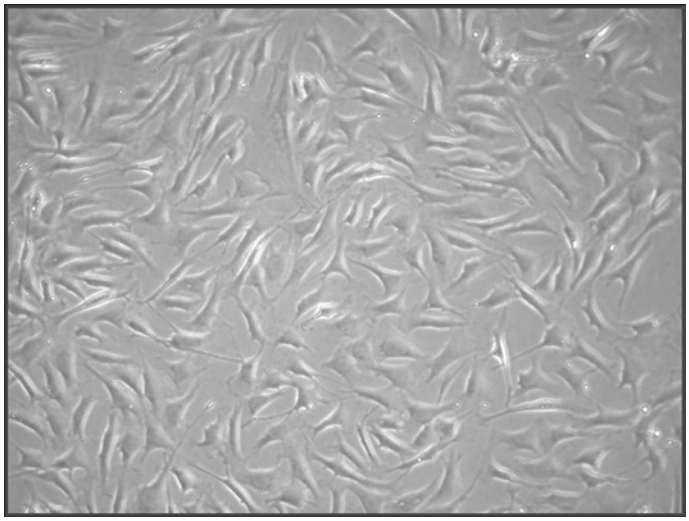

Figure 1E.2.2 Homogeneous population of amniotic fluid-derived mesenchymal cells after isolation, with the characteristic spindle-shaped morphology. (Magnification 100×)

Passage MSCs

9. When selected wells reach near confluence, aspirate medium and wash by adding 2 ml CMF-DPBS to each well and aspirating it.
10. Add 2 ml of 0.025% trypsin/0.04% EDTA, incubate 2 to 4 min to facilitate cell detachment, confirm detachment visually or under inverted microscope, and transfer each cell suspension into a separate 15-ml conical centrifuge tube.
11. Add 8 ml mesenchymal-20 medium to each tube.
12. Centrifuge each tube 5 min at 400 × *g*, room temperature.
13. Aspirate the supernatant carefully and resuspend the pellet in 6 ml mesenchymal-20 medium.
14. Dispense 1-ml aliquots of cell suspension into 10-cm tissue-culture dishes containing 9 ml mesenchymal-20 medium.
15. Grow cells to 80% to 90% confluence (replacing 9 to 10 ml of the medium every 3 days) and expand cultures (Support Protocol 1), or grow cells to ∼50% confluence and freeze (Support Protocol 2).

ALTERNATE PROTOCOL 1

ISOLATION OF MESENCHYMAL STEM CELLS FROM AMNIOTIC FLUID USING COVERSLIPS

The mechanical separation component of the isolation protocol described above is achieved by choosing only those wells that contain predominantly the characteristic mesenchymal-appearing cells. An alternative means of mechanical separation is to use coverslips, instead of distinct wells. To that end, process the amniotic fluid as in Basic Protocol 1 through step 4. Then, distribute as many 5-mm^2 coverslips (other sizes may also be used) as can fit on the surface of a single 10-cm collagen-coated plate and seed this plate at a cell density of 2 to 3 million cells per 150 cm^2 (see Phelan, 2006). The coverslips can also be precoated with collagen. (As for the well-based method, other options for coating the culture plates and coverslips are fibronectin and laminin.) After 48 hr, inspect each individual coverslip, select those containing the morphologically distinct, mesenchymal-like cells, and place them into separate 30-cm^2 culture plates containing

6 ml fresh mesenchymal-20 medium. Replace the medium every 3 days and resume the remainder of Basic Protocol 1, beginning at step 9.

ISOLATION OF MESENCHYMAL STEM CELLS FROM PLACENTA

BASIC PROTOCOL 2

Human placental tissue is typically procured from the whole organ, obtained at delivery, or from diagnostic chorionic villus sampling (CVS) specimens. Because the indications for CVS have progressively diminished recently and because of the sizeable variability of specimens produced by this method, the following text will refer to whole organ processing, i.e., from specimens obtained at delivery. Much of it can be adapted to the processing of CVS specimens, in accordance with the peculiarities of each individual CVS procurement. Once the placental tissue is cellularized (dissociated into a cell suspension), this protocol is identical to the one for amniotic fluid (Basic Protocol 1).

Materials

Placental sample
10% type 2 collagenase (Worthington Biochemical)
Dispase II (Roche Applied Science)
$CaCl_2$
Mesenchymal-20 medium (see recipe)
Dulbecco's phosphate-buffered saline (cation-free; CMF-DPBS; GIBCO)
0.025% (w/v) trypsin/0.04% (w/v) EDTA (Invitrogen)
Serum-free DMEM (Sigma)

15- or 50-ml conical centrifuge tubes (BD Biosciences)
100-μm mesh (Fisher Scientific)
Centrifuge
6-well culture plate precoated with collagen type I (BD Biosciences)
Inverted microscope
10-cm tissue culture dishes (BD Biosciences)

Process placental sample

1. Mechanically remove the maternal decidua by peeling it away from the remainder of the placenta, which contains the chorionic villi (each with a mesenchymal core), and discard.
2. Mince the residual specimen (chorionic villi with mesenchymal core) and transfer it to a 15-or 50-ml conical tube (depending on the volume of the sample).
3. Add a solution containing 10% (w/v) type 2 collagenase and 4.0 U dispase II/2.5 mmol $CaCl_2$ per liter to cover the tissue.

   The optimal amount of enzyme mixture per weight of tissue has not yet been established.
4. Filter the mixture through 100-μm mesh into a 15-ml conical centrifuge tube.
5. Centrifuge 15 min at 500 × *g*, room temperature.
6. Resuspend the pellet in 10 ml mesenchymal-20 medium.

   The placental samples that the authors have processed ranged in weight between 12 and 19 g, and the resulting pellet could be resuspended in 10 ml medium. The optimal volume of medium per weight of tissue has not yet been established.

Isolate MSCs

7. Plate 1 ml of the cell suspension into each well of a 6-well, collagen-coated culture plate, and place in incubator.

   Instead of collagen, other options for coating the wells are fibronectin and laminin.

8. After 48 hr, change the medium by aspirating and replacing 1 ml per well

   MSCs will adhere to collagen-coated plates by 48 hr; this step removes the nonadherent cells.

9. Aspirate and replace 1 ml of medium per well every 3 days thereafter.

Select MSCs

10. After 7 to 14 days, inspect individual wells for predominance of cells with the characteristic mesenchymal morphology (see Fig. 1E.2.2) and for absence of contamination. Select only these wells for expansion.

    This time period can be fairly variable. Accordingly, daily plate inspections are recommended.

    The typical mesenchymal morphology is of attached, spindle-shaped cells.

Passage cells

11. When selected wells reach near confluence, aspirate medium, add 2 ml CMF-DPBS to each well, and aspirate the buffer.
12. Add 2 ml of 0.025% trypsin/0.04% EDTA and incubate 2 to 4 min to facilitate cell detachment.
13. Confirm detachment visually or under an inverted microscope and transfer each cell suspension into separate 15-ml conical centrifuge tubes.
14. Add 8 ml mesenchymal-20 medium to each tube.
15. Centrifuge each tube 5 min at 400 × g, room temperature.
16. Aspirate the supernatant carefully and discard. Resuspend the pellet in 6 ml mesenchymal-20 medium.
17. Add 1 ml cell suspension to each of 10-cm tissue-culture dishes containing 9 ml of mesenchymal-20 medium.
18. Grow cells to 80% to 90% confluence (replacing 9 to 10 ml of the medium every 3 days) and expand cultures (Support Protocol 1), or grow cells to ∼50% confluence and freeze (Support Protocol 2).

ALTERNATE PROTOCOL 2

ISOLATION OF MESENCHYMAL STEM CELLS FROM PLACENTA USING COVERSLIPS

The mechanical separation component of the isolation protocol described above is achieved by choosing only those wells that contain predominantly the characteristic mesenchymal-appearing cells. An alternative means of mechanical separation is to use coverslips, instead of distinct wells. To that end, process the placental tissue as in Basic Protocol 2 through step 5. Then, distribute as many (usually, but not necessarily, 5 mm^2) coverslips as can be fit on the surface of a single 10-cm collagen-coated plate and seed this plate at a cell density of 2 to 3 million cells per 150 cm^2 (see Phelan, 2006). The coverslips can also be precoated with collagen. (As for the well-based method, other options for coating the culture plates and coverslips are fibronectin and laminin.) After 48 hr, inspect each individual coverslip, select those containing the morphologically distinct, mesenchymal-like cells, and place them into separate 30-cm^2 culture plates containing fresh 6 ml mesenchymal-20 medium (see recipe). Change the medium every 3 days and resume the remainder of Basic Protocol 2, beginning at step 11.

EXPANSION OF MESENCHYMAL STEM CELLS FROM AMNIOTIC FLUID OR PLACENTA

SUPPORT PROTOCOL 1

After isolation, cells will typically reach confluence in 3 to 5 days and require passage. For passages beyond p2, the authors typically lower the serum content of the medium from 20% to 10%, solely for cost savings because these cells expand quite rapidly even at 10%. This protocol describes the technique for passaging cells that have achieved 80% to 90% confluence.

Materials

MSC in culture at 80% to 90% confluence
Dulbecco's phosphate-buffered saline (cation-free; CMF-DPBS; Invitrogen)
0.025% (w/v) trypsin/0.04% (w/v) EDTA (Invitrogen)
Mesenchymal-10 medium (see recipe)

Inverted microscope
10-cm tissue culture dishes (BD Biosciences)

1. Aspirate medium from cultured cells, using a Pasteur pipet.
2. Wash the plate with 2 ml of either CMF-DPBS or 0.025% trypsin/0.04% EDTA: apply the solution gently along side of dish, swirl, and immediately aspirate and discard.
3. Add 3 ml 0.025% trypsin/0.04% EDTA and return the plate to the incubator for ~2 to 4 min to allow detachment of the cells.
4. Confirming detachment by observing under an inverted microscope.

5a. *If splitting cells 2:1:* Load 10-cm tissue culture dishes with 6 ml mesenchymal-10 medium. Add 5 ml mesenchymal-10 medium to the trypsinized plate, mix with gentle repeated pipetting for ~30 sec, and aliquot 4 ml into each of the two prepared dishes.

5b. *If splitting cells 3:1:* Load 10-cm tissue culture dishes with 7 ml mesenchymal-10 medium. Add 6 ml mesenchymal-10 medium to the trypsinized plate, mix with gentle repeated pipetting for ~30 sec, and dispense 3 ml into each of the three prepared dishes.

5c. *If splitting cells 4:1:* Load 10-cm tissue culture dishes with 8 ml mesenchymal-10 medium. Add 5 ml mesenchymal-10 medium to the trypsinized plate, mix with gentle repeated pipetting for ~30 sec, and dispense 2 ml into each of the four prepared dishes.

6. Return dishes to the incubator. Grow cells to 80% to 90% confluence (replacing 9 to 10 ml of the medium every 3 days) and passage by repeating the steps in this protocol, or grow cells to ~50% confluence and freeze (Support Protocol 2).

FREEZING AND THAWING MESENCHYMAL STEM CELLS

SUPPORT PROTOCOL 2

From early passage through any point during MSC expansion, these cells can be frozen for future use. The time span for their viability at −80°C has yet to be reliably determined, but it is certainly possible to store them for at least a year. Freezing of MSCs is optimally performed with exponentially growing cells, typically near 50% confluence.

Materials

MSC in culture, ~50% confluent
Dulbecco's phosphate-buffered saline (cation-free; CMF-DPBS; Invitrogen)
0.025% (w/v) trypsin/0.04% (w/v) EDTA (Invitrogen)
Mesenchymal-10 medium (see recipe)
Freezing solution: add 2 ml DMSO to 8 ml mesenchymal-10 medium DMSO (Sigma-Aldrich)

Pasteur pipet
Inverted microscope
15-ml conical centrifuge tube (BD Biosciences)
Centrifuge
2.0 ml cryogenic vials (Fisher Scientific)
–80°C freezer
37°C water bath
10-cm tissue culture dishes (BD Biosciences)

Freeze MSCs

1. Aspirate medium from the 50% confluent MSC culture, using a Pasteur pipet.
2. Wash the plate with 2 ml of either CMF-DPBS or 0.025% trypsin/0.04% EDTA: apply the solution gently along the side of dish, swirl, and immediately aspirate.
3. Add 3 ml 0.025% trypsin/0.04% EDTA and return the plate to the incubator for ~2 to 4 min to allow detachment of the cells.
4. Confirm detachment by observing under an inverted microscope.
5. Add 7 ml mesenchymal-10 medium to neutralize trypsin, bringing total volume to 10 ml, mix with gentle repeated pipetting for ~30 sec, and transfer the cell suspension to a 15-ml conical centrifuge tube.
6. Centrifuge 5 min at 200 × g, room temperature.
7. Aspirate the supernatant and discard. Resuspend the pellet in 1.5 ml mesenchymal-10 medium.
8. Add 1.5 ml freezing solution and mix with gentle repeated pipetting for ~30 sec.

   CAUTION: *DMSO is hazardous. When preparing the freezing solution, work in a fume hood and use gloves.*

9. Dispense 1-ml aliquots into each of three cryogenic vials.
10. Store up to 1 year at −80°C.

Thaw MSCs

11. Remove vial from the −80°C freezer and roll the vial between the hands for 10 to 15 sec until the outside of vial is frost free.
12. Hold the cryogenic vial in 37°C water bath until the contents are visibly thawed.

    To protect sterility do not submerge the cap.

13. Immerse the vial in 95% ethanol bath or spray with 70% ethanol to kill microorganisms from water bath and air dry in sterile hood.
14. Dispense the contents into a 10-cm tissue culture plate containing 9 ml mesenchymal-10 medium.

REAGENTS AND SOLUTIONS

For culture recipes and steps, use sterile tissue culture–grade water. For other purposes, use deionized, distilled water or equivalent in recipes and protocol steps. For suppliers, see ***SUPPLIERS APPENDIX****.*

Fetal bovine serum (FBS) stock

Divide samples of fetal bovine serum, MSC-qualified (Invitrogen) into 50 ml aliquots and store at −20°C. When ready for use, thaw a 50-ml aliquot of FBS in 37°C water bath.

Optional: Prior to adding to media, sterilize by passing through 0.22-μm polyethersulfone sterilizing, low protein–binding membrane (Corning), using a vacuum-driven filter system.

FBS purchased from Hyclone has traditionally been used in the authors' laboratory for MSC cultures, but recently it has been observed that MSC-qualified serum from Invitrogen leads to more robust cell growth. Batch testing of all serum is imperative to ensure optimal expansion of MSCs.

Avoid multiple freeze-thaw cycles.

Mesenchymal-10 and mesenchymal-20 medium

500 ml high-glucose DMEM containing L-glutamine (Sigma) supplemented with:
10% or 20% (v/v) FBS stock (see recipe)
5 ml 10,000 U/ml penicillin G sodium/10 mg/ml streptomycin sulfate antibiotic solution (Sigma; final concentration 100 U/ml PCN G and 0.1 mg/ml streptomycin)
50 μl 50 μg/ml rhFGF-basic stock (see recipe; final concentration 5 ng/ml)
Store up to 3 weeks at 4°C

The recipe name reflects the FBS concentration in the medium (10% or 20%).

rhFGF-basic stock, 50 μg/ml

Reconstitute 25 μg recombinant human, basic, fibroblast growth factor (Promega) in 500 μl sterile water. Divide into 50-μl aliquots and store up to 1 year at −20°C.

Avoid multiple freeze-thaw cycles.

COMMENTARY

Background Information

Ever since Friedenstein's original description of colony-forming, spindle-shaped stromal cells in the bone marrow with multipotent differentiation potential, MSCs have been intensively studied for over 40 years (Friedenstein et al., 1966, 1968, 1970). The plasticity, self-renewal, and multilineage potentials of MSCs (Prockop, 1997; Pittenger et al., 1999) have generated increasing interest in their use in an ever expanding variety of regenerative therapy applications.

At the same time, a plethora of reports have identified MSCs in diverse sources (Vaananen, 2005). Most of these sources, however, would not be compatible with perinatal cell-based therapies. In the perinatal setting, ethical objections to the isolation of amniotic fluid and placental MSCs should be obviated by the fact that amniocentesis and chorionic villus sampling are widely performed diagnostic procedures. An amniocentesis is the safest of any invasive prenatal diagnostic method, being associated with a less than 0.5% spontaneous abortion rate (Jauniaux and Rodeck, 1995). Furthermore, a mother carrying a fetus with a congenital anomaly on prenatal imaging is routinely offered a diagnostic amniocentesis. In this instance, a small extra aliquot of amniotic fluid could be obtained at that time for tissue engineering, gene therapy, or cell transplantation purposes without any additional risk to the mother or fetus.

Along these lines, the authors of this unit have recently introduced, and continued to develop, the concept of using MSCs derived from

amniotic fluid for tissue engineering strategies for the surgical repair of congenital anomalies in the perinatal period. Translated into the clinical context, from a simple amniocentesis grafts could be engineered in parallel to the remainder of gestation so that a newborn, or even a fetus, could benefit from having autologous tissue promptly available for surgical reconstruction, either before or at birth. Thus far, the authors have validated this concept in large animal models of diaphragmatic and tracheal repairs (Fuchs et al., 2004; Kunisaki et al., 2006a,b).

Cells from all three germ layers have been identified in the amniotic fluid for nearly 30 years (Milunsky, 1979; Hoehn and Salk, 1982; Gosden, 1983; Prusa et al., 2003). However, the isolation of progenitor cells was first reported only in 1993, when small, nucleated, round cells identified as hematopoietic progenitor cells were found therein before week 12 of gestation (Torricelli et al., 1993). The multilineage potential of nonhematopoietic stem cells was first suggested in 1996 by the demonstration of myogenic conversion of amniocytes (Streubel et al., 1996). Nonetheless, that study did not specify the identity of the cells that responded to the myogenic culture conditions, namely the supernatant of a rhabdomyosarcoma cell line. The proper characterization, differentiation potential, and therapeutic applications of mesenchymal amniocytes have started to be determined only quite recently (Kaviani et al., 2001; 2003; Int 't Anker et al., 2003; Fuchs et al., 2004; Kunisaki et al., 2006a,b,c).

Because of the mechanisms behind placental development, different cell types are found there at different gestational ages (Fauza, 2004). At approximately week 5 postconception, all placental villi are of the mesenchymal type. Much like mesenchymal amniocytes, placental mesenchymal cells have started to be well identified and explored only of late (Haigh et al., 1999; Kaviani et al., 2002).

In addition to the deliberately simple and easily reproducible methodology for isolation of amniotic fluid and placental MSCs described here, other methods that rely on oxygen tension, two-stage cultures, or alternative media formulations have been reported (Tsai et al., 2004; In 't Anker et al., 2004). Also, it remains to be determined whether amniotic stem cells recently obtained by somewhat different methods and described as more primitive than purely mesenchymal are actually the same cells commonly referred to as amniotic MSCs, which have been shown to be able to give rise to cells from more than one germ layer (Tsai et al., 2006; De Coppi et al., 2007). Finally, more recently, as a possible prerequisite to eventual clinical trials of amniotic MSC-based therapies, the authors have recently isolated and characterized human MSCs in the absence of fetal bovine serum (Kunisaki et al., 2007).

Critical Parameters and Troubleshooting

All experiments should be performed under sterile conditions in either Class II biological hazard flow hoods or laminar flow horizontal draft hoods. When working with human cells, Class II biological hazard flow hoods are recommended. Appropriate sterilization measures for reagents and containers are a necessity. Although amniotic MSCs, on the spectrum of cell culture, are fairly hardy cell lines, the author's experience has shown that strict adherence to sterile techniques are a must.

Batch testing of all serum is also a requirement to validate the purity of the batch and its ability to maintain undifferentiated MSCs in culture (characterized by their spindle shape and presence or absence of molecular markers; see below). Upon delivery, FBS should be tested, divided into aliquots, and stored at −20°C. In this manner, FBS should only be thawed once because multiple freeze/thaw cycles impede its efficacy for cell culture.

Media can typically be stored up to 2 or 3 weeks and should be inspected prior to each use. The color of the medium, as a reflection of pH, will evolve from pink-amber (fresh) to pink-magenta (old). In addition, evidence of crystallization or flocculation should prompt discarding.

When first performing MSCs isolation, it is prudent to confirm MSC identity. Such steps in stem cell characterization are outside the scope of this particular unit on isolation, but MSCs display a typical pattern of cell surface markers compatible with a multipotent mesenchymal progenitor lineage. The markers include CD73 (SH3), CD105 (SH2), CD44, CD29, CD90, CD13, CD10, and CD71. MSCs are also positive for human leukocyte antigens A, B, and C and negative for CD45, CD34, CD14, CD19, CD8, CD56, and CD31 (see Pittinger et al., 1999; Kaviani et al., 2001, 2003; Kunisaki et al., 2007).

Anticipated Results

MSC isolation via the protocols in this unit is fairly straightforward and consistently

reliable. However, as stated in the introduction, the cellular and chemical context of the amniotic fluid varies with gestational age, which may lead to variations in initial cell density between samples. Thus, there can be no "expected" or "typical" yield of MSCs from the initial isolation to be used as a guide. Regardless, the initial yield is not the rate-limiting step because amniotic fluid samples procured during pregnancy can generate sufficient numbers of MSCs within just a few generations.

Once derived, MSCs have lengthy lifespans and have been carried in the authors' laboratory for up to 45 passages. Nonetheless, siphoning off early-passage MSCs for freezing is routinely performed.

Time Considerations

The simplicity of these isolation protocols is among their key advantages. Overall time commitment for each step is not overburdening. Typically, it takes 2 to 3 weeks to generate ample amounts of MSCs from a small sample of amniotic fluid or placenta. The amounts of FBS and FGF added to the culture media have a direct impact on cell kinetics.

Placental dissection

The mechanical separation of the decidual layer from the remainder of the placental specimen, which contains the chorionic villi, generally takes about 1 hr but will depend on the familiarity of the operator with basic tissue dissection technique and placental anatomy (see Internet Resources). Dissolving, filtering, and resuspending the tissue into single cells can take up to another 1 hr.

Initial MSC isolation

The initial seeding of the amniotic or placental sample onto the precoated plates takes ~45 min.

MSC expansion

The transfer of selected MSCs from the precoated plates to the tissue culture dishes requires ~45 min.

Media preparation

Preparation of media typically takes 30 min and needs to be performed every 2 to 3 weeks.

Working with MSCs

Passaging and freezing each require ~30 min, depending on the number of plates being processed. Thawing requires 5 min, beyond the setup time for cleaning the workspace and bringing reagents to room temperature.

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Internet Resources

http://www.simba.rdg.ac.uk/Dave/Lit%20review.html

This Web site contains a useful drawing of placental structure.

Isolation of Mononuclear Cells from Human Cord Blood by Ficoll-Paque Density Gradient

Taina Jaatinen[1] and Jarmo Laine[1]

[1]Finnish Red Cross Blood Service, Helsinki, Finland

ABSTRACT

When preparing stem cell specimens from cord blood, pre-enrichment of mononuclear cells is highly recommended to improve the recovery of rare stem cells. Mononuclear cells are easily isolated by density gradient centrifugation. In Ficoll-Paque density gradient centrifugation, anticoagulant-treated and diluted cord blood is layered on the Ficoll-Paque solution and centrifuged. During centrifugation, erythrocytes and granulocytes sediment to the bottom layer. Lower density lymphocytes, together with other slowly sedimenting cells such as platelets and monocytes, are retained at the interface between the plasma and Ficoll-Paque, where they can be collected and subjected to subsequent isolation of hematopoietic stem cells or to the culture of mesenchymal stem cells. *Curr. Protoc. Stem Cell Biol.* 1:2A.1.1-2A.1.4. © 2007 by John Wiley & Sons, Inc.

Keywords: cord blood • fresh • cryopreserved • mononuclear cell • density gradient

BASIC PROTOCOL

When preparing stem cell specimens from cord blood, whole blood or mononuclear cell fractions can be used. However, pre-enrichment of mononuclear cells is highly recommended as it improves the recovery of rare stem cells. Mononuclear cells are easily isolated by density gradient centrifugation.

Enrichment of mononuclear cells by Ficoll-Paque density gradient centrifugation is used to improve the recovery of rare stem cells from whole blood. In Ficoll-Paque density gradient centrifugation, anticoagulant-treated and diluted cord blood is layered on the Ficoll-Paque solution and centrifuged. During centrifugation, erythrocytes and granulocytes sediment through the Ficoll-Paque to the bottom layer. Lower density lymphocytes, together with other slowly sedimenting cells such as platelets and monocytes, are retained at the interface between the plasma and Ficoll-Paque, where they can be collected and washed to remove platelets and residual Ficoll-Paque or plasma.

The isolation of pure mononuclear cell fractions from cord blood, and subpopulations thereof, brings about a special challenge. In Ficoll-Paque density gradient centrifugation, all erythroid cells do not necessarily sediment to the bottom layer as they are expected to. Some erythroid cells may instead be retained in the interface of plasma and Ficoll-Paque. These cells are nucleated progenitors that are not easily depleted and may hamper the subsequent selection of stem cell populations. In addition, erythrocytes may form aggregates and adhere to lymphocytes, thus causing unusual sedimentation of lymphocytes in the bottom layer. Further, when handling cryopreserved cord blood, cell aggregation may occur due to cell damage during thawing. Means to reduce these problems are presented in this unit.

Ficoll-Paque density gradient can be adapted to very small sample volumes. Thus, it is especially suitable for isolation of mononuclear cells from cord blood, as the sample size is often limited. Using density gradient centrifugation, mononuclear cells from a cord blood unit can be isolated within 2 hr. The mononuclear cell fractions prepared

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by density gradient centrifugation may be subjected to further selection procedures or mesenchymal stem cell cultures. Some cell loss may occur during density gradient centrifugation, but the method produces pure mononuclear cell fractions and removes nonviable cells effectively.

NOTE: All solutions and equipment coming into contact with live cells must be sterile, and proper aseptic technique should be used accordingly.

Materials

Anticoagulated cord blood (citrate dextrose, citrate phosphate dextrose, citrate, heparin, or EDTA)
PBS/EDTA: Phosphate-buffered saline (PBS; see recipe)/2 mM disodium EDTA
Ficoll-Paque Plus (Amersham Biosciences) or equivalent
PBS/0.5% (w/v) bovine serum albumin/2 mM EDTA

500-ml Erlenmeyer flask or similar container
50-ml centrifuge tubes
Centrifuge (preferably swinging-bucket rotor)

Collect cord blood cells

1. Transfer anticoagulant-treated cord blood into a 500-ml Erlenmeyer flask or similar container and determine the blood volume.
2. Dilute blood 1:2 or preferably 1:4 with PBS/EDTA.

   Aggregation of erythrocytes is reduced by diluting the blood. When working with cryopreserved cord blood, it is recommended that the EDTA is replaced by anticoagulant citrate dextrose solution, formula A (0.6% ACD/A, Baxter Healthcare), which reduces aggregation more effectively. If the aggregation is substantial, the thawed cord blood cells may be pelleted by centrifuging for 10 min at 600 × g, 18° to 20°C, and then suspended in 200 μl of 1 mg/ml DNaseI (Sigma-Aldrich) to digest the DNA released from dead cells. Then the cells can be suspended carefully in 100 ml PBS supplemented with 0.6% ACD/A..

Separate mononuclear cells

3. Place 15 ml Ficoll-Paque solution into a 50-ml centrifuge tube.
4. Carefully layer 30 ml of the diluted blood on Ficoll-Paque solution.

   Use as many tubes as needed for the total sample volume. Do not mix blood and Ficoll-Paque.

5. Centrifuge 40 min at 400 × *g*, 18° to 20°C, without brake.
6. Using a Pasteur pipet, collect the mononuclear cell fraction at the interface between plasma and Ficoll-Paque into a clean centrifuge tube.

   If there are many erythroid cells in the interface, treatment with 8% ammonium chloride or 3% diethylene glycol may be tested. Pellet cells by centrifuging 10 min at 700 × g, 18° to 20°C. Add 5 to 20 ml of lysis solution to the cell pellet, mix the suspension, and incubate 5 to 10 min at room temperature. Centrifuge 10 min at 700 × g, 18° to 20°C. Discard supernatant and proceed to step 7 (the depletion of erythrocytes may not be successful, as the nucleated erythroid progenitors are not easily lysed).

Wash the mononuclear fraction

7. Add 40 ml PBS/EDTA.
8. Centrifuge 10 min at 300 × *g*, 18° to 20°C, with brake.
9. Discard the supernatant and repeat the wash with 40 ml PBS/EDTA.
10. Centrifuge 10 min at 300 × *g*, 18° to 20°C, with brake.

11. Discard the supernatant and suspend the mononuclear cells in 5 to 10 ml of PBS/0.5% bovine serum albumin/2 mM EDTA and proceed with cell counting. Continue with isolation of hematopoietic stem cells (*UNIT 2A.2*) or with the culture of mesenchymal stem cells (*UNIT 2A.3*).

When working with cryopreserved cord blood, the aggregation may be so substantial that the cells need to be suspended in 200 μl of 1 mg/ml DNaseI (Sigma-Aldrich) to digest the DNA released from dead cells, thus preventing aggregation. Then PBS/0.5% bovine serum albumin/2 mM EDTA can be added in the appropriate volume (5 to 10 ml).

REAGENTS AND SOLUTIONS

For culture recipes and steps, use sterile tissue culture–grade water. For other purposes, use deionized, distilled water or equivalent in recipes and protocol steps. For suppliers, see ***SUPPLIERS APPENDIX****.*

Phosphate-buffered saline (PBS)

0.23 g NaH_2PO_4 (1.9 mM)
1.15 g Na_2HPO_4 (8.1 mM)
9.00 g NaCl (154 mM)
Add H_2O to 900 ml
If needed, adjust to desired pH (usually 7.2 to 7.4) with 1 M NaOH or 1 M HCl
Add H_2O to 1 liter
Sterilize by filtering through 0.22-μm filter or by autoclaving
Store indefinitely at 4°C
Without adjustment, pH is normally ~7.3.

COMMENTARY

Background Information

The isolation of mononuclear cells from human blood using a low-viscosity erythrocyte-aggregating agent was first described by Bøyum (Bøyum, 1964, 1968). There are many modifications for this method, but they all aim at easy, fast, and reproducible isolation of viable mononuclear cells. Several commercial solutions with proper density and viscosity are available for the density gradient centrifugation.

Critical Parameters and Troubleshooting

The blood volume and tube diameter determine the height of the blood sample, which is critical to the successful isolation of a pure mononuclear cell fraction. Increasing the height of the blood sample augments erythrocyte contamination in the mononuclear cell fraction. A larger blood volume can be separated in a tube with larger diameter.

Erythrocytes may also form aggregates and adhere to lymphocytes, thus leading to unusual sedimentation of lymphocytes in the bottom layer. This process can be reduced by diluting the blood. The more the whole blood is diluted, the better the separation of a pure mononuclear cell fraction. Also, a temperature of 18° to 20°C has been shown to give optimum results. All reagents should be adjusted to the temperature of 18° to 20°C.

Anticipated Results

Approximately a total of 1 to 3 × 10^8 mononuclear cells are obtained from one fresh cord blood unit. In fresh cord blood, the mean mononuclear cell concentration is 2.68 × 10^9/liter, whereas it is 5.14 × 10^9/liter in cryopreserved cord blood (Kekarainen et al., 2006). Cryopreserved cord blood is volume-reduced, which explains the increase in cell concentration—number of cells is not increased. In the Finnish Cord Blood Bank, the final processed cord blood product contains 20 ml cord blood and 5 ml DMSO. The viability of recovered mononuclear cells is typically at least 95%.

Time Considerations

The isolation of mononuclear cells from one cord blood unit is performed in 2 hr. The hands-on time is ~1 hr.

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Isolation of Hematopoietic Stem Cells from Human Cord Blood

Taina Jaatinen[1] and Jarmo Laine[1]

[1]Finnish Red Cross Blood Service, Helsinki, Finland

ABSTRACT

Enrichment of hematopoietic stem cells is based on the expression of certain surface antigens, such as CD34 and CD133, or on the lack of expression of lineage-specific antigens. Immunomagnetic positive selection of $CD34^+$ or $CD133^+$ cells is performed using paramagnetic microbeads conjugated to specific monoclonal antibodies (anti–human CD34 or anti–human CD133). In negative selection of lineage-negative (Lin^-) cells, the unwanted cells are labeled with antibodies against known markers for mature hematopoietic cells (CD2, CD3, CD14, CD16, CD19, CD24, CD56, CD66b, and glycophorin A) and retained in the column. Unlabeled cells pass through the column and are collected as the Lin^- cell fraction. Immunomagnetic cell sorting system MACS is a fast and gentle method to enrich hematopoietic stem cells. Viable and highly pure cells can be separated to be used in various downstream applications, such as flow cytometry and cell culture. *Curr. Protoc. Stem Cell Biol.* 1:2A.2.1-2A.2.9. © 2007 by John Wiley & Sons, Inc.

Keywords: cord blood • fresh • cryopreserved • hematopoietic • CD34 • CD133 • Lin

INTRODUCTION

Hematopoietic stem cells can be collected from peripheral blood, bone marrow, and cord blood mobilized by granulocyte colony-stimulating factor (G-CSF). Even though the cell content of cord blood is limited, it has a higher frequency of progenitor cells compared to peripheral blood or bone marrow (Broxmeyer et al., 1989; Grewal et al., 2003). Also, cord blood–derived $CD34^+$ cells have been shown to proliferate more rapidly than their counterparts from bone marrow (Hao et al., 1995), and the cells have been shown to possess increased engraftment potential when compared to cells from peripheral blood or bone marrow (Vormoor et al., 1994; Hogan et al., 1997).

Enrichment of hematopoietic stem cells is based on the expression of certain surface antigens, such as CD34 and CD133, or on the lack of expression of lineage-specific antigens. Immunomagnetic positive selection of $CD34^+$ or $CD133^+$ cells is performed using paramagnetic microbeads conjugated to specific monoclonal antibodies (anti-human CD34 or anti-human CD133). Labeled cells are enriched on a column placed in a magnetic field. Unlabeled cells pass through the column and the retained labeled cells can be eluted from the column after removal from the magnet. In negative selection or depletion, the unwanted cells are labeled with antibodies against known markers for mature hematopoietic cells and retained in the column. Unlabeled cells pass through the column and are collected as the lineage-negative (Lin^-) cell fraction. Mononuclear cells (*UNIT 2A.1*) are recommended as the starting material for immunomagnetic selection of hematopoietic stem cells. Aseptic cell processing is required if planning to perform cell culture experiments.

Both fluorescence-activated cell sorting and immunomagnetic selection systems utilize antibodies against cell surface antigens. The immunomagnetic cell sorting system MACS is a fast and gentle method to enrich hematopoietic stem cells. Viable and functionally active cells can be separated to be used in various downstream applications, such as

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flow cytometry and cell culture. Both labeled and unlabeled cells have good purity and recovery as well. The immunomagnetic selection can be made more effective using the AutoMACS system developed for high-speed automated cell sorting.

The MACS column matrix provides a strong magnetic field, allowing the selection of cells carrying only few specific antigens on their surface, and the method is well suited for isolating rare stem cells. These protocols can be used to enrich $CD34^{+/-}$, $CD133^{+/-}$, or $Lin^{-/+}$ cells with >90% purity from both fresh and cryopreserved cord blood.

NOTE: All solutions and equipment coming into contact with live cells must be sterile, and proper aseptic technique should be used accordingly.

BASIC PROTOCOL 1

ISOLATION OF $CD34^+$ OR $CD133^+$ CELLS FROM HUMAN CORD BLOOD

Positive selection is used to enrich known hematopoietic stem cell fractions from human cord blood. The most commonly used surface markers for hematopoietic stem cell selection are CD34 and CD133. In immunomagnetic separation, $CD34^+$ or $CD133^+$ cells are labeled with magnetic microbeads attached to specific antibodies. The magnetically labeled cells are then purified and enriched in a magnetic field using MS or LS MACS columns, which are optimized for positive selection of cells. Both $CD34^+$ and $CD133^+$ cells have been used in stem cell transplantation and the number of $CD34^+$ cells is used to depict the stem cell content of cord blood units in cord blood banking (Aroviita et al., 2005). A flow chart of the protocol steps is presented in Figure 2A.2.1.

Materials

Mononuclear cells from human cord blood (*UNIT 2A.1*)
Labeling buffer (see recipe), degassed
Direct CD34 Progenitor Cell Isolation Kit (no. 130-046-702, Miltenyi Biotec) or CD133 Cell Isolation Kit (no. 130-050-801, Miltenyi Biotec) containing:
FcR blocking reagent
MicroBeads

10-ml centrifuge tubes
Centrifuge (preferably a swinging-bucket rotor)
MACS columns (MS, no. 130-042-201 or LS, no. 130-042-401; Miltenyi Biotech)
MACS separator (MiniMACS, no. 130-042-102 or MidiMACS, no 130-042-302; Miltenyi Biotec)

Additional reagents and equipment for preparing mononuclear cell suspension (*UNIT 2A.1*)

Label the cells

1. Prepare mononuclear cell suspension (*UNIT 2A.1*) in a 50-ml centrifuge tubes.
2. Add 300 µl of labeling buffer per 10^8 mononuclear cells (for fewer cells use 300 µl of labeling buffer).

   Use degassed buffer only, as the gas bubbles may lead to clogging of the column and decrease the efficacy of immunomagnetic separation. EDTA in the labeling buffer may be replaced by other anticoagulants, such as 0.6% citrate dextrose or citrate phosphate dextrose. When working with higher cell numbers, scale up the labeling buffer and reagent volumes (e.g., 300 µl of labeling buffer per 10^8 mononuclear cells or 600 µl of labeling buffer per 2×10^8 mononuclear cells).

3. Add 100 µl of FcR blocking reagent and 100 µl of MicroBeads per 10^8 mononuclear cells. Use CD34 or CD133 microbeads to isolate $CD34^+$ or $CD133^+$ cells, respectively.

   Use cold reagents and solutions to avoid capping of antibodies. Blocking reagent (human IgG) is added to inhibit unspecific or Fc-receptor-mediated binding of the MicroBeads.

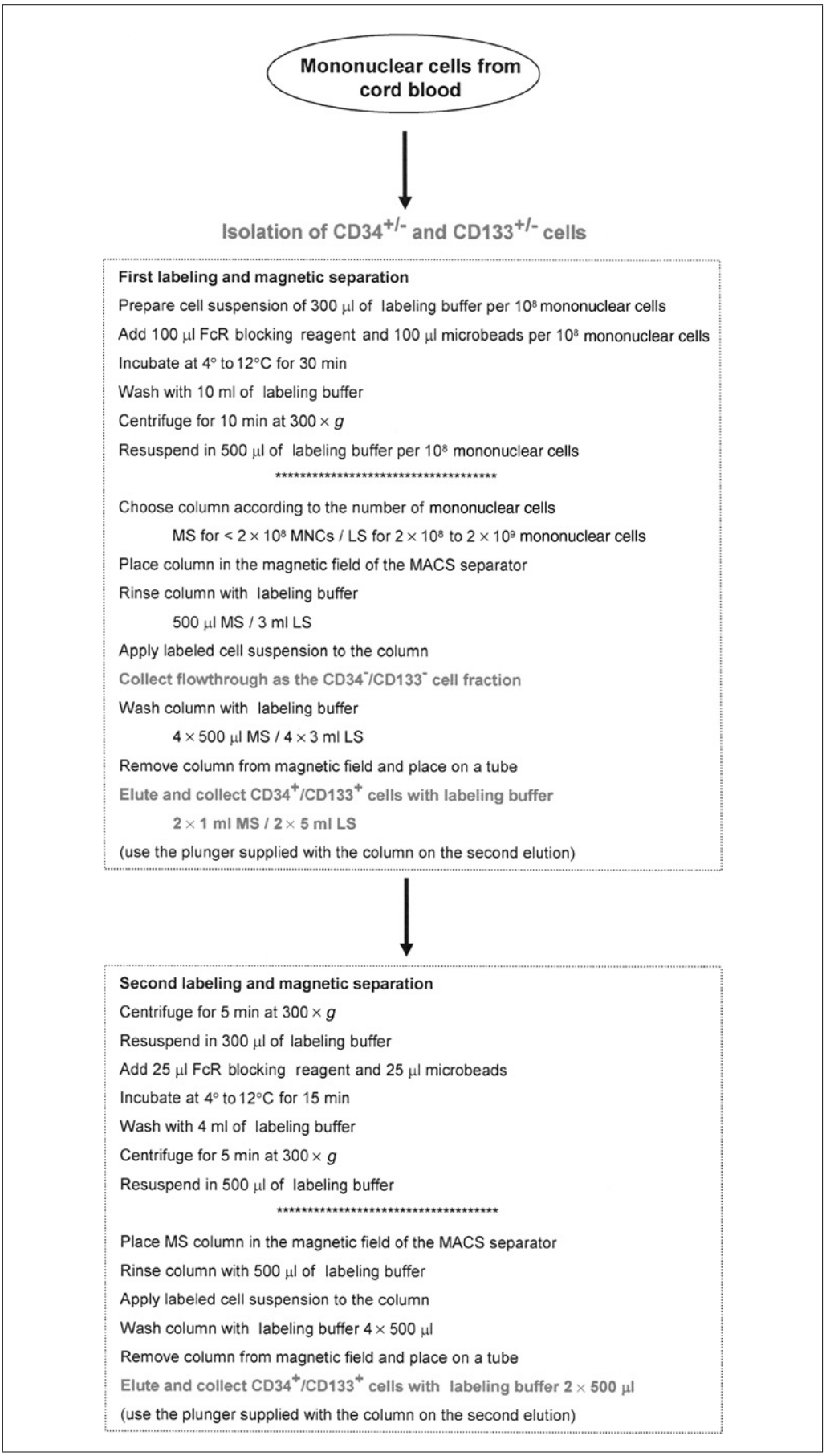

Figure 2A.2.1 Flow chart of the protocol steps to isolate $CD34^{+/-}$ or $CD133^{+/-}$ cells (Basic Protocol 1).

4. Mix well and incubate at 4° to 12°C for 30 min.
5. Add 10 ml of labeling buffer.
6. Centrifuge for 10 min at 300 × *g*, 18° to 20°C. Discard supernatant from the wash.
7. Resuspend cells in 500 µl of labeling buffer per 10^8 mononuclear cells.

Perform magnetic separation

8. Place the appropriate column in the magnetic field of the MACS separator.

   Choose the column type according to the number of mononuclear cells; MS column for <2 × 10^8 cells and LS column for 2 × 10^8 to 2 × 10^9 cells.

9. Rinse the column with labeling buffer: 500 µl for MS column and 3 ml for LS column.
10. Apply labeled cell suspension (from step 7) to the column.

    If cell aggregates are formed, the cell suspension may be filtered using 30-µm nylon mesh or preseparation filters (no. 130-041-407, Miltenyi Biotech) before applying the cell suspension to the column.

11. Allow unlabeled cells to pass through the column and collect the flowthrough as the $CD34^-$ or $CD133^-$ cell fraction.

    The $CD34^-$ or $CD133^-$ cells are often collected for control purposes.

12. Wash the column with labeling buffer, four times with 500 µl for MS column and four times with 3 ml for LS column.
13. Remove the column from the magnetic field and place on a new centrifuge tube.
14. Elute and collect the $CD34^+$ or $CD133^+$ cells with labeling buffer: two times with 1 ml for MS column and two times with 5 ml for LS column. Use the plunger supplied with the column on the second elution.

Label cells

15. Centrifuge the eluted cells for 5 min at 300 × *g*, 18° to 20°C.
16. Discard the supernatant and resuspend the cells in 300 µl of labeling buffer.
17. Add 25 µl of FcR blocking reagent and 25 µl CD34 or CD133 MicroBeads.
18. Mix well and incubate at 4° to 12°C for 15 min.
19. Add 4 ml of labeling buffer.
20. Centrifuge for 5 min at 300 × *g*, 18° to 20°C. Discard the supernatant from the wash.
21. Resuspend cells in 500 µl of labeling buffer.

Perform magnetic separation step

22. Place MS column in the magnetic field of the MACS separator.
23. Rinse the column with 500 µl of labeling buffer.
24. Apply labeled cell suspension to the column.
25. Wash the column four times with 500 µl of labeling buffer.
26. Remove the column from the magnetic field and place on a new centrifuge tube.
27. Elute and collect the $CD34^+$ or $CD133^+$ cells two times with 500 µl of labeling buffer. Use the plunger supplied with the column on the second elution.

    The cell suspension containing the $CD34^+$ and $CD133^+$ cells can be used in any laboratory assays or it can be frozen for later use.

ISOLATION OF LIN⁻ CELLS FROM HUMAN CORD BLOOD

Hematopoietic stem cells may be enriched based on the lack of lineage-specific antigens on their cell surface. To enrich progenitor cells, mononuclear cells are labeled with a cocktail of mouse monoclonal antibodies against known markers for mature human blood cells, such as CD2, CD3, CD14, CD16, CD19, CD24, CD56, CD66b, and glycophorin A. Magnetic iron particles are then attached to the antibodies and lineage-committed cells are depleted in a magnetic field using LD MACS columns. LD columns are recommended for depletion of unwanted cells and good depletion efficiency can be obtained even if the magnetic labeling of the cells is weak. MS and LS columns, designed for positive selection of cells, can also be used for depletion if the magnetic labeling of cells is strong. A flow chart of the protocol steps is presented in Figure 2A.2.2.

Materials

Mononuclear cells from human cord blood (*UNIT 2A.1*)
Labeling buffer (see recipe), degassed
StemSep Human Progenitor Enrichment Kit (StemCell Technologies) containing:

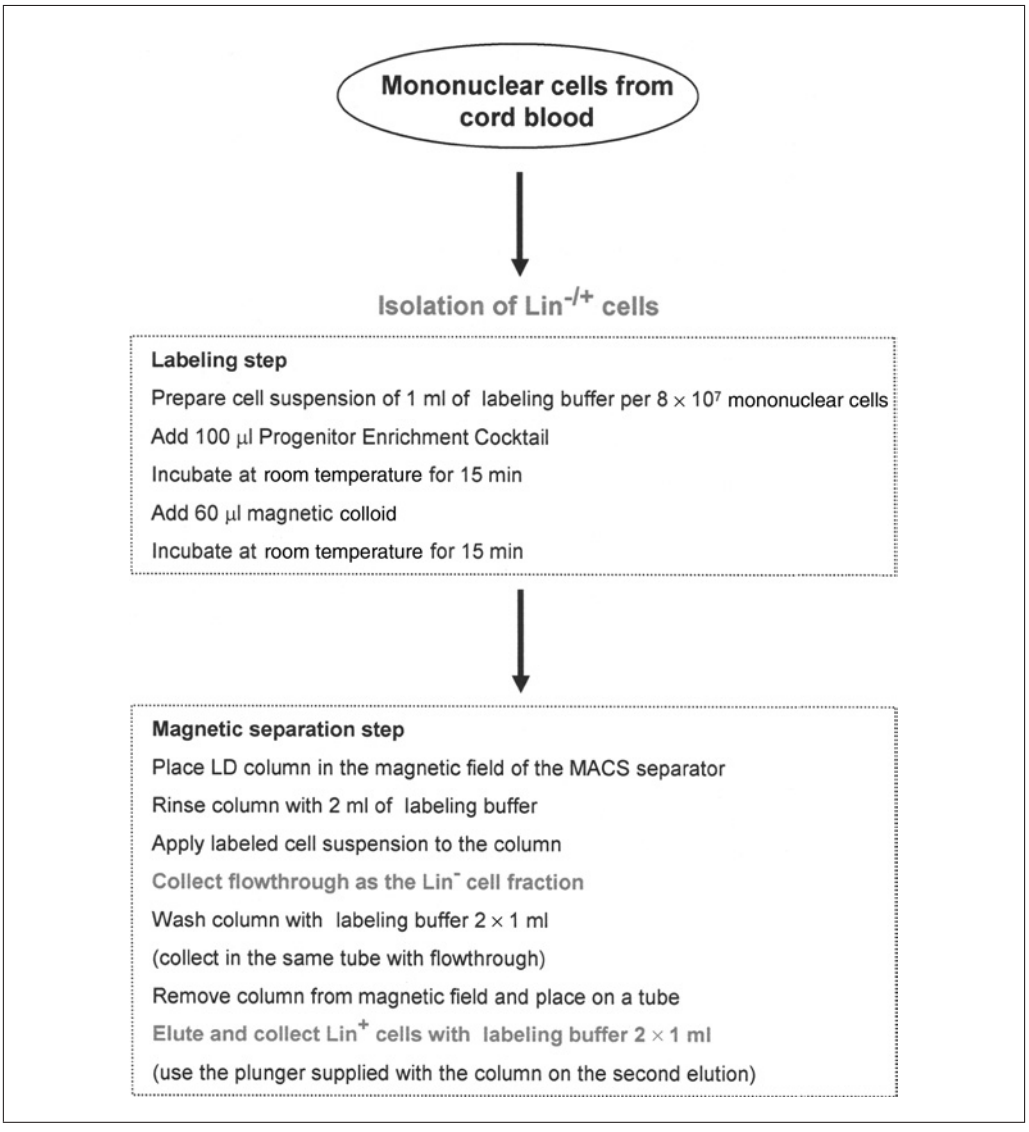

Figure 2A.2.2 Flow chart of the protocol steps to isolate Lin⁻ cells (Basic Protocol 2).

Progenitor Enrichment Cocktail with monoclonal antibodies against CD2, CD3, CD14, CD16, CD19, CD24, CD56, CD66b, and glycophorin A
StemSep magnetic colloid

MACS LD column (no. 130-042-901, Miltenyi Biotech)
Magnetic cell separator MidiMACS (no. 130-042-302, Miltenyi Biotec)
10 ml centrifuge tubes

Additional reagents and equipment for preparing mononuclear cell suspension (*UNIT 1E.2*)

Label cells

1. Prepare mononuclear cell suspension (*UNIT 2A.1*).
2. Add 1 ml of labeling buffer per 8×10^7 mononuclear cells (for fewer cells use 1 ml of labeling buffer).

   Use degassed buffer only, as the gas bubbles may lead to clogging of the column and decrease the efficacy of immunomagnetic separation. EDTA in the labeling buffer may be replaced by other anticoagulants, such as 0.6% citrate dextrose or citrate phosphate dextrose.

3. Add 100 µl of Progenitor Enrichment Cocktail.
4. Mix well and incubate at room temperature for 15 min.
5. Add 60 µl of StemSep magnetic colloid.
6. Mix well and incubate at room temperature for 15 min.

Perform magnetic separation

7. Place LD column in the magnetic field of the MACS separator.
8. Rinse the column with 2 ml of labeling buffer.
9. Apply labeled cell suspension to the column.

   If cell aggregates are formed, the cell suspension may be filtered using 30-µm nylon mesh or preseparation filters (no. 130-041-407, Miltenyi Biotech) before applying the cell suspension to the column.

10. Allow unlabeled cells to pass through the column and collect the flowthrough as the Lin^- cell fraction.
11. Wash the column two times with 1 ml of labeling buffer (collect in the same tube with the Lin^- cells from step 10).
12. Remove the column from the magnetic field and place on a new centrifuge tube.
13. Elute and collect the Lin^+ cells two times with 1 ml of labeling buffer. Use the plunger supplied with the column on the second elution.

    The cells are ready to be used in assays or experiments.

    The Lin^+ cells are often collected for control purposes and used as the positive counterparts for Lin^- cells.

REAGENTS AND SOLUTIONS

For culture recipes and steps, use sterile tissue culture–grade water. For other purposes, use deionized, distilled water or equivalent in recipes and protocol steps. For suppliers, see ***SUPPLIERS APPENDIX****.*

Labeling buffer

Phosphate-buffered saline (PBS; see recipe)
0.5% (w/v) bovine serum albumin
2 mM disodium EDTA
Degas by vacuum
Store up to 2 months at 4°C

Phosphate-buffered saline (PBS)

0.23 g NaH_2PO_4 (1.9 mM)
1.15 g Na_2HPO_4 (8.1 mM)
9.00 g NaCl (154 mM)
Add H_2O to 900 ml

Adjust to desired pH (usually 7.2 to 7.4) with 1 M NaOH or 1 M HCl
Add H_2O to 1 liter
Sterilize by filtering through a 0.22-μm filter or by autoclaving.
Store indefinitely at 4°C

Without adjustment, pH is normally ~7.3.

COMMENTARY

Background Information

MACS system was originally developed at the Institute of Genetics, University of Cologne in 1988 to pre-enrich cells for further sorting with flow cytometry. MACS cell separation is based on immunomagnetic selection of labeled cells. In positive selection, mononuclear cells are labeled with paramagnetic microbeads conjugated to specific monoclonal antibodies. Magnetically labeled cells are separated over a column placed in a magnetic field. The labeled cells are retained in the column, while unlabeled cells pass through and can be collected as the unlabeled fraction. The retained labeled cells are eluted from the column after removal from the magnet. Negative selection is a reversed technology, where the unwanted cells are labeled and retained in the column. Unlabeled cells pass through the column and are collected for subsequent applications.

The MACS column matrix provides a strong magnetic field to retain cells labeled with minimal amounts of magnetic material. The size of paramagnetic microbeads is small and only a few antigens attached to antibody conjugated microbeads are needed to separate a cell. Therefore, the method is well suited to isolate rare stem cells.

Immunomagnetic cell sorting enables fast and gentle separation of viable and highly pure hematopoietic stem cells. The immunomagnetically separated cells can be applied to various downstream applications, such as flow cytometry, cell culture, colony forming unit assay as well as microscopic, genetic, or molecular analysis.

Critical Parameters and Troubleshooting

In order to perform successful immunomagnetic separation for hematopoietic stem cells, one needs to make sure that the mononuclear cell suspension contains no aggregates that can clog the column. Aggregation can be especially problematic when working with cryopreserved cord blood. The mononuclear cells can be resuspended in 200 μl of 1 mg/ml DNaseI before continuing with the labeling. Also, a nylon mesh or commercial preseparation filters may be used to remove cell clumps.

Excess thrombocytes in the mononuclear cell population may cause clumping of cells, clog the column, and lower the purity of selected cell populations. The number of thrombocytes may be reduced by additional washing of the mononuclear cell fraction before continuing with the labeling. Use PBS supplemented with 2 mM EDTA or 0.6% citrate for washing and centrifuge for 10 min at 200 × *g*, 18° to 20°C.

If the antibody bound to the cell is crosslinking, it can form patches, i.e., precipitates of antigen-antibody complexes, resulting in uneven distribution of the antibody on the cell surface. This capping of antibodies can be avoided by using cold solutions and working quickly. However, working on ice is not recommended as it increases the incubation

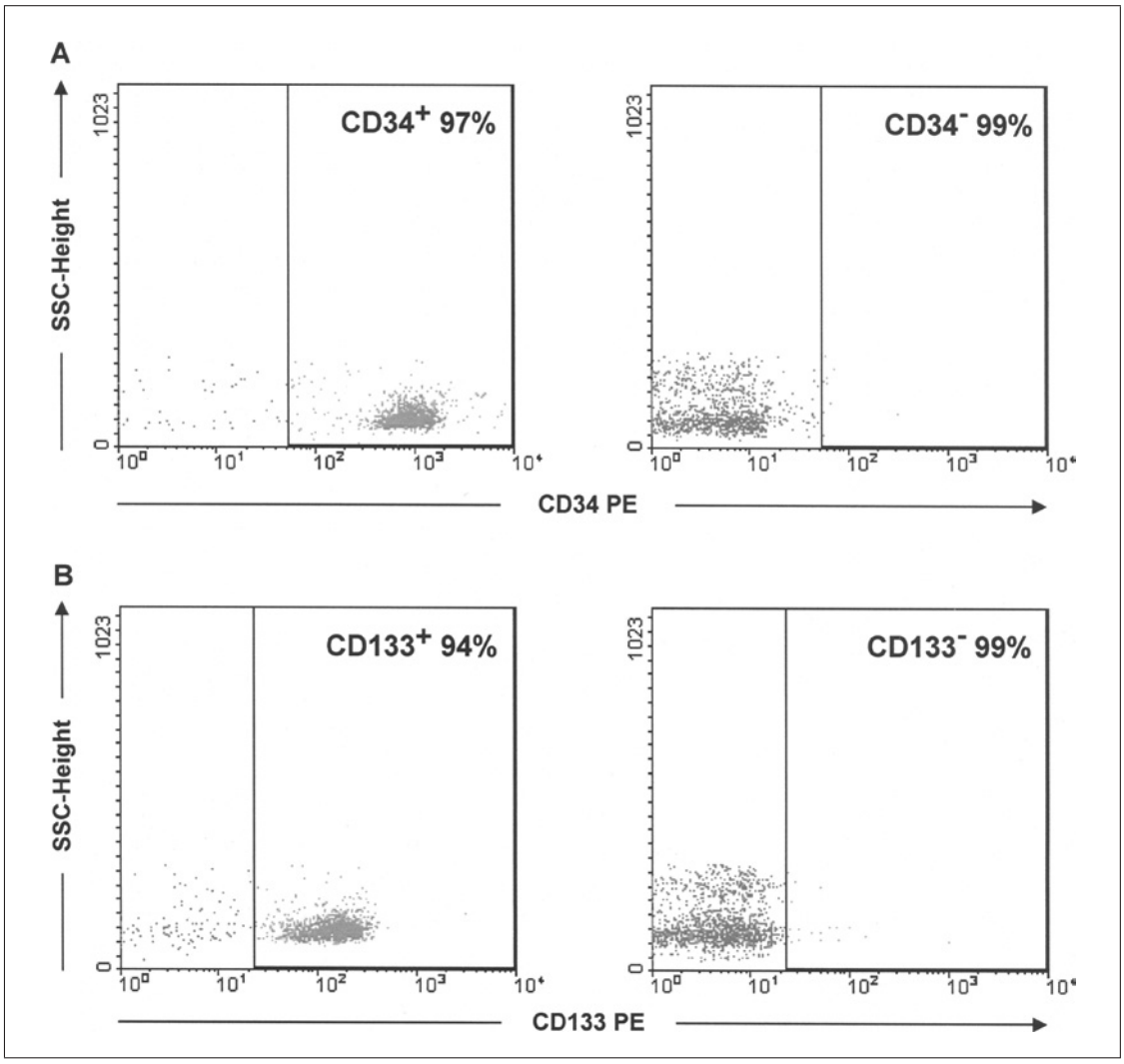

Figure 2A.2.3 Purity of $CD34^{+/-}$ and $CD133^{+/-}$ cell fractions (Basic Protocol 1) as determined by flow cytometry. (**A**) Representative sample of $CD34^+$ cells shows purity of 97%, and its negative counterpart ($CD34^-$ cells) is 99% pure. (**B**) Representative samples of $CD133^+$ and $CD133^-$ cells show purities of 94% and 99%, respectively. SSC, side scatter.

times. Increased temperature or unnecessary prolonged incubation time may lead to unspecific labeling.

Degassing the labeling buffer by vacuum is highly recommended, as the gas can form bubbles in the column matrix and lead to clogging of the column. This lowers the quality of the immunomagnetic separation and may even prevent the elution of the desired cell population. EDTA in the labeling buffer may be replaced by other anticoagulants (for example 0.6% citrate dextrose or citrate phosphate dextrose) if EDTA is expected to hamper subsequent applications such as cell culture.

Single column separation of $CD34^+$ or $CD133^+$ cells may result in low purity (typically <50%), yet a fairly large number of cells can be obtained. Two successive column separations with the additional labeling step (Basic Protocol 1) increase the purity to >90%, but lowers the yield. Highly pure cell fractions are often needed for subsequent applications, so pooling of samples may need to be considered to obtain sufficient cell numbers. Hence, enrichment of hematopoietic stem cells requires balancing between yield and purity, especially when working with a limited source of sample, such as cord blood.

The purity of $CD34^+$, $CD133^+$, or Lin^- cell fractions may be determined by flow cytometry using anti-human CD34, anti-human CD133, or anti-mouse immunoglobulin specific antibodies (as the Lin^- cells are depleted using mouse monoclonal antigens), respectively. Typical purities for selected cell fractions are presented in Figures 2A.2.3 and 2A.2.4.

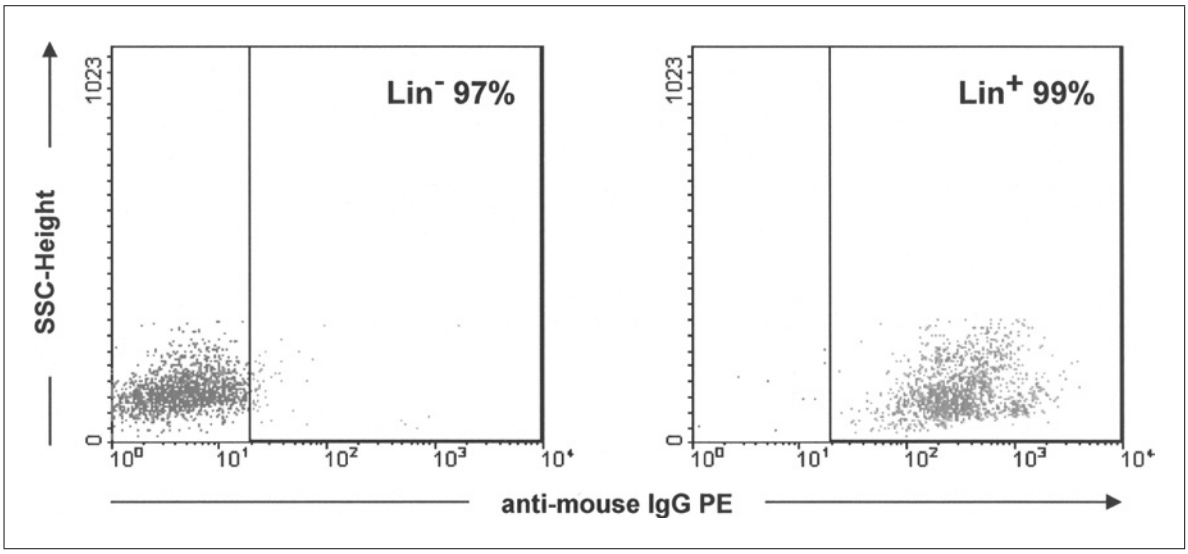

Figure 2A.2.4 Purity of Lin$^{-/+}$ cell fractions (Basic Protocol 2) as determined by flow cytometry. Representative sample of Lin$^-$ cell fraction is 97% pure. The Lin$^+$ cell fraction is 99% pure. SSC, side scatter.

Anticipated Results

Generally, the recovery of CD34$^+$, CD133$^+$, and Lin$^-$ cells from cord blood mononuclear cells is 0.86%, 0.21%, and 0.29%, respectively (Kekarainen et al., 2006). The recovery is slightly higher from fresh cord blood when compared to cryopreserved cord blood. The purity of immunomagnetically separated stem cell fractions (CD34$^+$, CD133$^+$, and Lin$^-$) is over 90%, and their control cell populations (CD34$^-$, CD133$^-$ and Lin$^+$) are nearly 100% pure (Figs. 2A.2.3 and 2A.2.4). The CD34$^+$ and CD133$^+$ cells have similar colony forming potential (84.5 and 80.0 total cfus per 1000 cells, respectively), whereas Lin$^-$ cells possess lower capacity to form colonies in CFU assay (57.3 total cfus per 1000 cells).

Time Considerations

Isolation of CD34$^+$ or CD133$^+$ cells (Basic Protocol 1) is performed in ~1.5 hr and the operation time for isolation of Lin$^-$ cells (Basic Protocol 2) is typically 45 min.

Literature Cited

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Isolation of Mesenchymal Stem Cells from Human Cord Blood

Anita Laitinen[1] and Jarmo Laine[1]

[1]Finnish Red Cross Blood Service, Helsinki, Finland

ABSTRACT

Cord blood is a rich source of stem cells especially for hematopoietic stem cells. Recently, mesenchymal stem cells (MSCs) have also been shown to exist in cord blood. However, these fibroblast-like multipotent progenitor cells are rather rare in cord blood. Many different methods have been used for their culture. This unit describes one method to obtain MSCs from cord blood and another method to differentiate these cells into osteoblasts, which is one of the lineages that mesenchymal stem cells are capable of differentiating into. The starting material for the protocol is cord blood–derived mononuclear cells. As cord blood contains a great number of erythroid precursors, the glycophorin A–positive cells are depleted using magnetic cell separation to reduce their presence in MSC culture. Osteoblast differentiation and a method to demonstrate the result of the differentiation are also described in this unit. *Curr. Protoc. Stem Cell Biol.* 1:2A.3.1-2A.3.7. © 2007 by John Wiley & Sons, Inc.

Keywords: cord blood • mesenchymal stem cells • glycophorin A • osteoblasts • von Kossa staining

INTRODUCTION

Cord blood is one of the latest sources for mesenchymal stem cells (MSCs). Because MSC populations lack specific cell surface markers many isolation protocols are based on negative selection or rely on culturing of the cells as primary cultures without any other selection methods. MSC isolation is traditionally based on the ability of the cells to adhere to plastic surfaces. Bone marrow (BM) has been the prime source of MSCs. Cord blood differs from BM as the starting material and extra steps are helpful before plating the cells.

As cord blood contains a large number of erythroid progenitors that do not necessarily sediment to the bottom layer in Ficoll-Paque density gradient centrifugation, the mononuclear cell fraction can be purified from these contaminating cells by magnetic cell separation (also see *UNIT 2A.2*). The erythroid progenitor cells express a single-pass transmembrane glycoprotein glycophorin A (GlyA) on their plasma membrane that can be used in cell selection. The GlyA negative ($GlyA^-$) cells can then be cultured without the confounding presence of erythroid progenitors.

This unit presents a protocol for MSC isolation (see Basic Protocol 1) and a protocol for MSC differentiation into osteoblasts (see Basic Protocol 2). The first protocol describes a method for growing MSCs from cord blood mononuclear cells isolated using magnetic cell separation (also see *UNIT 2A.2*). The second protocol gives a method for differentiating the MSCs into osteoblasts, which is one of the lineages that the MSCs can be differentiated to. The Support Protocol describes a staining method to demonstrate the differentiation of MSCs to osteoblasts.

NOTE: All solutions and equipment coming into contact with live cells must be sterile, and proper aseptic technique should be used accordingly.

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NOTE: All incubations are performed in a humidified 37°C, 5% CO2 incubator unless otherwise specified.

BASIC PROTOCOL 1

ISOLATION OF MESENCHYMAL STEM CELLS FROM HUMAN CORD BLOOD MONONUCLEAR CELLS

This protocol describes one method for obtaining MSCs from fresh human cord blood. Mononuclear cells from fresh cord blood isolated using Ficoll-Paque density gradient centrifugation are used as the starting material (*UNIT 2A.1*). The GlyA$^-$ cell selection and the culture of the cells to obtain for MSCs is described in this protocol (also see *UNIT 2A.2*). The GlyA$^-$ cell selection is performed using immunomagnetic selection. The mononuclear cells are labeled with magnetic microbeads attached to an antibody specific to GlyA. The magnetically labeled cells are then depleted in a magnetic field using LD MACS columns. These GlyA$^-$ cells are then cultured in conditions that support mesenchymal stem cell growth.

Materials

Mononuclear cells from fresh human cord blood (*UNIT 2A.1*)
Labeling buffer (see recipe), degassed
Glycophorin A MicroBeads (Miltenyi Biotec)
MSC culture medium (see recipe)
Phosphate-buffered saline (PBS; see recipe)
0.25% (w/v) trypsin/1 mM EDTA solution
Freezing medium: 50% MSC culture medium/40% (v/v) fetal bovine serum/10% (v/v) dimethyl sulfoxide (DMSO), chilled

10-ml and 50-ml centrifuge tubes
Centrifuge (preferably swinging-bucket rotor)
MACS LD columns (Miltenyi Biotech)
Magnetic cell separator (MidiMACS, Miltenyi Biotec)
Fibronectin-coated 6-well plates (see recipe)
Cryovials
Cell-freezing container
Liquid nitrogen

Additional reagents and equipment for preparing mononuclear cell suspension (*UNIT 2A.1*) and cell counting (Phelan, 2006)

Separate GlyA$^-$ cells

1. Prepare mononuclear cell suspension (*UNIT 2A.1*) in a 50-ml centrifuge tube. Add 80 µl labeling buffer per 10^7 mononuclear cells (for fewer cells use 80 µl of labeling buffer).

   Use degassed labeling buffer only, as the gas bubbles may lead to clogging of the column and hamper the immunomagnetic separation. When working with higher cell numbers, scale up the buffer and reagent volumes (e.g., 80 µl of labeling buffer per 10^7 mononuclear cells or 1600 µl of labeling buffer per 2×10^8 mononuclear cells).

2. Add 20 µl of Glycophorin A MicroBeads per 10^7 mononuclear cells.

   Use cold reagents and solutions to avoid capping of antibodies on cell surface.

3. Mix well and incubate at 4° to 8°C for 15 to 30 min.

4. Add 10 vol of labeling buffer to wash cells/beads.

5. Centrifuge for 10 min at $300 \times g$, 20°C. Discard the supernatant from the wash.

6. Resuspend the cells in 500 µl of labeling buffer per 10^8 mononuclear cells.

7. Place the LD column in the magnetic field of the MACS separator.
8. Rinse the column with 2 ml labeling buffer.

Collect GlyA$^-$ cells

9. Place a new 10-ml tube under the column and apply the labeled cell suspension to the column.
10. Allow unlabeled cells to pass through the column.

    The effluent of the unlabeled cell suspension will be clear. The GlyA$^+$ cells remaining in the column gives the red color of the mononuclear cell suspension described in previous steps.

11. Wash the column twice with 1 ml labeling buffer and collect in the same tube with the GlyA$^-$ cells from step 10.

    The total effluent contains the GlyA$^-$ cell fraction.

    If the GlyA$^+$ cell fraction is to be collected remove the column from the magnetic field and place it on a new centrifuge tube. Apply 3 ml of labeling buffer to the column. Use the plunger supplied with the column to flush out the magnetically labeled cell fraction. The color of the eluted cell suspension will be red.

Establish MSC cultures

12. Centrifuge the eluted GlyA$^-$ cell fraction for 5 min at 300 × *g*, room temperature.
13. Resuspend the cells in 5 ml of MSC culture medium and count the cells (Phelan, 2006).
14. Adjust the cells to a concentration of 3.2×10^6/ml of MSC culture medium.
15. Plate the cells on fibronectin-coated 6-well plates, 3 ml of cell suspension per well (cell density 10^6/cm^2).
16. Incubate the cells overnight.
17. The next day, remove nonadherent cells by changing the medium.

    The nonadherent cells that are not removed at the first medium change will be removed in subsequent medium changes. Do not wash the plates at this point because the desired cells might not have adhered properly yet.

Maintain MSC cultures

18. Incubate the cells changing the medium twice a week.

    Monitor the wells for the cell proliferation. The colonies of fibroblast-like cells should be seen within 3 weeks (Fig. 2A.3.1A). If no colonies have appeared within 3 weeks the plates can be discarded.

19. When cell colonies are visible, let the cells grow to 50% to 80% confluency.

Passage cells

20. Wash the wells with PBS. Add 150 µl of trypsin/EDTA solution and incubate at room temperature for 5 min.
21. Neutralize the trypsin with 1 ml of MSC culture medium containing serum and collect the cells into a 50-ml centrifuge tube. Centrifuge for 5 min at 300 × *g*, room temperature.

    The cells should loosen easily from the plastic. The cells that are not detached might be unwanted monocyte lineage cells.

22. Replate the cells on 6-well plates at density of 2000 to 3500/cm^2 using 3 ml of MSC medium per well.

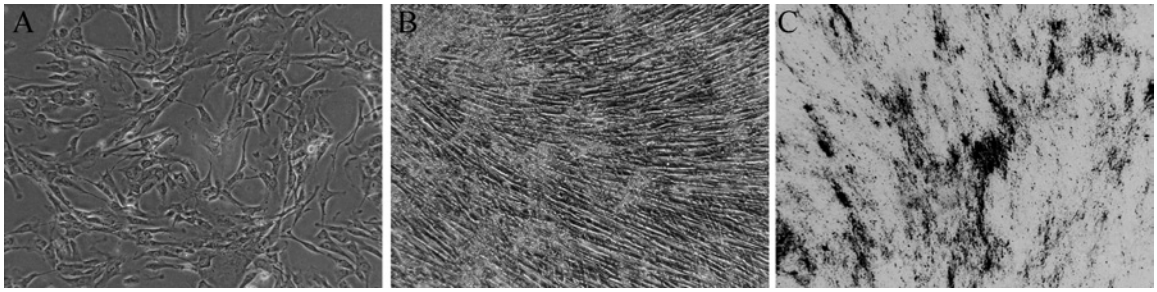

Figure 2A.3.1 Morphology of the mesenchymal stem cells and von Kossa staining of the osteoblast differentiated cells. (**A**) Proliferating mesenchymal stem cells from cord blood (100× magnification; Basic Protocol 1). (**B**) Mesenchymal stem cells differentiated 3 weeks to osteoblasts (100× magnification; Basic Protocol 2). (**C**) von Kossa stained cells differentiated 3 weeks to osteoblasts (40× magnification; Support Protocol).

At the first passage, if there are only few cells, the cells might be replated to lower density without counting the cells. Just trypsinize the cell colony and plate the cells on a new 6-well plate well or wells.

23. Continue to culture the cells until a sufficient number have been obtained for all necessary experiments or for cryopreservation.

24. Passage the cells when they are ∼80% confluent and if needed change the medium so that the cells will have fresh medium at least twice a week.

Freeze aliquots of MSC

25. To prepare frozen stocks, remove the cells from the plate, centrifuge, and resuspend the cells in chilled freezing medium at 10^6 cells/ml.

26. Pipet the cell suspension into a cryovial. Place the cryovials into a cell freezing container and place the container in −70°C freezer and then later into liquid nitrogen.

BASIC PROTOCOL 2

DIFFERENTIATION OF MESENCHYMAL STEM CELLS TO OSTEOBLASTS

Osteoblast differentiation of MSCs is used to test their differentiation potential. Osteoblast differentiation is usually verified using von Kossa staining method (see Support Protocol). This protocol describes a basic method to differentiate MSCs to osteoblasts using differentiation medium containing dexamethasone, β-glycerophosphate, and ascorbic acid-2-phosphate, which induce the MSCs differentiation into osteoblasts.

Materials

Mesenchymal stem cells (MSCs; Basic Protocol 1)
MSC culture medium (see recipe)
Differentiation medium (see recipe)
24- or 6- well tissue culture plates

Additional reagents and equipment for preparation of mesenchymal stem cell suspension (Basic Protocol 1) and von Kossa staining (Support Protocol)

1. Prepare MSC suspension in MSC culture medium containing 6–10 × 10^3 cells/ml for 24-well plates or 9.6–16 × 10^3 cells/ml for 6-well plates.

2. Pipet 1 ml of cell suspension per well for 24-well plate or 3 ml per well for 6-well plate to give a final cell density 3–5 × 10^3 per cm^2.

3. Let the cells adhere well overnight in tissue culture incubator.

4. Next day, aspirate the culture medium and add the same volume of differentiation medium to the cells.
5. Change the medium twice a week.
6. Monitor the cell differentiation.

   The cells will start to form mineralized matrix that is seen as deposit on the cells (Fig. 2A.3.1B).

7. Let the cells differentiate 2 to 5 weeks.
8. Check the differentiation at time points of interest (e.g., 2, 3, 4, and 5 weeks) by von Kossa staining (Support Protocol).

VON KOSSA STAINING FOR OSTEOBLASTS

SUPPORT PROTOCOL

Von Kossa staining is a basic staining method used to demonstrate mineralization by staining the calcium phosphate matrix formed by osteoblasts.

Materials

Differentiated cells (Basic Protocol 2)
Phosphate-buffered saline (PBS; see recipe)
4% (w/v) paraformaldehyde (pH 7.2; see recipe)
Deionized water
1% (w/v) silver nitrate
2.5% (w/v) sodium thiosulfate

Mirror or aluminum foil
UV light source or 60-W lamp

1. Wash the differentiated cells (Basic Protocol 2) with PBS (use 1 ml/well for the 24-well plate and 3 ml/well for the 6-well plate).
2. Fix the cells with 4% paraformaldehyde for 10 min, room temperature. Use 500 µl/well for the 24-well plate and 1 ml/well for the 6-well plate.
3. Aspirate the paraformaldehyde and wash the wells once with PBS and twice with deionized water.
4. Apply 1% silver nitrate solution to the wells (use 500 µl/well for the 24-well plate and 1 ml/well for the 6-well plate).
5. Place the plates on a mirror or aluminum foil and illuminate them with UV light (or use 60-W lamp) for 30 min.

   The calcium-containing matrix is stained black (Fig. 2A.3.1C).

6. Wash the wells three times for 5 min with deionized water each wash.
7. Apply sodium thiosulfate to the wells (use 500 µl/well for the 24-well plate and 1 ml/well for the 6-well plate) and incubate for 5 min to stop the reaction.
8. Rinse the wells with deionized water.
9. Air dry the cells.
10. Photograph the plates. Semiquantitatively score the staining based on von Kossa staining intensity.

REAGENTS AND SOLUTIONS

For culture recipes and steps, use sterile tissue culture–grade water. For other purposes, use deionized, distilled water or equivalent in recipes and protocol steps. For suppliers, see ***SUPPLIERS APPENDIX****.*

Differentiation medium

α-MEM (Invitrogen)
15% (v/v) fetal bovine serum (Invitrogen)
20 mM HEPES
1× L-glutamine (Invitrogen)
1× penicillin-streptomycin (Invitrogen)
0.1 μM dexamethasone
10 mM β-glycerophosphate
0.05 mM L-ascorbic acid-2-phosphate
Prepare fresh medium weekly
Store the medium up to 1 week at 4°C

Fibronectin coating of culture surfaces

Prepare 5 ng/ml fibronectin in PBS (see recipe). Pipet 1 ml/well of solution into 6-well plates. Incubate plates for ≥2 hr at 37°C. Aspirate the solution before use. The plates can be stored packaged in an air tight container up to 2 weeks at 4°C.

Labeling buffer

Phosphate-buffered saline (PBS; see recipe) containing:
0.5% (w/v) bovine serum albumin
2 mM disodium EDTA
Degas by vacuum
Store up to 1 month at 4°C

MSC culture medium

41% (v/v) DMEM, low glucose (Invitrogen)
40% (v/v) MCDB 201 (Sigma-Aldrich)
15% (v/v) fetal bovine serum (Invitrogen)
1× penicillin-streptomycin (Invitrogen)
1× ITS (insulin-transferrin-selenium) liquid supplement (Sigma-Aldrich)
1× linoleic acid–BSA (Sigma-Aldrich)
5×10^{-8} M dexamethasone (Sigma-Aldrich)
0.1 mM L-ascorbic acid-2-phosphate (Sigma-Aldrich)
Store up to 1 week at 4°C

Paraformaldehyde, 4% (w/v), pH 7.2

Dissolve 20 g paraformaldehyde in ~450 ml of PBS (see recipe). Heat the mixture to ~70°C while stirring. Add 1 M NaOH until the solution clarifies. Cool and adjust the pH to 7.2 using 1 M HCl. Adjust the volume to 500 ml with phosphate-buffered saline (PBS, see recipe). Divide into 10-ml aliquots and store up to 3 months at −20°C, thaw just before use.

Phosphate-buffered saline (PBS)

0.23 g NaH_2PO_4 (1.9 mM)
1.15 g Na_2HPO_4 (8.1 mM)
9.00 g NaCl (154 mM)
Add H_2O to 900 ml
Adjust to desired pH (usually 7.2 to 7.4) with 1 M NaOH or 1 M HCl, if necessary
Add H_2O to 1 liter
Sterilize by filtering through 0.22-μm filter or autoclaving
Store indefinitely at 4°C
Store up to 1 month at 4°C

COMMENTARY

Background Information

Isolation of mesenchymal stem cells (MSCs) was initially described from mouse bone marrow (Friedenstein et al., 1976). MSCs have been isolated from different tissues including adipose tissue, peripheral blood, and cord blood. MSCs can be isolated from cord blood using many different protocols. The MSCs from cord blood are typically isolated without any cell selection method using mononuclear cells as starting material. However, as cord blood contains a large number of erythroid progenitors, their potential confounding effect on MSC isolation can be avoided by magnetic cell separation of $GlyA^+$ cells.

The method described here is based on the protocol to isolate multipotent adult progenitor cells (MAPCs) from bone marrow as described by Reyes et al. (2001). The protocol to isolate MAPCs from cord blood was tested, but instead of MAPCs it gave rise to MSCs. The protocol was further developed to give a better yield of MSC. Serum concentration was 15%, which is much higher than has been used to isolate MAPCs from bone marrow. In lower serum concentration the efficiency rate for growing MSCs from cord blood proved to be only 11%.

MSCs are capable of differentiating into mesenchymal cell types like osteoblasts, chondroblasts, and adipocytes (Pittenger et al., 1999). The osteoblast differentiation occurs under appropriate tissue culture conditions demonstrated by Jaiswal et al. (1997). The reagents supporting osteoblast differentiation in vitro are dexamethasone, β-glycerophosphate, and ascorbic acid-2-phosphate. The osteoblast differentiation of the cells has traditionally been demonstrated by so-called von Kossa staining, in which the mineralized calcium matrix of the osteoblasts is stained with silver nitrate.

Critical Parameters and Troubleshooting

As the MSCs are very rare in cord blood and they tend to adhere to plastic, it is important to work fast or use siliconized glassware whenever possible. The time between the collection of cord blood and starting the culture procedure should be kept as short as possible. To get better yield of MSCs from cord blood, the cord blood units should be processed within 15 hr (Bieback et al., 2004). All work must be carried out aseptically.

Anticipated Results

The number of $GlyA^-$ cells obtained from one cord blood unit varies between 1×10^8 and 3×10^8. The efficiency to grow mesenchymal stem cells from fresh cord blood using the described protocol is ~40% to 50%. In practice, a single cord blood unit may give rise to 1 to 2 MSC colonies that can be expanded and cultured for 5 to 20 passages.

Time Considerations

The isolation of $GlyA^-$ cells is performed in ~1.5 hr. Setting up the culture of $GlyA^-$ cells takes ~45 min including preparation of medium. Obtaining MSC colonies is a very slow process that usually takes between 8 and 21 days.

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Guidelines for the Conduct of Human Embryonic Stem Cell Research

ISSCR International Human Embryonic Stem Cell Research Task Force[1]

[1]International Society for Stem Cell Research, Northbrook, Illinois

ABSTRACT

The ISSCR calls for due consideration and appropriate oversight of human stem cell research to ensure transparent, ethical, and responsible performance of scientific experiments. These Guidelines, prepared by the ISSCR Task Force comprised of international representatives, are meant to emphasize the responsibility of scientists to ensure that human stem cell research is carried out according to rigorous standards of research ethics, and to encourage uniform research practices that should be followed by all human stem cell scientists globally. *Curr. Protoc. Stem Cell Biol*. 1:A.1A.1-A.1A.15. © 2007 by John Wiley & Sons, Inc.

1. JUSTIFICATION FOR STEM CELL RESEARCH AND GOALS

1.1. Stem cell research, with foundations in the fields of cell and developmental biology and genetics, seeks to answer basic questions about the nature of tissue formation and maintenance. The first stem cells to be isolated were from developed tissues and organs, and are now used in tissue and organ regeneration experimentally and clinically.

1.2. The rapid growth of the field of stem cell research follows numerous recent seminal discoveries, including the isolation of human embryonic stem cells and stem cells of various types that have the potential to generate many different cells and tissues.

1.3. Stem cell research encompasses new approaches for the elucidation of disease mechanisms, offers promise for discovery of novel drugs that act on stem cells, and may yield cell replacement therapies for a multitude of devastating and widespread genetic, malignant, and degenerative diseases that are currently untreatable. Stem cell research is certain to advance fundamental knowledge and to have a profound impact on medicine. The goals of stem cell research are widely accepted in the biomedical research community and endorsed by diverse scientific societies worldwide. Long-term goals include improvements in human health and the relief of disease, infirmity, and human suffering through advances in knowledge and new clinical tools that can be made available and affordable throughout the world.

1.4. The International Society for Stem Cell Research (ISSCR) endorses the goals of stem cell scientists and exists to promote innovation in research, education, and the free exchange of scientific ideas and research materials.

2. MISSION OF TASK FORCE

2.1. Scientific, cultural, religious, ethical, and legal differences across international borders affect how early stages of human development are viewed, and how research on human embryos and embryonic stem cells is conducted. ISSCR calls for due consideration and appropriate oversight of human stem cell research to ensure transparent, ethical, and responsible performance of scientific experiments.

2.2. The ISSCR Task Force is charged with formulating guidelines that articulate ethical principles and rules of behavior for the performance of human stem cell research.

2.3. These Guidelines are meant to emphasize the responsibility of scientists to ensure that human stem cell research is carried out according to rigorous standards of research ethics, and to encourage uniform research practices that should be followed by all human stem cell scientists globally.

3. COMMENT ON SCIENTIFIC TERMINOLOGY

3.1. The ISSCR is dedicated to the use of precise and accurate terminology in stem cell research, and to educating researchers and the public on the meaning of terms and their proper usage in the discourse on stem cell research. Discussion of the merits of stem cell research requires that the discussants share a common understanding about the meaning

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and usage of specific terms, and the biological implications of the terminology. We also acknowledge the limitations of descriptive terminology in the practice of a fast-moving field of science, and endeavor to apply the most precise terms in the proper context. We have endeavored to employ the most accurate terms throughout the document, as defined in an appended glossary.

4. SCOPE OF GUIDELINES

4.1. Fundamental ethical requirements in research include review and approval of projects by a panel that is independent of the investigators, and voluntary and informed consent from any human participants. Well-established guidelines and regulations governing the use of human subjects are already in place throughout the world. These principles have been articulated in internationally recognized research ethics guidelines including, but not limited to, the Nuremburg Code of 1947, the Declaration of Helsinki of 1964 and amendments, the Belmont Report of 1979, the Council for International Organizations of Medical Sciences (CIOMS) International Ethical Guidelines for Biomedical Research Involving Human Subjects of 2002, and the UNESCO Universal Declaration on Bioethics and Human Rights of 2005. Regulations for use of animals and hazardous materials in research have also been well established and are in wide use. This Guideline document focuses on issues unique to stem cell research that involves pre-implantation stages of human development, research on the derivation or use of human pluripotent stem cell lines, and on the range of experiments whereby such cells might be incorporated into animal hosts.

4.2. These Guidelines pertain to the procurement, derivation, banking, distribution, and use of cells and tissues taken from pre-implantation stages of human development; to procurements of gametes and somatic tissues for stem cell research; and to the use of human totipotent or pluripotent cells or human pluripotent stem cell lines.

4.3. These Guidelines assert that researchers involved in human stem cell research must adhere to ethical and transparent practices for performing research and sharing research materials.

4.4. These Guidelines assign criteria for defining categories of research that are nonpermissible, that are permissible under currently mandated review processes, and research that is permissible yet should be subjected to an added level of oversight. These Guidelines prescribe the nature of regulatory review and oversight for each of the permissible research categories.

4.5. These Guidelines do not pertain to research on animal stem cells, or on classes of human somatic stem cells that remain restricted in tissue potential and are not known to possess totipotent or pluripotent potential. Research pertaining to these classes of stem cells does not raise the same sets of issues as dealt with in these Guidelines.

4.6. In their current form, these Guidelines are incomplete in their potential application to various types of fetal stem cells, which raise a unique set of issues around their procurement. Future revised versions of the Guidelines may incorporate more specific information pertinent to fetal stem cells.

5. RESPONSIBILITY FOR CONDUCT

5.1. International scientific collaboration and mutual trust among researchers are vital to the success and advancement of science and should be encouraged. Collaborations between scientists in different jurisdictions will raise issues due to the differences in the laws and regulations that govern stem cell research. An underlying principle of these guidelines is that any and all stem cell research shall be conducted in accordance with any applicable laws and regulations of the country or region where such research takes place, recognizing and respecting that certain laws and regulations may be applicable to individual researchers, regardless of where the research will take place.

5.2. Researchers must assume the responsibility for compliance with local statutes and adherence to guidelines. Institutions sponsoring stem cell research must take steps to ensure that education and appropriate training takes place to make researchers aware of regulations and professional guidelines. If warranted, institutions should obtain legal opinions on any issues of concern on behalf of their researchers.

5.3. Scientists and clinicians must be transparent and truthful about issues relating to human stem cell research and its potential to advance medicine. To guard against the creation of unrealistic expectations of success and to safeguard patients from serving prematurely as experimental subjects in human stem cell research, scientists and clinicians must clearly articulate the distinct goals of basic research, preclinical studies, and clinical trials. Investigators must assume the responsibility to

educate the public about the many steps required to garner the scientific and clinical evidence to establish treatments as safe and effective.

5.4. Scientific trainees and technical staff who have a conscientious objection to aspects of stem cell research should not be required to participate in research, and should be free of retribution or undue discrimination in assessments of professional performance. Clinical personnel who have a conscientious objection to stem cell research should not be required to participate in providing donor information or securing donor consent for research use of embryos, gametes, or somatic cells; that privilege should not extend to the clinical care of a donor.

6. STATEMENT ON REPRODUCTIVE CLONING

6.1. Human reproductive cloning is defined as the act of seeking to establish either a pregnancy or the birth of a child by gestating or transferring into a uterus human embryos that have been derived in vitro by nuclear transfer or nuclear reprogramming. Given current scientific and medical safety concerns, attempts at human reproductive cloning should be prohibited.

7. ISSUES PERTINENT TO INTERNATIONAL COLLABORATIONS AND THE ROLE OF ISSCR

7.1. In the context of international collaborations, issues will arise that relate to ownership and custodianship of intellectual property. As a general principle, the ISSCR stands for the open exchange of scientific ideas and materials to maximize exploration, to promote innovation and to increase the probability of public benefit through affordable advances made possible by human stem cell research. Given the need to respect the varying laws and regulations of different jurisdictions that may apply to any international collaboration, intellectual property issues are best left to be negotiated among the collaborating parties, taking into consideration the protection regimes or other relevant laws and regulations of their respective jurisdictions. Nonetheless, we endorse in the strongest possible terms the principle that research with human materials is valuable to all, and that the proper practice of science requires unhindered distribution of research materials to all qualified investigators engaged in noncommercial research and the dissemination of its benefits to humanity at large on just and reasonable terms.

7.2. Pluripotent human stem cell lines are important tools for research and replication of experimental data and scientific collaboration are vital to scientific advancement. The ISSCR recommends that institutions engaged in human stem cell research, whether public or private, academic or otherwise, develop procedures whereby research scientists are granted, without undue financial constraints or bureaucratic impediment, unhindered access to these research materials for scientifically sound and ethical purposes, as determined under these Guidelines and applicable laws. The ISSCR urges such institutions, when arranging for disposition of intellectual property to commercial entities, to take all possible care to preserve nonexclusive access for the research community, and to promote public benefit as their primary objective. The ISSCR endorses the principle that as a prerequisite for being granted the privilege of engaging in human stem cell research, researchers must agree to make the materials readily accessible to the biomedical research community for noncommercial research. Administrative costs such as shipping and handling should be borne by the receiving party so as not to pose a severe financial burden on the researcher providing the cells.

7.3. The ISSCR encourages scientists conducting human stem cell research to submit any human stem cell lines they derive to national or international depositories that allow open distribution in order to facilitate the wider dissemination of these valuable research tools across national boundaries. Scientists and stem cell bio-banks should endeavor to work together to harmonize standard operating procedures to facilitate international collaboration.

7.4. The process of identifying international ethical standards and practices for the conduct of human stem cell research should include concerted efforts to engage people throughout the world in honest and realistic conversations about the science and ethics of stem cell research and its emerging applications.

8. RECOMMENDATIONS FOR OVERSIGHT

8.1. All experiments pertinent to human embryonic stem cell research that involve preimplantation stages of human development, human embryos or embryonic cells, or that entail incorporating human totipotent or pluripotent cells into animal chimeras, shall be subject to review, approval and ongoing monitoring by a special oversight mechanism

or body equipped to evaluate the unique aspects of the science. Investigators should seek approval through a process of Stem Cell Research Oversight (SCRO).

8.2. Review can be performed by an oversight mechanism or body at the institutional, local, regional, national, or international level, or by some coordinated combination of those elements provided that the review as a whole occurs effectively, impartially and rigorously. Multi-institutional arrangements for coordinated review, which involve delegation of specific parts of this review, shall be permitted as long as they meet that standard. A single review rather than redundant review is preferable as long as the review is thorough and pertains to the uniquely sensitive elements of human stem cell research. Unless the review is specifically designed to be comprehensive, the SCRO process shall not replace other mandated reviews such as institutional reviews that assess the participation of human subjects in research, or the oversight for animal care, biosafety, or the like. Institutions engaged in stem cell research must establish procedures to ensure that research conducted under their auspices have been subject to appropriate review.

8.3. Review must include assessment of:

i. Scientific rationale and merit of proposal. Research with human embryonic material, or totipotent or pluripotent cells requires that scientific goals and methods be scrutinized to ensure scientific rigor. Appropriate scientific justification for performing the research using the specified materials is required.
ii. Relevant expertise of investigators. Appropriate expertise and/or training of the investigators to perform the stated experiments must be ascertained in order to ensure the optimal use of precious research materials. For derivation of new human cell lines or experiments that involve use of human embryonic materials, relevant expertise would include prior experience with embryonic stem cell derivation in animal systems and competence in the culture and maintenance of human embryonic stem cells.
iii. Ethical permissibility and justification. Research goals must be assessed within an ethical framework to ensure that research proceeds in a transparent and responsible manner. The project proposal should include a discussion of alternative methods, and provide a rationale for employing the requested human materials, the proposed methodology and for performing the experiments in a human rather than animal model system.

8.4. The mechanism or body that provides SCRO function is responsible for interpreting Guidelines, defining research practices, and monitoring compliance.

8.4a. The SCRO function should assume responsibility for monitoring and periodic review and re-approval of ongoing research proposals.

8.4b. The SCRO function has the responsibility for defining whether a research proposal constitutes permissible or nonpermissible research.

8.5. Recommendations for composition of participants to be engaged in providing SCRO function; appropriate expertise, objectivity and responsibility.

8.5a. Scientists and/or physicians with relevant expertise, including representation from scientists that are not directly engaged in the research under consideration. Relevant expertise includes areas of stem cell biology, assisted reproduction, developmental biology, and clinical medicine.

8.5b. Ethicists with ability to interpret the moral justifications and implications of the research under consideration.

8.5c. Members or advisors familiar with relevant local legal statutes governing the research.

8.5d. Community members, unaffiliated with the institution through employment or other remunerative relationships, who are impartial and reasonably familiar with the views and needs of research subjects, patients, and patient communities who could be benefited by stem cell research, and community standards.

8.5e. Those responsible for formulating the mechanism or body to provide SCRO function must be cognizant of the potential for conflicts of interest that might compromise the integrity of the review process, and attempt to eliminate such conflicts. Potential participants in the SCRO process should be selected based on the capacity for impartiality and freedom from political influence.

8.6. Each institution, academic or commercial, that engages in human stem cell research shall determine an appropriate SCRO procedure, either internal or external, by which their researchers will be subject to review, approval, and monitoring of their human stem cell research activities.

9. MECHANISMS FOR ENFORCEMENT

9.1. The development of consensus in ethical standards and practices in human stem cell research through thoughtful and transparent dialogue is a critical catalyst for international collaboration to proceed with confidence, and for research from anywhere in the world to be accepted as valid by the scientific community. These standards and practices should be incorporated in a comprehensive code of conduct applicable to all researchers in the field. Senior or corresponding authors of scientific publications should specifically be charged with the responsibility of ensuring that the code of conduct is adhered to in the course of conducting human stem cell research and of supervising junior investigators that work in their respective organizations or projects. Institutions where such research is undertaken shall strive to provide to researchers working on any such projects under their auspices, particularly junior investigators, with up-to-date information on such standards and practices on an ongoing basis.

9.2. Journal editors should require a statement of compliance with the ISSCR 'Guidelines for the Conduct of Human Embryonic Stem Cell Research' or adherence to an equivalent set of guidelines or applicable regulations, and a statement that the research was performed after obtaining approvals following a suitable SCRO process.

9.3. Grant applicants, in particular the individual scientists undertaking the research, should undertake to provide funding bodies with sufficient documentation to demonstrate that the research for which funding is requested is ethically and legally in accordance with relevant local and national regulations and also in compliance with the ISSCR 'Guidelines for the Conduct of Human Embryonic Stem Cell Research.' Funding organizations should pledge to comply with these Guidelines or their equivalent and require entities whose research is funded by such organizations to do the same.

9.4. In order to facilitate the adoption of uniform standards and practice of human stem cell research, the ISSCR will make available for download on the ISSCR website examples of informed consent documents for obtaining human materials for stem cell research (gametes, embryos, somatic tissues), and a Material Transfer Agreement for the sharing and distribution of materials (see Internet Resources).

10. CATEGORIES OF RESEARCH

To ensure that stem cell research is proceeding with due consideration, to ensure consistency of research practices among scientists globally, and to specify the nature of scientific projects that should be subject to SCRO review, we propose specific categories of research.

10.1. Category 1: Experiments that are permissible after review under existing mandates and by existing local committees, and are determined to be exempt from full SCRO review. These will include experiments with pre-existing human embryonic stem cell lines that are confined to cell culture or involve routine and standard research practice, such as assays of teratoma formation in immune-deficient mice. We recommend that all institutions pursuing such research establish a mechanism capable of determining that a) these projects can be adequately reviewed by committees with jurisdiction over research on human tissues, animals, biosafety, radiation, etc. and b) that full review by a SCRO mechanism or body is not required. This mechanism should include a determination that the provenance of the human embryonic stem cell lines to be used has been scrutinized and deemed acceptable according to the principles outlined in this document, and that such research is in compliance with scientific, legal, and ethical norms.

10.2. Category 2: Forms of research that are permissible only after additional and comprehensive review by a specialized mechanism or body established to address the issues pertinent to stem cell research (i.e., the SCRO function). Such forms of research will require provision of greater levels of scientific justification, consideration of social and ethical aspects of the research and justification for not pursuing alternative methods to address the same experimental goals. If the research requires obtaining informed consent from human subjects, the research will require review to ensure that treatment of human subjects is consistent with international norms and local laws, and any other applicable regulations or guidelines. Review of such forms of research should consider the protection of genetic and medical privacy of donors; such a review is typically done by a local institutional review board or its equivalent, but could also be performed as part of the SCRO process, with the SCRO exercising due regard for the authority of the institutional review board and avoiding duplication of its functions.

10.2a. Forms of research that involve the derivation of new human pluripotent cell lines by any means.

10.2b. Forms of research in which the identity of the donors of blastocysts, gametes, or somatic cells from which totipotent or pluripotent cells are derived is readily ascertainable or might become known to the investigator.

10.2c. Forms of research in which human totipotent cells or pluripotent stem cells are mixed with pre-implantation human embryos. In no case shall such experiments be allowed to progress for more than 14 days of development in vitro, or past the point of primitive streak formation, whichever is first.

10.2d. Clinical research in which cells of totipotent or pluripotent human origin are transplanted into living human subjects.

10.2e. Forms of research that generate chimeric animals using human cells. Examples of such forms of research include, but are not limited to introducing totipotent or pluripotent human stem cells into nonhuman animals at any stage of post-fertilization, fetal, or postnatal development.

i. We note that chimeric animal research has a long history and has been a scientifically essential and valid procedure for understanding cellular, tissue, and organ function, and has also served as a key preclinical stage of research in the evaluation of therapeutics.
ii. There are two main points of concern with chimeric animals containing human cells: the degree of the resulting chimerism and the type of tissues that are chimerized. The earlier that human cells are introduced during animal development, the greater the potential for their widespread integration during development. Introduction of a greater number of cells later in development may have an equivalent effect. In general, chimerism of the cerebral cortex or the germ-line are of greatest concern.
iii. In reviewing forms of research of this type, the SCRO mechanism or body should communicate with the appropriate mechanism or body that oversees research involving animal subjects, and give special attention to a number of issues including: A) the probable pattern and effects of differentiation and integration of the human cells into the nonhuman animal tissues; and B) the species of the animal, with particular scrutiny given to experiments involving nonhuman primates. Experiments that generate chimerism of the cerebral cortex or germ-line should be subjected to especially careful review. Although it is highly unlikely that any viable fertilization event of an animal gamete by a human gamete generated in an animal would occur, chimeric animals should typically not be allowed to produce offspring, whether by natural or artificial means. If there is a very strong scientific rationale for deriving offspring from such animals, then review committees should consider whether such an experiment might be appropriate to pursue. In any case, interbreeding of such chimeras should not be allowed, to preclude the possibility of inadvertent human-human fertilization events.

10.3. Category 3: Research that should not be pursued at this time because of broad international consensus that such experiments lack a compelling scientific rationale or raise strong ethical concerns. Such forms of research include:

10.3a. In vitro culture of any post-fertilization human embryos or organized cellular structures that might manifest human organismal potential, regardless of derivation method, for longer than 14 days or until formation of the primitive streak begins, whichever occurs first.

10.3b. Research in which any products of research involving human totipotent or pluripotent cells are implanted into a human or nonhuman primate uterus.

10.3c. Research in which animal chimeras incorporating human cells with the potential to form gametes are bred to each other.

11. PROCUREMENT OF MATERIALS

The procurement of human gametes, preimplantation embryos, and somatic cells are integral to the conduct of human stem cell research. The international community of professional scientists conducting human stem cell research must ensure that human biological materials are procured in a manner according to globally accepted principles of research ethics. Chief among the ethical principles applicable to the conduct of human stem cell research are that persons should be empowered to make voluntary and informed decisions to participate or to refuse to participate in research. In the case of human embryonic stem cell research, the public participates by providing necessary human biological materials. Persons should be afforded a fair opportunity to participate in research, and they must

be treated justly and equitably. Furthermore, privacy and confidentiality of personal information should be protected with the utmost care. Caution must also be taken to ensure that persons are not exploited during the procurement process, especially individuals who are vulnerable due to their dependent status or their compromised ability to offer fully voluntary consent. Consistent with well-established principles of justice in human subject research, there must be a reasonable relationship between those from whom such materials are received and the populations most likely to benefit from the research. Finally, the voluntary nature of the consent process must not be undermined by undue inducements or other undue influences to participate in research.

11.1. Institutional review for procurement of materials: Rigorous review, whether at the local institutional, regional, or national level, must be performed prior to the procurement of all gametes, embryos, or somatic cells that are destined for use in stem cell research. This will include the procurement of oocytes and embryos in excess of clinical need from infertility clinics, fertilized oocytes, and embryos generated by IVF specifically for research purposes, and oocytes, sperm, or somatic cells donated for development of totipotent cells or pluripotent stem cell lines by parthenogenesis, androgenesis, nuclear transfer, or other means of somatic cell reprogramming. Review at all levels must ensure that vulnerable populations are not exploited due to their dependent status or their compromised ability to offer fully voluntary consent, and that consent is voluntary and informed, and that there are no undue inducements or other undue influences for the provision of human materials.

11.2. Contemporaneous consent for donation: Consent for donation of materials for research should be obtained at the time of proposed transfer of materials to the research team. Only after a rigorous review by a SCRO mechanism or body can permission be granted to use materials for which prior consent exists but for which reconsent is prohibitively difficult. Consent must be obtained from all gamete donors for use of embryos in research. Donors should be informed that they retain the right to withdraw consent until the materials are actually used in research.

11.3. Informed consent: Researchers should exercise care in communicating the concept of "informed consent" to ensure that such consent has actually been obtained. The informed consent process should take into account language barriers and the educational level of the subjects themselves. In order to facilitate the adoption of sound and uniform standards of informed consent for the procurement of materials for human stem cell research, the ISSCR has made sample documents available to researchers by download from the ISSCR website (*http://www.isscr.org*). The samples will need to be customized for use in specific research studies.

11.3a. The informed consent document and process should cover, at a minimum, the following statements (adapted to the particular research project):

i. that the materials will be used in the derivation of totipotent or pluripotent cells for research.
ii. that the materials will be destroyed during the process of deriving totipotent or pluripotent cells for research (unless the specific research protocol aims to preserve the integrity of the research material, as in the case of embryo biopsy for procurement of blastomeres for human embryonic stem cell generation. In this circumstance, disclosure that the materials "may be destroyed" rather than "will be destroyed" would be appropriate).
iii. that derived cells and/or cell lines might be kept for many years and used for future studies, many of which may not be predictable at this time.
iv. that cells and/or cell lines might be used in research involving genetic manipulation of the cells or the generation of human-animal chimeras (resulting from the mixing of human and nonhuman cells in animal models).
v. that the donation is made without any restriction or direction regarding who may be the recipient of transplants of the cells derived, except in the case of autologous transplantation.
vi. whether the donation is limited to specific research purposes and not others or is for broadly stated purposes, including research not presently anticipated, in which case the consent shall notify donors, if applicable under governing law, of the possibility that permission for broader uses may later be granted and consent waived under appropriate circumstances by an ethical or institutional review board. The consent process should explore whether donors have objections to the specific forms of research outlined in the research protocol.

vii. disclosure of what donor medical or other information and what potential donor identifiers will be retained; specific steps taken to protect donor privacy and the confidentiality of retained information; and whether the identity of the donor will be readily ascertainable to those who derive or work with the resulting stem cell lines, or any other entity or person, including specifically any oversight bodies and government agencies.

viii. disclosure of the possibility that any resulting cells or cell lines may have commercial potential, and whether the donor will or will not receive financial benefits from any future commercial development.

ix. disclosure of any present or potential future financial benefits to the investigator and the institution related to or arising from proposed research

x. that the research is not intended to provide direct medical benefit to anyone including the donor, except in the sense that research advances may benefit everyone.

xi. that neither consenting nor refusing to donate materials for research will affect the quality of care provided to potential donors.

xii. that there are alternatives to donating human materials for research, and an explanation of what these alternatives are (e.g., donation for fertility treatment, discard, etc.).

xiii. (for donation of embryos) that the embryos will not be used to produce a pregnancy, and will not be allowed to develop in culture in vitro for longer than 14 days from conception.

xiv. (for experiments in embryonic stem cell derivation, somatic cell nuclear transfer, somatic cell reprogramming, parthenogenesis, or androgenesis) that the resulting cells or stem cell lines derived would carry some or all of the DNA of the donor and therefore be partially or completely genetically matched to the donor.

11.4. Separation of informed consent for research donation from clinical treatment. To facilitate free and voluntary choice, decisions related to the donation of gametes or creation of embryos for fertility treatment should be free of the influence of investigators who propose to derive or use human embryonic stem cells in research. Wherever possible, the treating physician or infertility clinician should not also be the investigator who is proposing to perform research on the donated materials.

11.5. Additional guidelines for procurement of specific research materials:

11.5a. For donating embryos or gametes generated in the course of clinical treatment. Except when specifically authorized by the SCRO process, no reimbursement of direct expenses or financial considerations of any kind may be provided for donating embryos or gametes that have been generated in the course of clinical treatment and are in excess of clinical need or deemed of insufficient quality for clinical use. Researchers may not request that members of the infertility treatment team generate more embryos or harvest more oocytes than necessary for the optimal chance of reproductive success. People who elect to donate stored materials for research should not be reimbursed for the costs of storage prior to the decision to donate. Reimbursement for direct expenses incurred by donors as a consequence of the consent process may be determined during the SCRO process.

11.5b. For provision of oocytes for research, when oocytes are collected outside the course of clinical treatment. In locales where oocyte donation for stem cell research is allowed, the SCRO mechanism or body is responsible for conducting rigorous review of any protocol to ensure the safety and the free and informed choice of oocyte providers, according to the following principles:

i. There must be monitoring of recruitment practices to ensure that no vulnerable populations, for example, economically disadvantaged women, are disproportionately encouraged to participate as oocyte providers for research.

ii. In locales where reimbursement for research participation is allowed, there must be a detailed and rigorous review to ensure that reimbursement of direct expenses or financial considerations of any kind do not constitute an undue inducement.

iii. At no time should financial considerations of any kind be given for the number or quality of the oocytes themselves that are to be provided for research.

iv. Oocyte procurement must be performed only by medically qualified and experienced physicians, and nonaggressive hormone stimulation cycles and frequent monitoring must be used to reduce the

risk of ovarian hyperstimulation syndrome (OHSS).

v. Due to the unknown long-term effects of ovulation induction, women should not undergo an excessive number of hormonally induced ovarian stimulation cycles in a lifetime, regardless of whether they are induced for research or assisted reproduction. The limits should be determined by thoughtful review during the SCRO process, which should be informed by the latest available scientific information about the health risks.

vi. There should be a provision to pay for the cost of any medical care required as a direct and proximate result of a woman's provision of oocytes for research.

vii. An infertility clinic or other third party responsible for obtaining consent or collecting materials should not be paid specifically for the material obtained, but rather for specifically defined cost-based reimbursements and payments for professional services.

11.5c. For provision of sperm for research. Reimbursement for direct expenses incurred by donors as a consequence of the consent process may be determined during the SCRO process.

11.5d. For provision of somatic cells for research.

Reimbursement for direct expenses incurred by donors as a consequence of the consent process may be determined during the SCRO process.

i. In the case that the somatic cell donor is a child or a decisionally incapacitated adult, consent must be provided by a legal parent or guardian or other person authorized under applicable law.

ii. Contemporaneous consent is not necessary if researchers procure somatic cells from a tissue bank. However, somatic cells may be procured from a tissue bank only if the tissue bank's informed consent documents specifically designate nuclear transfer or other reprogramming methods for stem cell research as one of the possible uses of the donor's tissues, and only if researchers use somatic cells from tissue samples whose donors have clearly consented to this possible use.

11.6. Steps to enhance the procurement process: Attempts should be made to improve the informed consent process for human materials procurement. The informed consent document is but one aspect of this process. The purpose of the informed consent document is to record that all the ethically relevant information has been discussed. The informed consent document alone can never take the place of an interactive dialogue between research staff and providers of human materials. Researchers are thus encouraged to focus on enriching the informed consent process itself, in addition to ensuring that the informed consent document includes all of the ethically relevant information. The informed consent process can be enhanced in the following ways:

i. Whenever possible, the person conducting the informed consent dialogue should have no vested interest in the research protocol. If members of the research team participate in the informed consent process, their role must be disclosed and care must be taken to ensure that information is provided in a transparent and accurate manner.

ii. Empirical research has shown that informed consent is most effective as a dynamic, interactive, and evolving process as opposed to a static, one-time disclosure event. Thus, researchers should provide ample opportunities for providers of human materials to discuss their involvement in the research protocol.

iii. Counseling services should be made available upon request to any providers of human materials prior to procurement.

iv. Procurement procedures should be revised in light of a) ongoing studies of the long-term risks associated with oocyte retrieval; and b) research on informed consent for all types of human biological materials procurement.

v. Researchers should consider on a regular basis, subject to annual review, the possible use of alternatives to hormonally induced oocytes procured solely for stem cell research, such as oocytes derived from pluripotent stem cells, in vitro maturation of oocytes from ovariectomy samples, and egg sharing programs offered through infertility clinics.

12. PRINCIPLES FOR DERIVATION, BANKING, AND DISTRIBUTION OF HUMAN PLURIPOTENT STEM CELL LINES

Proposals for derivations of new human pluripotent stem cell lines should be scientifically justified and executed by scientists with

appropriate expertise. Hand-in-hand with the privilege to perform derivations is the obligation to distribute the cell lines to the research community. A clear, detailed outline for banking and open access to the new lines should be incorporated into derivation proposals. New pluripotent stem cell lines should be made generally available as soon as possible following derivation and first publication. The ISSCR encourages researchers to deposit lines early into centralized repositories where the lines will be held for release and distribution upon publication.

12.1. Derivation of New Lines:

12.1a. Proposals to attempt derivation of new totipotent cells or pluripotent stem cell lines from donated pre-implantation human embryos, embryonic cells, or via nuclear reprogramming must be approved by a SCRO process. New derivations by necessity involve procurement of materials from human subjects and, therefore, will need to be approved by institutional oversight bodies with specific responsibility for protection of human subjects, as well as by the SCRO process. In some jurisdictions, the SCRO process will be formulated in a manner that encompasses all human subjects and stem cell oversight responsibilities.

12.1b. The scientific rationale for the need to derive new totipotent cells or pluripotent stem cell lines must be provided by the researcher, with justification of the numbers of pre-implantation embryos to be used. For proposals that incorporate nuclear transfer or reprogramming, an explicit scientific justification is needed and the numbers of trials to be attempted must be justified.

12.1c. Researchers must demonstrate appropriate expertise or training in the culture and maintenance of existing human embryonic stem cell lines and expertise or training in the derivation of pluripotent nonhuman stem cell lines before being granted permission for attempts at derivations of new human stem cell lines.

12.1d. Investigators performing derivations should have a detailed, documented plan for characterization, storage, banking and distribution of new lines.

12.1e. Embryos made via nuclear transfer, parthenogenesis, androgenesis, or other in vitro mean of embryo production shall not be transferred to a human or nonhuman uterus or cultured in vitro intact as embryos for longer than 14 days or until formation of the primitive streak, whichever occurs first.

12.1f. Investigators performing derivations should propose a plan to safeguard the privacy of donor information.

12.2. Banking of stem cell lines: The ISSCR encourages the establishment of national and international repositories, which are expected to accept deposits of newly derived stem cell lines and to distribute them on an international scale. In order to facilitate easy exchange and dissemination of stem cell lines, repositories should strive to form and adhere to common methods and standards; at a minimum, each repository must establish its own clear guidelines and make those available to the public. Repositories must have a clear, easily accessible material transfer agreement (MTA) [a link to a sample MTA is available in the Internet Resources and can be downloaded from the ISSCR website]. Each repository may have its own criteria for distribution. The repository has right of refusal if a cell line does not meet its standards.

12.2a. Repository must have clear, publicly available protocols for deposit, storage and distribution of hESC lines and related materials.

12.2b. For deposits, repository must receive documentation pertinent to the depositor's SCRO process. These documents should be kept on file at the repository. This will include, but is not limited to, proof of institutional and/or SCRO approval of the process for procurement of research materials according to ethical and legal principles of procurement as outlined in these Guidelines, approval of protocols for derivation of new lines, copies of the donor informed consent documents and what, if any, reimbursement of direct expenses or financial considerations of any kind were provided to the donors.

12.2c. Repository should obtain all technical information from depositor. For example, methods used in the derivation of lines, culture conditions, infectious disease testing, passage number and characterization data. Repository will make this information publicly available. If repository modifies depositor's protocols or obtains additional data this will also be made available.

12.2d. Repository should engage in, but is not limited to, the following:

i. Reviewing and accepting deposit applications
ii. Assigning unique identifiers (catalogue number) to deposits
iii. Characterizing cell lines

iv. Human pathogen testing
v. Expansion, maintenance, and storage of hESC lines
vi. Quality assurance and quality control of all procedures
vii. Maintenance of website with pertinent characterization data, protocols and availability of hESC lines
viii. Tracking distributed cell lines
ix. Posting a clear cost schedule for distribution of materials. Repositories should distribute internationally and charge only the necessary costs, which include shipping and handling.

12.3. Provenance of stem cell lines: Owing to the nature of the materials involved in the generation of human stem cell lines, appropriate safeguards should be used to protect the privacy of donors and donor information. In order for the stem cell lines to be as useful as possible and so as not to preclude future potential therapeutic applications, as much donor information as possible should be maintained along with the cell line, including, but not limited to: ethnic background, medical history, and infectious disease screening. Subject to local laws, donor samples and cell lines should be de-identified (anonymized) and coded using internationally accepted standards for maintaining privacy. Informed consent and donor information will be gathered and maintained by the repository, including whatever reimbursement of direct expenses or financial considerations of any kind were provided in the course of the procurement. Documentation of the provenance of the cell lines is critical if the cell lines are to be widely employed in the research community, and the provenance must be easily verified by access to the relevant documents.

12.4. Maintenance of a database of human stem cell lines and verification of provenance: The ISSCR will curate and maintain a website listing of human stem cell lines that testifies to independent validation of the provenance of the cell lines. It will become the responsibility of the ISSCR Standards Committee to scrutinize the documents relevant to the derivation of stem cell lines to vouch for the provenance of the cell lines, according to the principles laid out in these Guidelines.

13. DISPUTE RESOLUTION

Any conflicts of interest or other conflicts or disputes that may arise in the course of any international collaboration, for example, disagreements or difference of opinions between researchers from different countries involved in common projects, may be resolved in accordance with an agreed-upon dispute resolution mechanism in a forum with international representation from countries doing research and clinical trials in human stem cells. Members of the forum will, as appropriate, seek guidance from experts in the fields of science, ethics, law and medicine from different national, social and religious backgrounds. It is recommended that all international collaboration agreements incorporate a dispute resolution provision providing that any disputes or differences shall be settled through mediation or arbitration by international forum, and this provision shall stipulate whether or not any decision made by the forum will be binding on the relevant parties.

14. ONGOING REVIEW OF GUIDELINES: CALL FOR FORMATION OF A NEW INTERNATIONAL GUIDELINES COMMITTEE OF THE ISSCR

14.1. These guidelines should be revised and updated on a regular basis to accommodate scientific advances and to address specific scientific issues in the order of priority based on the degree of urgency. These guidelines should be applicable to stem cell research internationally and should continue to address the challenges of international collaboration.

14.2. New ethical challenges in the conduct of stem cell research that are on the horizon must be addressed in a timely manner to ensure that our science proceeds in a socially responsible and ethically acceptable fashion. To enhance the likelihood that the international scientific research community will be bound together by a common set of principles governing the performance of stem cell research, we recommend the creation, under the auspices of the ISSCR, of a committee that includes members with expertise in various aspects of stem cell biology, including but not limited to the scientific issues pertinent to human embryo and pluripotent stem cell research and the ethical considerations of such research, supplemented by external contributors with supplemental and complementary expertise as needed. This committee will evaluate the Guidelines on a yearly basis, updating and revising, and providing guidance and counsel on the ethical, social and legal aspects as stem cell science evolves. The committee will coordinate with other national and international efforts to promote a uniform set of governing principles for the field.

15. ACKNOWLEDGMENTS

The establishment of these Guidelines represent the fruits of a global conversation among researchers, ethicists, and legal experts from 14 countries and a diversity of scientific and intellectual perspectives. No such document stands independent of documents and discussions that preceded it. Indeed, these Guidelines borrow extensively from the principles and the language established by the Committee on Guidelines for Human Embryonic Stem Cell Research (2005) of the National Research Council and Institute of Medicine of the National Academy of Sciences of the USA. Other important source materials include the Medical and Ethical Standards Regulations of the California Institute for Regenerative Medicine and the Hinxton Group's Consensus Statement (2006). The thoughtful deliberations that characterized these earlier efforts provided a sound foundation upon which to launch our own inquiry into the subject. The ISSCR wishes to acknowledge the financial support of The Genetics and Public Policy Center of John Hopkins University (Director: Kathy Hudson, PhD), the Norwegian Research Council, Sung Chull Junn Esq., and WilmerHale.

The members of the Task Force and ad hoc contributors are listed below:

George Daley, Chair
Lars Ahrlund-Richter
Jonathan Auerbach
Nissim Benvenisty
R.Alta Charo
Grace Chen
Hongkui Deng
Lawrence Goldstein
Kathy Hudson
Insoo Hyun
Sung Chull Junn
Jane Love
Eng Hin Lee
William Lee
Anne McLaren
Christine Mummery
Norio Nakatsuji
Catherine Racowsky
Janet Rossant
Hans Schoeler
Jan Helge Solbakk
Patrick Taylor
Alan Trounson
Irving Weissman
Ian Wilmut
John Yu
Laurie Zoloth

Ad hoc:
Richard Hynes
M.William Lensch
Leonard Zon

ISSCR staff:
Michael Hagedorn
Heather Rooke
Nancy Witty

Full member affiliations can be found at *http://www.isscr.org/guidelines/index.htm*

16. APPENDICES

16.1. Definitions and Discussion of Scientific Terminology as Used in These Guidelines

16.1a. The term 'embryo' and other terms used to describe early stages of development

Embryo: The term 'embryo' has been defined and used differently in various biological contexts as discussed below.

In this document, the term 'embryo' is used generically to describe all stages of development from the first cleavage of the fertilized ovum to nine weeks of gestation in the human (and to term in the mouse). More precise terms have been used to describe specific stages of embryogenesis; for example, the two, four and eight cell stages, the compacting morula and the blastocyst all describe particular stages of early pre-implantation embryonic development.

Prior to implantation, the embryo represents a simple cellular structure with minimal cellular specialization, but soon after implantation a defined axis of development called the primitive streak begins to form. After this time twinning of the embryo can no longer occur as there is irreversible commitment to the development of more complex and specialized tissues and organs.

Classical embryology used the term embryo to connote different stages of post-implantation stages of development (e.g., the primitive streak and onwards to fetal stages). Indeed, Dorland's Illustrated Medical Dictionary (27th edition, 1988 edition, W. B. Saunders Company) provides the definition "in animals, those derivatives of the fertilized ovum that eventually become the offspring, during their period of most rapid development, i.e., after the long axis appears until all major structures are represented. In man, the developing organism is an embryo from about 2 weeks after fertilization to the end of seventh or eighth week." An entry in Random House Webster's College Dictionary reads "in humans, the stage approximately from attachment of the fertilized egg to the uterine wall until about the eighth week of pregnancy." However, the nomenclature is often extended by modern embryologists for the human to include the stages from first cleavage of the fertilized ovum onwards to seven to nine weeks of gestation, after which the term fetus is used.

Zygote: The fertilized single cell pronuclear ovum (egg), typically observed in humans between 20 to 35 hours after insemination with sperm.

Cleavage Stage Embryo (pre-implantation stage): The cleaving or dividing zygote; precise terms include the 2- cell, 4-cell, 8-cell and 16-cell embryo. In humans, each cleavage division consumes around 18 to 24 hr.

Morula: The compacting grape-like cluster of 16-cells, typically formed 4 days after fertilization.

Blastocyst: The embryonic stage formed from 64 cells onward, defined by the pumping of fluid into an internal space. The blastocyst is surrounded by a ring of differentiated trophectoderm cells, and encloses a nest of 10 to 25 cells termed the Inner Cell Mass (ICM). The trophectoderm cells attach the embryo to the uterine wall, and the ICM forms the embryo proper. The blastocyst forms 5 to 7 days after fertilization. The blastocyst hatches from the zona pellucida (glycoprotein shell) around days 6 to 7 after fertilization. Thereafter, and coupled to implantation, which provides requisite signals for the maturation of the embryo, the ICM of the blastocyst begins to organize itself into a long axis with anterior and posterior orientation. If isolated from the trophectoderm, the ICM never adopts an axis, and after this time it is not possible for the cultured ICM to form an organism, but instead will form a teratoma (disorganized differentiating cell body) when transplanted.

Gastrula: The embryonic stage of formation of the trilaminar embryonic disc, leading to the specification of ectoderm, endoderm, and mesoderm.

Neurula: The embryonic stage when the neural plate is closing to form the neural tube.

Parthenogenetic embryo: Activation of the unfertilized mammalian ovum can result in embryonic development, and embryonic stem cells can be derived from the ICMs of parthenogenetic blastocysts. After uterine transfer, parthenogenetic embryos can progress to a fetal stage, but further development is compromised by an underdeveloped placental system that prevents normal gestation.

Gynogenesis: A particular form of parthenogenesis in which an embryo is created from the genetic contributions (female pronuclei) of two different fertilized oocytes.

Androgenesis: The creation of an embryo that incorporates the male pronuclei from two different fertilized oocytes.

Nuclear Transfer: The insertion of a nucleus of a cell into an ovum from which the nuclear material (chromosomes) has been removed. The ovum will reprogram (incompletely) the cell nucleus to begin development again. Embryos created by nuclear transfer are typically abnormal and often die during development, but rarely are capable of development to term. ICMs from blastocysts derived by nuclear transfer will form apparently normal embryonic stem cells.

Altered Nuclear Transfer: A process whereby the genetic material of the donor cell is altered prior to nuclear transfer, such that implantation and subsequent development of an embryo is not possible. Pluripotent stem cells can be derived following the process of altered nuclear transfer.

This technique was employed in the mouse by knock-down of expression of the *cdx2* gene. The *cdx2* gene product is critical for the formation of the trophoblast which is critical for post-implantation development. When expression of cdx2 was knocked-down by short hairpin RNAs in the donor cell nucleus prior to nuclear transfer, the developmental potential of the nuclear transfer embryo was compromised; the trophoblast was unable to develop and support post-implantation development. However, ICM structures formed, and embryonic stem cells were successfully generated.

Meissner, A. and Jaenisch, R. (2006). Generation of nuclear transferderived pluripotent ES cells from cloned Cdx2-deficient blastocysts. *Nature* 439:212-215.

Fetus: In this document, the term ‘fetus’ is used to describe post-embryonic stages of prenatal development, after major structures have formed. In humans, this period is from seven to nine weeks after fertilization until birth.

16.1b. Terminology relating to developmental potential

Totipotent: The state of a cell that is capable of giving rise to all types of differentiated cells found in an organism, as well as the supporting extra-embryonic structures of the placenta. A single totipotent cell could, by division in utero, reproduce the whole organism.

Pluripotent: The state of a single cell that is capable of differentiating into all tissues of an organism, but not alone capable of sustaining full organismal development, because for instance, it lacks competency to generate the supporting extra-embryonic structures of the placenta.

16.1c. The term ‘chimera’ in stem cell research

Trace Chimeras: The simplest form of chimera is one in which a limited number of human cells are introduced at any stage of

pre-or post-natal development, and where incorporation into any lineage or tissue is likely to be minimal. An example is the use of an immuno-deficient mouse as a host to study tumor formation from a human cancer cell line. Such chimeras require oversight appropriate to animal use and biosafety (among others as deemed appropriate by local regulatory bodies), and typically will not raise significant concerns unique to human stem cells. Any trace human/animal chimera that carries human germ-lineage cells bears special concern.

Interspecies Chimeras: Interspecies chimeras are those animals containing extensive and integrated cellular contributions from another species. There are two types of true human/animal chimeras bearing special concern: those formed at the earliest stages of development and those formed later but contributing a significant degree of chimerism to the central nervous system and/or germline. Human/nonhuman primate chimeras formed at any stage of development warrant particular attention. Human/nonhuman chimeras bearing central nervous system chimerism also warrant particular attention.

Hybrids: Animals formed in which each of the individual cells carry roughly equal genetic contributions from two distinct species resulting from inter-breeding of species or fusion of genetic material. Examples include the mule (horse bred to a donkey). Hybrids are only likely to survive if the genetic contributions derive from closely related species. The greatest concern would be for experiments that entail creation of hybrids between humans and closely related nonhuman primates.

16.2. Web links: International, National, and Local Regulations and Reports Pertinent to Stem Cell Research

Belmont Report (1979)
Ethical Principles and Guidelines for the Protection of Human Subjects of Research
http://ohsr.od.nih.gov/guidelines/belmont.html

Council for International Organizations for Biomedical Sciences (CIOMS): International Ethical Guidelines for Biomedical Research Involving Human Subjects (2002)
http://www.cioms.ch/frame_guidelines_nov_2002.htm

Guidelines for Human Embryonic Stem Cell Research
Authored by the Committee on Guidelines for Human Embryonic Stem Cell Research, NATIONAL RESEARCH COUNCIL AND INSTITUTE OF MEDICINE OF THE NATIONAL ACADEMIES (USA)
http://www.nap.edu/books/0309096537/html

Hinxton Group's Consensus Statement (2006)
http://hinxtongroup.org/consensus/consensus.html

Human Fertilisation and Embryology Authority (HFEA)
Home Page
http://www.hfea.gov.uk/cps/rde/xchg/hfea
Code of Practice
http://www.hfea.gov.uk/cps/rde/xchg/SID-3F57D79BF6DA3CC3/hfea/hs.xsl/411.html
Research license application
http://www.hfea.gov.uk/cps/rde/xchg/SID-3F57D79BF6DA3CC3/hfea/hs.xsl/376.html
EU Tissues and Cells Directives
http://www.hfea.gov.uk/cps/rde/xchg/SID-3F57D79BF6DA3CC3/hfea/hs.xsl/493.html

Nuremburg Code (1947)
http://www.nihtraining.com/ohsrsite/guidelines/nuremberg.html

Regulations of the California Institute for Regenerative Medicine
Medical and Ethical Standards Regulations
http://www.cirm.ca.gov/laws/default.asp

UNESCO Universal Declaration on Bioethics and Human Rights (2005)
http://portal.unesco.org/shs/en/ev.php-URL_ID=1883&URL_DO=DO_TOPIC&URL_SECTION=201.html

World Medical Association Declaration of Helsinki (1964 and updates)
Ethical Principles for Medical Research Involving Human Subjects
http://www.wma.net/e/policy/b3.htm

INTERNET RESOURCES

16.3. URL Links to Sample Consent Documents for Procurement of Human Biological Research Materials for Stem Cell Research

http://www.isscr.org/guidelines/CFeggsresearch.doc
Egg donation for stem cell research; provided directly and solely for stem cell research
http://www.isscr.org/guidelines/CFeggsexcessofclinical.doc

Egg donation for stem cell research; collected during the course of fertility treatment and in excess of clinical need
http://www.isscr.org/guidelines/CFembryos.doc
Embryo donation for stem cell research; created for fertility purposes and in excess of clinical need
http://www.isscr.org/guidelines/CFsomatic.doc
Somatic cell donation for stem cell research
http://www.isscr.org/guidelines/CFsperm.doc
Sperm donation for stem cell research

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16.4. URL Link to Consent Document for Sample Material Transfer Agreement (MTA)

[Adapted and modified from the uniform biological material transfer agreement published in the Federal Register, February 18, 1995]

http://www.isscr.org/guidelines/mta.doc
Sample Material Transfer Agreement (MTA) document

Acknowledgment. The sample material transfer agreement document was prepared by task force member **Patrick Taylor, JD.**

Standard Laboratory Equipment

ABSTRACT

This appendix lists pieces of equipment that are standard in the modern cell biology laboratory, i.e., items used extensively in this manual and thus not usually included in the individual materials lists. *Curr. Protoc. Stem Cell Biol.* 1:A.2.1-A.2.2. © 2007 by John Wiley & Sons, Inc.

INTRODUCTION

Listed below are pieces of equipment that are standard in the modern stem cell biology laboratory, i.e., items used extensively in this manual and thus not usually included in the individual materials lists. No attempt has been made to list all items required for each procedure in the Materials list of each protocol; rather, those lists note those items that might not be readily available in the laboratory or that require special preparation. See *SUPPLIERS APPENDIX* for contact information for commercial vendors of laboratory equipment.

Applicator, cotton-tipped and wooden
Autoclave
Bag sealer
Balances, analytical and preparative
Beakers
Bench protectors, plastic-backed (including "blue" pads)
Biohazard disposal containers and bags
Biosafety cabinet, tissue culture or laminar flow hood; filters air and maintains air flow pattern to protect cultured cells from investigator and vice versa
Bottles, glass, plastic, and squirt
Bunsen burners
Centrifuges, low-speed (to 20,000 rpm) refrigerated, ultracentrifuge (20,000 to 80,000 rpm), large-capacity low-speed, tabletop, with appropriate rotors and adapters
Centrifuge tubes and bottles, plastic and glass, various sizes
Clamps
Conical centrifuge tubes, plastic and glass
Containers, assortment of glass and plastic, for gel and membrane washes
Coplin jars, glass, for 25 × 75–mm slides
Cryovials, sterile (e.g., Nunc)
Cuvettes
Desiccator and desiccant
Dry ice
Electrophoresis equipment, agarose and acrylamide, full-size and mini, with power supplies
Film developing system and darkroom
Filtration apparatus
Forceps
Fraction collector
Freezers, −20°C, −70°C, and liquid nitrogen
Fume hood
Geiger counter
Gel dryer
Gloves, disposable plastic and heat resistant
Graduated cylinders
Heating blocks, thermostatically controlled for test tubes and microcentrifuge tubes
Hemacytometer and/or electronic cell counter
Homogenizer
Humidified CO_2 incubator
Ice bucket
Ice maker
Immersion oil for microscopy
Lab coats
Laboratory glassware
Light box
Liquid nitrogen
Lyophilizer
Magnetic stirrer, with and without heater, and stir bars
Markers, including indelible markers, china-marking pens, and luminescent markers
Microcentrifuge, Eppendorf-type with 12,000 to 14,000 rpm maximum speed
Microcentrifuge tubes, 0.2-, 0.5-, 1.5-, 2-ml
Microscope slides, glass, 25 × 75–mm, and coverslips
Microscope with camera, upright, inverted, fluorescence, phase-contrast, dissecting
Microtiter plate reader
Mortar and pestle
Ovens, drying and microwave
Paper cutter, large
Paper towels

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Parafilm
Pasteur pipets and bulbs
PCR thermal cycler and tubes
pH meter
pH paper
Pipets, graduated
Pipettors, adjustable delivery, 0.5- to 10-µl, 10- to 200-µl, and 200- to 1000-µl
Polaroid camera or video documentation system
Power supplies, 300-V for polyacrylamide gels, 2000- to 3000-V for other applications
Racks, test tube and microcentrifuge tubes
Radiation shield, Lucite or Plexiglas
Radioactive waste containers for liquid and solid wastes
Refrigerator, 4°C
Ring stand and rings
Rubber policemen or plastic scrapers
Rubber stoppers
Safety glasses
Scalpels and blades
Scintillation counter, β
Scissors
Shakers, orbital and platform, room temperature or 37°C
Spectrophotometer, visible and UV range
Speedvac evaporator
Syringes and needles
Tape, masking, electrician's black, autoclave, and Time tape
Test tubes, glass and plastic, various sizes, with and without caps
Timer
Toolbox with common tools
Trays, plastic and glass, various sizes
Tubing, rubber and Tygon
UV light sources, long- and short-wavelength
UV transilluminator
UV transparent plastic wrap (e.g., Saran Wrap)
Vacuum desiccator
Vacuum oven
Vacuum supply
Vortex mixers
Waring blendor
Water bath with adjustable temperature
Water purification system
X-ray film cassettes and intensifying screens