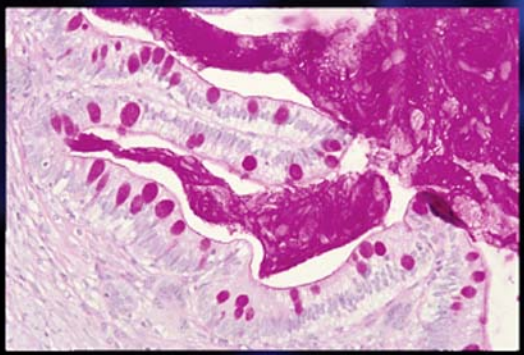
Human Stem Cell Manual

A LABORATORY GUIDE



JEANNE F. LORING
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A Laboratory Guide

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Foreword

Stem cells have the unique ability to generate more than one type of specialized cell. Each daughter cell has the potential to either remain a stem cell or to begin to differentiate into a more specialized cell, such as a nerve cell, a liver cell, or a heart muscle cell. Human stem cells offer scientists a remarkable platform for understanding the fundamental mechanisms of differentiation. What we learn from stem cells can help us develop novel ways to treat diseases and injuries, including correcting birth defects, reprogramming adult cells to generate multipotent cells, generating tissues for transplantation, and growing human cells in culture to test potential new drugs for toxicity and efficacy. While it is difficult to predict exactly how stem cell science will inform the medicine of the future, there is no doubt that regenerative medicine will revolutionize patient intervention and treatment strategies.

Except for blood-forming stem cells, the field of human stem cell research is still in its infancy. Scientists must build a strong foundation of basic knowledge before they can develop safe and effective therapies using the stem cells themselves or with the knowledge that we gain from them. Challenges that are still before us include developing better methods for sorting, identifying, and culturing the cells, determining their long-term stability, understanding genes and growth factors that control their specialization, regulating the cell cycle, and understanding the interactions between stem-cell derived tissues for transplantation and the host.

It is my hope that research scientists will use this book to overcome these challenges and develop stem cell technologies that improve human health.

James F. Battey, Jr, MD, PhD
Director, National Institute on Deafness and,
Other Communication Disorders;
Chair, NIH Stem Cell Task Force, Bethesda, MD

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Foreword

While we live in a society that places great importance on movie stars and athletes, it really is you scientists who are the untold heroes. You are the ones embarking into a new scientific frontier, one that holds such hope and promise to the millions who suffer from diseases. The overwhelming victory of Proposition 71, The Stem Cell Initiative in California, will go down in history as one of the great grass roots efforts. Millions of Californians cast their ballots to support their belief in the value of embryonic stem cell research. It sent a loud message to Washington, DC that Californians disagree with the current Administration's policy and that we are willing to take on a great financial burden in return for the potential of therapies that will improve the lives of those in California, the nation and the world. And the world is watching!

While exploring the use of stem cells in identifying drug delivery systems, developing new models of disease, new methods of toxicological screening, high-throughput drug discovery systems and tissue regeneration, I implore you to stay focused on the patient. While basic research for the sake of basic research is important, keep in mind that the end purpose is the treatment of the patient. Remember those who are desperately waiting for a therapy – not necessarily a cure – but a treatment for debilitating symptoms and thus improve their lives.

I applaud you and all involved who will explore this very exciting field of stem cell research. You are like the brave explorers of the past challenging knowledge of the known world as you embark on penetrating this new scientific frontier. Your efforts hold enormous hope and promise for the millions who suffer from yet untreatable diseases.

Jeannie Fontana, MD, PhD, Bel Air, California
ALS Research and Patient Advocate,
Founder of the Cedars Sinai ALS Center

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Preface

It's appropriate that I'm writing this preface while I'm at a meeting at Cold Spring Harbor Laboratory, because it was my experience at CSH courses that gave me the inspiration for our human embryonic stem cell training courses, and it was Tom Maniatis' "Molecular Cloning-A Laboratory Manual" that inspired my decision to write this book. CSH has changed since I took the Developmental Neurobiology course here in 1980, but the essence is still there – a quiet, idyllic, and exciting place for biologists to meet and learn from each other.

Every book, even a laboratory manual, has a personal story behind it; briefly, this is mine. On Friday morning, August 10, 2001, I picked up the newspaper from my doorstep in Foster City, California, and read on the front page that at 9:01 pm Thursday, President George W. Bush had announced that the NIH would begin supporting research on any human embryonic stem cells that existed at that moment.

To my surprise, those 60 existing hESC "derivations" included nine that I had initiated myself. The 60 grew to 78 as more scientists reported in, but as I finish this book, the number has dwindled to 21. Fifty-seven of the 78 were like mine: early stage experimental cultures that didn't further expand into robust lines.

NIH management, especially Jim Battey, lost no time in establishing funding mechanisms for hESC research. One of the first was a request for applications to develop an hESC training course. In 2002, two people at the NIH, John Thomas and Arlene Chiu (who was one of my classmates at the memorable 1980 Developmental Neurobiology course at CSH) introduced me to Phil Schwartz, and he and I decided to write the grant together. After we learned that the grant was funded, in the spring of 2003, we became serious about finding a location for the course. Luckily, that summer Evan Snyder invited me to be the inaugural speaker for his monthly Southern California Stem Cell Consortium at the Burnham Institute, and I visited the campus for the first time. I noticed immediately that the Burnham campus had some of the ambience of Cold Spring Harbor – isolated, quiet and full of smart people.

By January 2004, I had joined the Burnham faculty and convinced my old friend, Robin Wesselschmidt, who is the best in the world at mouse ES cell culture, to come from St. Louis to help direct the course. We got rid of the boxes in an unused lab at the Burnham, and, with the grant funds and nearly $200,000 in donated and loaned equipment and reagents, we held the first course for 12 students in March 2004. It was modeled after the CSH courses – a total immersion in hESC techniques for ten days. We invited outside scientists to give lectures about things we wanted to learn about, and a dozen postdocs, graduate students and faculty from the Burnham Institute joined in to help us. By the end of the course, both the students and faculty were exhausted but elated, just as we'd hoped!

During the first course, we started writing the protocols that would become the basis for this book – often finishing a chapter just as the students arrived in the lab to do the experiments. By the time we ran the second course, at Phil's place, the Children's Hospital of Orange County, I realized that we were walking in Maniatis' footsteps, and should follow through and write a laboratory manual. Elsevier Press liked the idea, and Robin, Phil, and I began recruiting experts to write the first drafts of the chapters – asking specifically for postdoc-mentor pairs – one who knew the hardcore methods and the other to provide context. Over the next two years we wrote, rewrote, and edited the chapters, and I refined the text to evoke a common voice throughout the book. During our fourth course this spring, we made the final edits to the galley proofs.

I could not have found better partners for either the course or the book than Robin and Phil. The three of us, with our different styles and points of view, challenge each other, and both of them are people who "under-promise and over-deliver." I advise students to choose an hESC course that has more than one director – there are so many points of view in this field that it would be unfortunate to learn only one.

Writing this book has taught me a great deal – I now know how to read a karyotype, how to select human IVF embryos for transfer, and how to do SNP genotyping. I hope that the readers of this book will find that it expands their understanding of human ES cells and directs their research toward new discoveries. I hope that the readers will tell us when they find errors or better methods, so that our next edition can be better.

Most of all, I hope that the classmates from our courses stay in touch with each other for years and years, as Arlene and I and the others from the 1980 Cold Spring Harbor course have done, and that they become colleagues for life, as we have.

Jeanne Loring, Spring 2007

Preface

The publication of *Human Stem Cell Manual: A Laboratory Guide* is the culmination of countless hours of reading writing and experimentation at the laboratory bench by the experts who contributed the protocols used in their own laboratories to the timely publication of this lab manual. It is our hope that even in the rapidly evolving field of human embryonic stem cell research most of these protocols will remain useful and that this manual will provide a ready and reliable reference source that is opened, read, and re-read by both the novice and the experienced stem cell investigator.

We assembled the techniques that are most commonly used to maintain, validate, and differentiate stem cells, especially human embryonic stem cells, in one manual. We expect that assembling these protocols in this straightforward "how-to" format will aid those working in the field, especially those new to embryonic stem cell research, and help move the field forward.

Our goal is that this manual will find a place at the lab bench and have many dog-eared corners, sticky notes, and hand-written "notes to self" in the margins.

I have truly enjoyed being part of the NIH-sponsored human embryonic stem cell training courses that were the seeds from which this manual grew, the students, the instructors, and the expert faculty that made those annual courses successful have been great fun and thoroughly enjoyable both personally and professionally.

I thank Jeanne Loring and Phil Schwartz, my co-editors, without whom this manual would not have been possible.

And to Jeanne, who recognized the broader impact our training course notes and protocols could have in helping those entering the field and was the powerhouse that drove the transformation of these protocols into this formalized laboratory manual, a very special thanks.

Robin L. Wesselschmidt

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Preface

This book is one product of a relatively short but very interesting journey. The journey for me began in 1999 when I teamed with Theo Palmer and Rusty Gage to see if we could reliably harvest human neural stem cells from post-mortem human brains, particularly those from patients with neurogenetic disease. As a result of the success of those efforts, the focus of my scientific life shifted entirely to the study of neural stem cells. A major part of this new line of scientific inquiry was to establish, in early 2001, a repository of human neural stem cells so that I could share these cells with other scientists.

I approached Arlene Chiu, who was then at the NINDS, to see what kind of funding might be available to support this resource, and she came to visit my laboratory in mid-2002. At about the same time, the Office of the Director at the NIH, knowing of my stem cell repository operations, asked me to consider adding human embryonic stem cells, then in the possession of Jeanne Loring, to my collection, for distribution to other scientists. Jeanne and I met and agreed to move forward with this if legal obstacles could be overcome. More importantly, Arlene, knowing Jeanne and of my new scientific relationship with her, suggested that I partner with Jeanne and apply for one of the NIH's T15 grants for human embryonic stem cell training.

We offered the first course in 2004, and it became obvious immediately that the courses were mammoth undertakings and we had to generate everything, including all the written materials, from scratch. It was shortly after the first course that we began to write the first comprehensive laboratory book on human stem cell culture, a book whose primary focus was human embryonic stem cells but which also included other stem cells and most pertinent associated laboratory techniques. This book, although carefully and painstakingly shepherded through the production process by the editors, really is the result of the participation of many exemplary stem cell scientists as chapter authors and of fine-tuning by the students, course instructors, and experiences of the last three courses.

I am humbled by the quality of this book and am very proud to have played a part in its production. My sincerest hope is that beginning stem cell scientists find this book useful for their laboratories and that it helps propel them to new frontiers in stem cell research.

Philip H. Schwartz

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PART

I

Basic Methods in Stem Cell Culture

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CHAPTER

1

Human Embryonic Stem Cell Culture

Rudolfo Gonzalez, Robin L. Wesselschmidt, Philip H. Schwartz, and Jeanne F. Loring

INTRODUCTION

Culturing human embryonic stem cells (hESCs) requires a significant commitment of time and resources. It takes weeks to establish a culture, and the cultures will require daily attention. Once hESC cultures are established, they can, with skill and the methods described below, be kept in continuous culture for years.

A word of caution for those with experience culturing mouse embryonic stem cells: they are not the same! Both mouse and human ESCs are diploid, they are pluripotent, and they are relatively stable in culture. However, the stability of mouse ESC lines is regularly measured because the objective of almost all genetic manipulation is to make new lines of mice. If an ESC line can generate a mouse, as we term it, "go germline," we know that it is clearly pluripotent. This has given us an operational definition for pluripotence and stability in culture for mouse ESCs.

hESC lines were originally derived using very similar culture medium and conditions as those developed for the derivation and culture of mouse ESC lines. However, these methods were suboptimal for hESCs, and have evolved considerably in the years since hESC lines were derived. Compared with mouse ESCs, hESCs are very difficult to culture – they grow slowly, and most importantly, since we have no equivalent

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assays for germline competence, we cannot assume that the cells that we have in our culture dishes are either stable or pluripotent. This makes it far more critical to assay the cells frequently, using characterization methods such as the karyotyping, immunocytochemistry, gene expression analysis, and fluorescence activated cell sorting (FACS) methods provided in this manual.

Overview

In this chapter we outline protocols for the culture of hESCs, starting as one would usually do, by being handed a culture by an experienced colleague. Other chapters focus on cryopreservation and establishing hESC cultures from frozen stocks, and on the variety of culture conditions, including the preparation of various types of feeder layers, conditioned medium, and extracellular matrix substrata.

The methods we recommend are those that are the most straightforward and have worked well in our hands; these are offered as the recommended methods and reagents. We also offer alternative methods and reagents that work but are not routinely used in most laboratories. The key variables that we outline in this chapter are:

- Culture medium
  - Basal medium
  - Serum or serum substitute
- Passaging cells
  - Manual passage
  - Non-enzymatic dissociation
  - Enzymatic dissociation.

While optimizing and standardizing conditions in your lab, it is important to keep in mind that changing one thing in a system may have unexpected impact on the entire system.

Procedures

Tips for successfully culturing hESCs

- Feed cells *every day*, except for 1 or 2 days following passage.
- Examine the cultures *every day* under 4× and 10× phase contrast. This will allow you to become familiar with the morphologies of undifferentiated and differentiated cells and colonies.
- When they are cultured on feeder layers some hESC lines tend to undergo spontaneous differentiation in the centers of the colonies. When passaging, take care to avoid passaging these differentiated "centers" to the new culture.
- Most hESC lines double every 31–35 h.
- Store medium at 4°C and discard any unused medium after 10 days. Best results are achieved when medium is prepared in small batches once a week.

Recognizing hESC morphology

The single most important skill in successful culturing of hESCs may be the ability to recognize the morphology of undifferentiated cells under a variety of conditions (Figure 1.1).

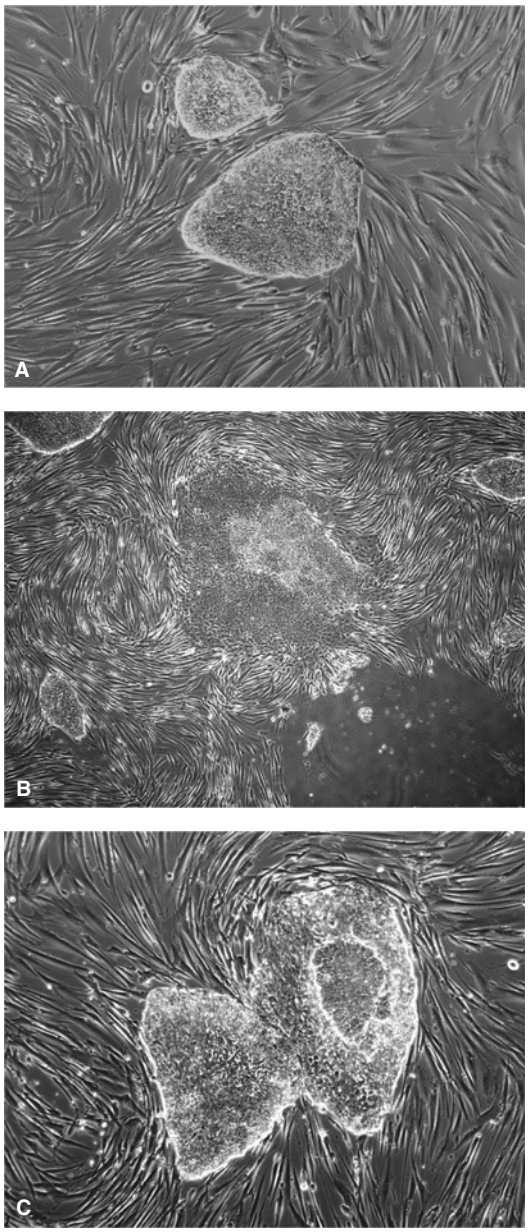

FIGURE 1.1 Phase contrast micrographs from the same culture, 4 days after it was passaged onto a feeder layer (human foreskin fibroblasts, ATCC HS27). (A) Typical colonies with smooth, phase-bright edges, with the fibroblast feeder layer forming whorls around the colonies (10× magnification). In contrast, in the same culture there are colonies with obvious differentiation at the edges (B – 4× magnification) and in the center. (C – 10× magnification). In selecting colonies for passage and expansion, only the ones shown in (A) would be acceptable. The others should not be passaged to the next culture dish.

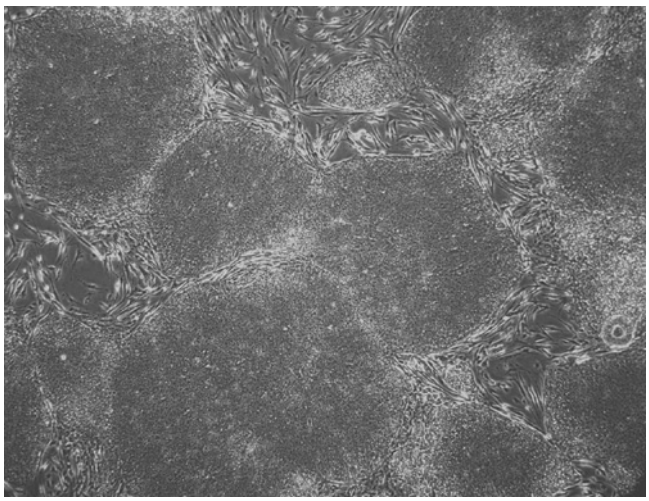

FIGURE 1.2 hESCs can be cultured to high density.

For routine expansion of hESCs, we recommend that the cells be cultured at a relatively low density so that individual colonies can be easily monitored and selected against differentiation. hESCs can be cultured to high density (Figure 1.2), but a higher proportion of differentiated cells must be expected.

Passaging hESCs

hESCs, unlike mouse ESCs, do not survive well when dissociated to single cells. Therefore, the most reliable method for passaging undifferentiated hESC cultures is manual dissection of the colonies. This method may seem tedious, but it is virtually foolproof and we recommend that novices use this method until they have familiarity with the cells and can easily recognize differentiation in the culture. We also recommend manual passaging for producing cell banks of low-passage hESCs. Enzymatic dissociation methods are provided in Alternative Procedures.

NOTE: Using the number of passages as a measure of the age of an hESC line is an unfortunate historical accident. Because of the inconsistencies in hESC culture procedures in different labs, cells are passaged at different time intervals, ranging from 4 to 7 days. Therefore the number of passages for one line might be twice that of another, even though the cells have been in culture for exactly the same amount of time. For example, in a year of continuous culture, a cell line could be passaged as few as 52 and as many as 90 times. A better measure would be the number of doublings, but to count the number of cells in a culture is difficult since the cells form tight clusters and are not passaged as single cells.

General guidelines

- The cells should be passaged at about 1:3 every 5–7 days.
- Prepare the feeder layer or extracellular matrix (ECM) substrata the day before passaging.
- Depending on the cell line, passaging on Friday may be a good routine. The cells can usually be left undisturbed for 2 days following passaging, which allows them to settle down on the substrata, attach and begin dividing before the medium is changed.

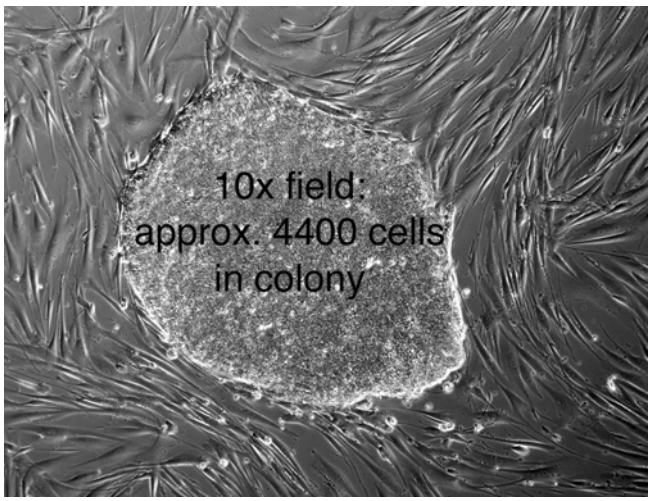

FIGURE 1.3 A colony about half the diameter of the 10× field contains about 4400 cells.

- There will be considerable variation in the size of colonies in a single dish. Compared with their mouse counterparts, hESCs do not substantially pile up on each other, and their colonies can grow to a large diameter while remaining undifferentiated. Culture conditions affect the flatness of the colonies, but as an approximation, they are ready to split when the diameter fills the 10× field when observed under the microscope. As shown in Figure 1.3, a colony about half the diameter of the 10× field contains about 4400 cells. A colony filling the field would contain about 15 000 cells.
- For routine passaging by any method, *do not make a single-cell suspension*; dissociate the colonies into smaller colonies of a few hundred cells.
- Examine the culture daily for colony morphology under the phase contrast or dissecting microscope.
- With experience, one can get a good overview of colony morphology by holding the dish up to a light and looking at the bottom of the dish. The differentiated colonies will have ragged edges and hollow centers.
- On the bottom of the dish, mark colonies that are badly differentiated or parts of the colony that you do not wish to transfer to a new culture dish.
- To be certain that the colonies selected are undifferentiated, it is advisable to dissect the colonies while viewing the dish under a dissecting microscope with illumination from the base. But this is not absolutely necessary, and some prefer to passage the cells without magnification.

Mechanical dissociation

1. Evaluate the culture under 4× or 10× phase contrast optics.
2. The cells can be split among 3–6 dishes of the same size as the original culture, depending on the density of the original culture. If you wish to put the cells in

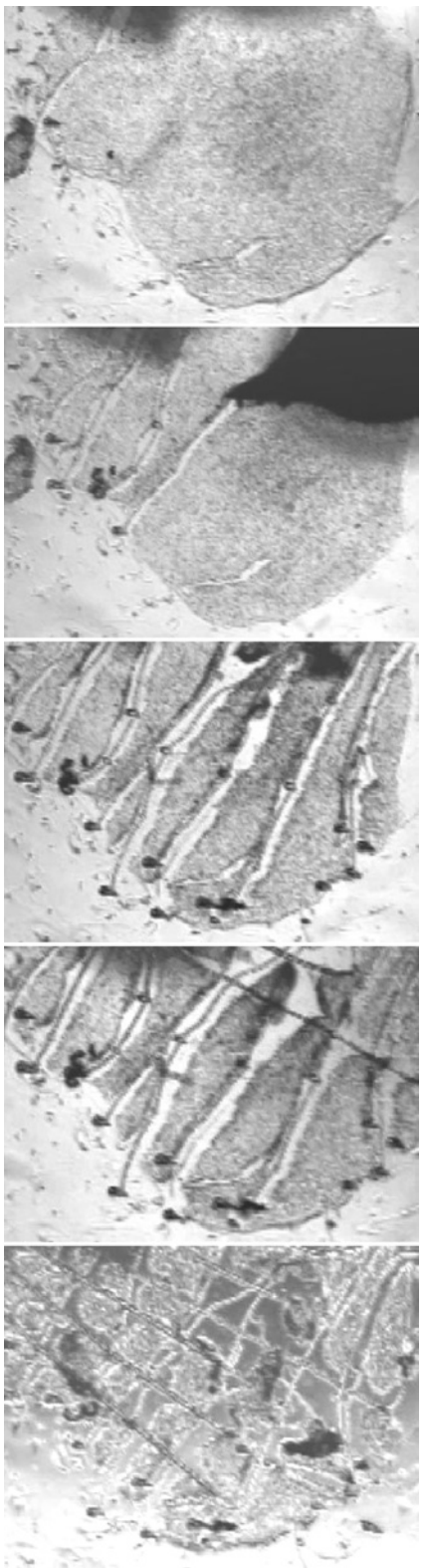

FIGURE 1.4 Frames from a movie, showing the cutting up of an hESC colony for passaging.

different-sized dishes, calculate the amount of volume to add based on surface area of each type of dish.

3. Mark (or remove) overtly differentiated colonies so as not to disturb these during the dissociation process.
4. Remove the medium from the dish and replace with fresh hESC medium.
5. Dissect the colonies by hand, either under a low-power dissecting microscope (in a horizontal flow hood) or without a microscope, in the tissue culture hood.

NOTE: Several implements can be used to slice up or break up the colonies. Because they are inexpensive and sterile, we recommend either a 20 µL pipettor which has a sterile filter tip attached, or a sterile 23G needle.

6. Figure 1.4 shows the method used for slicing the colonies into about 100 pieces. The colony is cut into strips, and then into squares. Each piece of the colony has a few hundred cells.
7. Break up each colony by moving the tip around and across each colony in a crosshatch or a spiral motion.

NOTE: Since the colonies are large at the time of passage, it is relatively easy to see individual colonies on the plate and, with practise, one can quickly dissociate an entire plate in less than 20 min.

8. After all of the colonies are dissected, use a 5 mL pipette to transfer the culture medium containing the dissected colonies to a 15 mL conical tube. Rinse the plate with hESC medium and add this to the same 15 mL tube.
9. Bring up the final volume in the tube to 8–10 mL with hESC medium.
10. Gently triturate the cell clumps using a sterile 10 mL pipette and divide the suspension into the prepared culture dishes on feeder layer or ECM-coated plates. *Do not* make a single-cell suspension; triturate gently, trying to achieve a relatively uniform suspension of cell clumps containing a few hundred cells each.

Alternative Procedures

Enzymatic dissociation

Enzymatic dissociation methods vary widely, and the exact conditions need to be developed for each laboratory. Most importantly, cultures that have been maintained by manual passaging cannot be passaged by enzymatic dissociation unless exceptional care is taken to adapt and monitor the cells.

The type of enzyme used for dissociation is critical. For example, passaging with trypsin appears to put more selective pressure on the cultures than other methods, resulting in a higher incidence of drift of hESC lines toward aneuploidy. But some hESC lines have been derived using trypsin from the outset; these lines can be rountinely passaged using whatever enzymatic technique is provided by the supplier.

Microbial collagenase is preferred by many laboratories, perhaps because of the way in which it is used. Collagenase is used to loosen the hESC colonies from the dishes, not to dissociate them to single cells, and the cell clumps have to be further dissociated by trituration.

NOTE: Keep in mind that enzymes are not highly purified recombinant products, and they may contain animal products. Trypsin is prepared from porcine (pig) tissue, and collagenase is a crude microbial product.

Collagenase dissociation

1. Remove the culture medium.
2. Rinse culture with Dulbecco's PBS (D-PBS).
3. Treat the culture with 200 U/mL of collagenase IV for 5–10 min at 37°C until the edges of the colonies start to curl up – observe the culture under the microscope.
4. Remove the collagenase and replace with 2 mL of hESC medium (if using a six-well or 35 mm dish).
5. Using a 5 mL pipette, gently dislodge the "good" colonies from the plate and place them in a 15 mL conical tube. *Alternatively, one could remove the differentiated colonies prior to treating the culture dish with collagenase.*
6. Gently triturate the cell clumps using a sterile 10 mL pipette and plate on feeder layer- or ECM-prepared dishes. Try to achieve a relatively uniform suspension of cell clumps containing several hundred cells each.
7. The cells can be split among 3–6 dishes of the same size as the original culture, depending on density of the original culture. If you wish to put the cells in different sized dishes, calculate the dilution based on surface area of each type of dish.

Non-enzymatic cell dissociation

Ca^{2+}- and Mg^{2+}-free saline solutions containing EDTA or EGTA have not been as widely used for hESC dissociation as the methods described above, but they should offer advantages for assays that require intact cell surface proteins such as flow cytometry and immunocytochemistry. Commercial formulations are available, such as Cell Dissociation Buffer (Invitrogen catalog no. 13150016), which contains glycerol as well as a proprietary mixture of salts and chelators.

If you decide to try this method, remove all of the protein-containing medium and rinse the cells briefly with the dissociation buffer. Add enough buffer to cover the cells and monitor them under the microscope until the edges of the colonies begin to lift, then triturate the cells gently to dissociate. If the cells are to be recultured, don't dissociate them into single cells, and be certain to check the karyotype of the cells after 10 passages; until you prove otherwise, you should assume that any untested passaging method is selecting for chromosomal abnormalities.

Other enzymes

Accutase and Accumax (Millipore/Chemicon catalog no. SCR005 and SCR006)

These products are proprietary mixtures of proteolytic and collagenolytic enzymes in EDTA that the manufacturer states is free of mammalian- or bacterial-derived products. Accumax also contains DNAse. If you test this method, start with a 5-minute room temperature incubation and monitor the cells under the microscope. While the manufacturer indicates that inactivation of the enzymes with protein is not necessary, we recommend that protein-containing medium be used to dilute out the enzyme after the cells are dissociated, to prevent clumping and sticking of the cells to the pipettes.

Trypsin-like Enzyme (TrypLE Select, Invitrogen catalog no. 12563-029)

This is a single enzyme, a recombinant fungal serine protease with trypsin-like activity. Anecdotal reports suggest that hESC line that have been mechanically passaged can be successfully transitioned to single-cell enzymatic passaging using TrypLE Select. If you decide to try this method, we recommend a saline rinse, then a 5-minute incubation in the 1× enzyme solution as provided by the manufacturer. Monitor the cells under the microscope and add protein-containing medium to the culture before triturating.

HyQTase (HyClone catalog no. SV30030.01)

This is a cell detachment solution in D-PBS with EDTA. The composition is proprietary. According to the manufacturer, HyQTase is composed of a naturally derived complex of proteolytic and collagenolytic enzymes in D-PBS containing EDTA. According to the manufacturer it can be used for either serum-containing or serum-free cultures. The manufacturer states that it does not contain mammalian or bacterial derived products and is non-recombinant.

Pitfalls and Advice

Monitoring drift in hESC cultures

Since hESC cultures are often kept in continuous culture for months, even years, it is very important to monitor for drift in the cultures. The best way to avoid drift is to generate a large bank of frozen cells as soon as possible after the cultures are first expanded. The importance of this cannot be overemphasized; the value of discoveries based on hESC cells depends on the reproducibility of results. See Chapter 26 for methods for setting up an hESC lab.

Genetic drift

We know that hESCs acquire chromosomal abnormalities over long periods of culture, so karyotyping or other genetic analysis methods must be performed on a regular basis. For detailed information about how to monitor genetic drifts, see Chapters 5–7 and 26.

Keep in mind that changes during the time the cells are cultured in your lab can only be detected if you analyze the cells very soon after you obtain them.

Developmental drift

hESCs can also drift toward a more differentiated state over periods of extended culture. Since there is no assay for pluripotence equivalent to germline transmission of mouse ESCs, surrogate markers, such as antibody markers, should be routinely checked, especially if the morphology of the cells seems to be different from the earlier cultures.

The gold standard for measuring the pluripotency of an hESC line is to transplant it to an immune-deficient mouse to form a teratoma tumor (Chapters 12 and 13). Keep in mind that it will require histological expertise to identify cell types and tissues in the tumors.

In vitro, differentiation of hESCs using embryoid body culture will allow at least a cursory analysis of hESC differentiation potential. However, embryoid bodies never achieve the maturity of cells that develop in teratomas, and since the methods used to assess differentiation *in vitro* usually involve a small number of markers assayed by PCR (Chapter 10) or immunocytochemistry (Chapter 9), it is more difficult to judge the full range of pluripotence.

The best approach to monitoring developmental drift is to pick a particular method and differentiated cell type to check periodically (see Chapters 14 on embryoid body and neuroepithelial differentiation, as well as the specific chapters on neuronal, cardiac, and hematopoietic cells, Chapters 15–18).

Contamination of cultures

hESCs are usually cultured without antibiotics; with good culture technique, bacterial contamination should not be a problem. However, we recommend that antibiotics be used while new investigators are being trained in the techniques. Antibiotics such as penicillin and streptomycin *do not* have any effect on mycoplasma. Mycoplasma is a serious problem in laboratories that culture multiple cell lines or have inadequately trained personnel. Cultures must be monitored for mycoplasma on a regular basis, and contaminated cultures destroyed. Methods for mycoplasma detection are provided in the quality control section of this chapter, and in Chapter 26.

Equipment

- Tissue culture hood: Class II A/B3
- Tissue culture incubator, 37°C, 5% CO_2, in humidified air
- Inverted phase contrast microscope with 4×, 10×, and 20× objectives
- Centrifuge, low speed 300–1000 rpm
- Water bath, 37°C
- Pipettors, such as Eppendorf p-2, p-20, p-200, p-1000
- Pipette aid, automatic pipettor for use in measuring and dispensing media
- Aspirator in the hood, with flask
- Refrigerator, 4°C
- Freezers: –20°C, –80°C, and –140°C.

Supplies and Reagents

Supplies

- 5 mL, 10 mL, 25 mL sterile disposable pipettes
- Six-well culture dishes
- 15 mL sterile conical tubes
- 50 mL sterile conical tubes
- Sterile 9″ Pasteur pipettes
- Pipette tips for Eppendorf or similar pipettor.

Recommended reagents

Item	Supplier	Catalog no.	Notes
Basal medium			
D-MEM/high glucose, L-glutamine (Dulbecco's modified Eagle's medium)	Invitrogen	11965-092	
D-MEM (high glucose, Glutamax [dipeptide L-alanyl-L-glutamine])	Invitrogen	10566-016	
Knockout D-MEM (lower osmolarity and lower bicarbonate for optimal pH at 5% CO_2)	Invitrogen	10829-018	Contains NO L-glutamine. Add 2 mM before use
D-MEM/F12 1:1 liquid (Dulbecco's modified Eagle's medium/Ham's F12) contains HEPES buffer, L-glutamine, pyridoxol HCl)	Invitrogen	11330-032	
D-MEM/F12 1:1 (Glutamax, no HEPES)	Invitrogen	10565-018	
Serum components			
KnockOut™ serum replacement (contains bovine products) (KSR)	Invitrogen	108280-028	
Fetal bovine serum (FBS)	HyClone	SH30070.03	
L-Glutamine 200 mM	Invitrogen	25030-081	
MEM non-essential amino acids (100× = 10 mM)	Invitrogen	11140-050	
2-Mercaptoethanol			
Solution: 55 mM in D-PBS	Invitrogen	21985-023	
Concentrated: 14.3 M	Sigma	M7522	Make 1000× stock by diluting 35 μL of 14.3 M into 5 mL PBS (0.1 M)
D-PBS (Dulbecco's phosphate-buffered saline without calcium and magnesium)	Invitrogen	14190-144	
Basic FGF (FGF2)			
Recombinant human bFGF	Chemicon	GF003	
Recombinant human bFGF	Invitrogen	13256-029	
Collagenase, type IV, microbial	Invitrogen	17104-019	
Pen-strep (100×)	Invitrogen	15070-063	Optional

Recipes

Note: Glassware should be dedicated to tissue culture only. If glassware is to be used instead of pre-sterilized plasticware, do not expose bottles to detergent.

Stock solutions

Human FGF2 (10 μg/mL, 1 mL)

Component	Amount	Stock concentration
Human bFGF	10 μg	10 μg/mL PBS with 0.2% BSA

1. Dissolve 10 μg of human basic FGF in 1 mL PBS containing 0.2% BSA.
2. Aliquot in 50–100 μL samples.
3. Store thawed aliquots at 4°C for up to two weeks.
4. Store frozen aliquots at –20°C or –80°C.

Note: For all growth factors, pre-wet all pipette tips, tubes, and filters with PBS + 0.2% BSA to lessen the loss of the growth factor.

Additional information

- Other designations: basic fibroblast growth factor; basic fibroblast growth factor; bFGF; fibroblast growth factor 2; heparin-binding growth factor 2 precursor; prostatropin

Collagenase IV (200 units/mL, 100mL)

Component	Amount	Stock concentration
Collagenase IV (Invitrogen catalog no. 17104-019)	20 000 units (typically)	200 U/mL in D-MEM

1. Dissolve 20 000 units of collagenase IV in 100 mL of D-MEM. This is usually 1 mg/mL.
2. Add to a 250 mL filter unit and filter.
3. Aliquot in 5–10 mL tubes and store at –20°C until use.

Additional information

- Collagenase is isolated from *Clostridium histolyticum*. Type IV is selected because of its low tryptic activity, and is recommended for isolation of pancreatic islets.
- This is a crude product, so expect lot-to-lot variation. EDTA inhibits this enzyme's activity.
- A unit is defined as the amount of enzyme required to liberate 1 μM of L-leucine equivalents from collagen in 5 h at 37°C pH 7.5.

KnockOut™ serum replacement (Invitrogen catalog no. 108280-028)

This product has a short shelf-life and should be aliquoted into 50 mL tubes and stored at –20°C. Thaw at 37°C just prior to use.

Additional information
KnockOut serum replacement (KSR) is a brand name for an Invitrogen product that is composed of BSA, transferrin, insulin, and other protein and non-protein components. The exact formulation is proprietary, but its composition was published (July 16, 1998) in an International Application Published under the Patent Cooperation Treaty (PCT), designated WO98/20679. See Epoline (ofi.epolin.org) to view the entire patent application.

L-Glutamine (200 mM)

L-Glutamine (Invitrogen catalog no. 25030-081) is unstable and must be stored frozen at –20°C. Thaw the bottle completely just prior to use and aliquot in 10 mL tubes. Do not refreeze tubes, store at –4°C and discard unused glutamine after two weeks.

2-Mercaptoethanol

2-Mercaptoethanol (2-ME) has been used in ESC culture media since the first derivation of mouse ESCs in 1981. Originally included as a reducing agent because of concern about oxidation of culture components, it continues to be used in hESC media. Since the final concentration is 0.1 mM, and the pure solutions of 2-ME are 14.3 M, it is necessary to start with a stock solution.

Several companies sell diluted solutions of 2-ME; the 55 mM solution in PBS (Invitrogen catalog no. 21985-023) is a convenient concentration for a stock.

If you wish to make your own stock, we suggest that you make a 1000× stock from the generally available concentrated solution (14.3 M).

For 1000× stock: dilute 35 μL of 14.3 M 2-ME (Sigma catalog no. M7522) into 5 mL of PBS to make a 0.1 M stock solution. Filter before use.

Embryonic stem cell medium (100mL)

Component	Amount	Final concentration
D-MEM/F12 (containing glutamine or Glutamax)	80 mL	
KSR or FBS	20 mL	20%
100× MEM non-essential amino acids (10 mM)	1 mL	0.1 mM
100× L-Glutamine (if D-MEM/F12 lacks glutamine)	1 mL	2 mM
55 mM 2-Mercaptoethanol or *0.1 M (1000×) stock*	182 μL of 55 mM *(0.1 mL of 0.1 M)*	0.1 mM
Human bFGF (1 μg/mL, 10 ng/μL)	40–200 μL	4–20 ng/mL

1. Prepare all media in the tissue culture hood using aseptic techniques.
2. Combine the basal medium and the serum component in the filter top of a 0.22 μm, low protein binding filter unit, then add the other supplements.

Quality Control Methods

Lot-to-lot variability of reagents

It is important to keep in mind the actual source of the materials and reagents used in the culture and maintenance of hESCs. Since many are derived from animal sources, there is inherent lot-to-lot variability of the product. While vendors make every effort to control the variability by setting production specifications, these are usually ranges and as long as the product falls within the approved range, the product passes inspection and is distributed.

Ideally, you should have your own quality control methods to test new lots of products. At the very least, record the lot numbers of reagents used; if an experimental result cannot be replicated, or a cell line fails to thrive, you will save considerable time if the problem is traceable to a bad reagent.

Monitoring for mycoplasma contamination

Mycoplasma are the smallest forms of bacteria (0.2–0.3 μm in diameter) and they can pass through the typical microbiology 0.2 μm filter used in cell culture and do not produce the characteristic turbid growth shown by other bacteria. Because they lack cell walls, they are unaffected by the standard antibiotics used in culture media (penicillin and streptomycin that act on bacterial cell walls). Serious infections can be detected in cultures by DAPI or Hoescht staining for DNA; stained cell nuclei will be surrounded by fluorescing structures in the cytoplasm.

Mycoplasma infections drastically affect cell metabolism, gene expression and antigenicity, and can be devastating to a hESC laboratory. Infections are difficult to get rid of once they take hold, and some tissue culture collections recommend that contaminated cells be destroyed as soon as mycoplasma are detected.

Mycoplasma are highly infectious and cross-contamination commonly occurs when new cells are introduced into laboratories. The ATCC (American Type Culture Collection) estimates that 16% of cell cultures are contaminated by mycoplasma. The bacteria can also come from tissue culture reagents such as serum and media supplements and from laboratory staff.

The best defense against mycoplasma contamination is good aseptic technique, and the laboratory should not allow inexperienced or careless workers to share cell lines, solutions, or equipment. As a precaution, the cell lines should be tested at least four times a year. Testing for mycoplasma can be done by enzymatic, polymerase chain reaction (PCR), fluorescent staining, or culture methods (see list below).

Service organizations

ATCC (www.atcc.org)
Corielle (www.coriell.org)

Enzymatic assays

Cambrex Corp. (www.cambrex.com) MycoAlert® Mycoplasma Detection Assay
Invitrogen (www.invitrogen.com) MycoTect™ Kit

PCR

Stratagene (www.stratagene.com), MycoSensor™ PCR Assay Kit

Fluorescence

Sigma (www.sigmaaldrich.com) Mycoplasma Stain Kit
Invitrogen (www.Invitrogen.com) MycoFluor™ Mycoplasma Detection Kit

ELISA

Roche Applied Science (www.roche-applied-science.com) Mycoplasma Detection Kit

Reading List

Feeder-free culture

Rosler ES, Fisk GJ, Ares X, Irving J, Miura T, Rao MS, Carpenter MK (2004). Long-term culture of human embryonic stem cells in feeder-free conditions. *Dev Dyn* 229: 259–274.

Xu C, Inokuma MS, Denham J, Golds K, Kundu P, Gold JD, Carpenter MK (2001). Feeder-free growth of undifferentiated human embryonic stem cells. *Nat Biotechnol* 19: 971–974.
These papers describe the methods of transferring hESCs to feeder-free culture.

Aneuploidy in hESC culture

Cowan CA, Klimanskaya I, McMahon J, Atienza J, Witmyer J, Zucker JP, Wang S, Morton CC, McMahon AP, Powers D, Melton DA (2004). Derivation of embryonic stem-cell lines from human blastocysts. *N Engl J Med* 350: 1353–1356.

Draper JS, Smith K, Gokhale P, Moore HD, Maltby E, Johnson J, Meisner L, Zwaka TP, Thomson JA, Andrews PW (2004). Recurrent gain of chromosomes 17q and 12 in cultured human embryonic stem cells. *Nat Biotechnol* 22: 53–54.

Ludwig TE, Levenstein ME, Jones JM, Berggren WT, Mitchen ER, Frane JL, Crandall LJ, Daigh CA, Conard KR, Piekarczyk MS, Llanas RA, Thomson JA (2006). Derivation of human embryonic stem cells in defined conditions. *Nat Biotechnol* 24: 185–187.

Zeng X, Chen J, Liu Y, Luo Y, Schulz TC, Robins AJ, Rao MS, Freed WJ (2004). BG01V: a variant human embryonic stem cell line which exhibits rapid growth after passaging and reliable dopaminergic differentiation. *Restor Neurol Neurosci* 22: 421–428.
Many investigators have discovered that their cell lines develop aneuploidy with time, and several lines of hESC have been shown to be aneuploid from the beginning of their derivation.

Mycoplasma in tissue cultures

Anon. (1999). Mycoplasma testing kits and services. *The Scientist* 13: 21.
This article underlines the pervasiveness of mycoplasma in cell cultures and reviews tests used to detect it.

CHAPTER

2

Human Feeder Cells, Feeder-free, and Defined Culture Systems

Robin L. Wesselschmidt and Jeanne F. Loring

INTRODUCTION

Feeder cells support the growth of cells in culture by contributing an as yet undefined and complex mixture of extracellular matrix (ECM) components and growth factors. Feeder cells used for the co-culture of embryonic stem cells (ESCs) are usually fibroblasts and are usually (but not always) mitotically inactivated so that they remain viable but cannot replicate and overgrow the ESC culture.

Mouse embryonic fibroblasts (MEFs) are the most commonly used feeder cell type and have reliably served as feeder cells for co-culture with mouse embryonic stem cells (mESC) since they were first derived in 1981. Following the methods published in the early 1980s, most researchers used MEFs (or other mouse fibroblastic cells) to derive new lines of human and other primate ESCs, and they continue to be the most frequently used feeder cell for culturing hESCs. Even though they are primary cells, MEFs are inexpensive to prepare or purchase, and when properly cultured provide

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ISBN: 978-0-1237-0465-8.

excellent support for growth of undifferentiated hESCs. See Chapter 3 for a description of methods for producing MEFs.

The search for improved methods and efforts to develop non-xenogenic culture systems has led to the use of various human-derived feeder cells, extracellular matrix components, and growth factors. The development of fully defined culture systems is an important milestone for the hESC field, which will greatly improve the usefulness of hESCs in both basic science programs and, over the longer term, their use in human therapeutic applications. The use of defined culture systems will eliminate much of the inherent variability in culture media, whose components are sourced from animals, and are likely to improve our ability to predictably and reliably direct differentiation. The acceptance of such systems is not solely dependent on identification of an ideal mix of factors and chemicals; if the cost of the perfect medium is too high, it will price most researchers out of the field. The movement now is therefore toward reasonable compromises that combine the best possible components at an affordable cost.

Overview

In this chapter we provide examples of alternatives to culture of hESC on MEF feeder layers. These methods are still the focus of a great deal of research and testing, and no ideal culture system has been developed to fulfill the needs of most researchers. The direction is toward animal product-free systems with all components defined, but a word of caution is warranted. The methods that have been most well tested are either not completely animal-free nor defined, are too expensive for most laboratories, and/or simply do not work for multiple hESC lines. We provide two practical approaches: feeder-free systems and human feeder cell-based systems.

The most well-documented feeder-free culture system uses MEFs to condition the hESC culture medium and a BD Matrigel™ substratum, which is derived from a mouse sarcoma cell line. While this feeder-free system eliminates the direct co-culture of hESCs with feeder cells, it includes animal products and relies on a commercial product that is not completely predictable.

An alternative to building a culture system from a combination of purified human factors and animal-derived products is to use human fibroblast feeder layers instead of MEFs. This solves the problem of the animal origin of unknown conditioning factors provided by MEF feeder layers. But simply using human feeder layers does not resolve the issue of completely animal-free culture, since the culture medium used generally contains animal products. Nor does it solve the problem of defining the composition of the medium since the human fibroblasts are still an undefined component.

Procedures

Mouse embryo fibroblast (MEF)-conditioned medium/BD Matrigel culture system

This feeder-free hESC culture system consists of a MEF-conditioned hESC medium and a BD Matrigel substratum. This has been used for years of continuous culture of hESCs

without loss of pluripotency markers or the development of karyotypically abnormal clones in the hESC cultures.

MEF-conditioned medium (MEF-CM)

MEF feeders are cultured and inactivated as described in Chapter 3. MEFs inactivated by gamma irradiation or mitomycin C work equally well.

1. Plate inactivated MEFs at 5×10^5 cells/cm^2 in MEF medium and allow them to attach to the flask overnight in the incubator.

NOTE: MEF medium contains fetal bovine serum (FBS), and MEFs will not attach well to the tissue culture flask in hESC medium without FBS.

2. The next day replace MEF medium with hESC medium (0.5 mL/cm^2) containing 4 ng/mL hFGF2. Allow MEFs to condition the hESC medium for 24 h.

3. Medium/flask guide:
   - 38 mL of medium/75 cm^2 flask
   - 75 mL of medium/150 cm^2 flask
   - 112 mL of medium/225 cm^2 flask.

4. Collect the conditioned hESC medium (MEF-CM) from feeder cell flasks daily, for up to 7 days.

NOTE: Harvest MEF-CM about the same time each day in an effort to minimize the variability of the medium.

5. Replace the medium in the flasks with hESC medium.

6. Filter MEF-CM through a 2 μm low protein binding filter and aliquot in 10 mL, 25 mL, and 50 mL aliquots in labeled and dated sterile tubes. Store at −20°C. MEF-CM is stable for least six months at −20°C.

NOTE: Prior to using MEF-CM to feed hESC cultures, supplement with an additional 8 ng/mL of hFGF2.

BD Matrigel

BD Matrigel is a derived from the Engelbreth-Holm-Swarm mouse tumor cell line. It is very rich in extracellular matrix components and comprises approximately 60% laminin, 30% collagen IV, 8% entactin, heparan sulfate proteoglycan, and low levels of growth factors.

It is a liquid at 4°C, but polymerizes quickly room temperature.

Stock solution (1:2)

1. Prior to preparation of gel, thaw Matrigel slowly on ice at 4°C overnight.

NOTE: Keep on ice until use. When preparing gel, use pre-cooled pipettes, plates, and tubes. BD Matrigel Matrix will gel rapidly at 22–35°C.

2. Keep on ice and add 10 mL of cold knockout D-MEM to the bottle containing 10 mL of Matrigel.
3. Keeping the mixture on ice, mix well by pipetting up and down with a 10 mL pipette.
4. Aliquot 1–2 mL into pre-chilled tubes (on ice) and store aliquots at −20°C.

Preparation of culture plates

1. Slowly thaw the frozen aliquots at 4°C on ice.
2. Dilute BD Matrigel aliquots 1:15 in cold knockout D-MEM (1:30 final).
3. Add 1 mL of diluted BD Matrigel/well of a six-well plate.
4. Incubate the plates for at least 1 h at room temperature or overnight at 4°C.

NOTE: If stored at 4°C, coated plates can be used for up to one week after coating.

5. Allow the plate containing the Matrigel to sit at room temperature for at least 10 min before removing excess Matrigel solution, in order for the gel to polymerize.

Culture of hESCs – collagenase passaging

1. Observe cultures daily.
2. Change medium daily. Feed hESCs with 4 mL of MEF-CM supplemented with additional 8 ng/mL of hFGF2.
3. Passage when cells are confluent, removing differentiated cells.

NOTE: In the MEF-CM/Matrigel system, hESCs are maintained at high density and passaged at 1:3 to 1:6 every week.

4. Aspirate medium and add 1 mL of collagenase IV (1 mg/mL) per well of six-well plate.
5. Incubate 5–10 min at 37°C. Monitor the culture during the incubation and stop collagenase treatment when the edges of the colonies begin to curl up and slightly loosen from the plate.
6. Aspirate the collagenase and rinse with 2 mL of PBS. Take care not to wash off loosened colonies.
7. Add 2 mL of MEF-CM to each well.
8. Gently scrape the well using a cell scraper or 10 mL pipette.
9. Collect most of the cells from the well and transfer to a 15 mL conical tube.
10. Gently pipette the cells to break up the clumps into groups of 50–100 cells, but do not make single-cell suspension.

11. Remove excess Matrigel from the new plates.
12. Seed the cells onto new plates at 1:3 to 1:6 dilutions. Place the plates in the incubator.
13. Observe the cultures and change medium daily.

NOTE: See Chapter 1 for other passaging methods.

Animal product-free culture conditions

Most hESC lines have been derived and maintained using medium containing bovine sera (FBS) or bovine-serum-derived products (such as Invitrogen's KnockOut™ serum replacement, KSR) and co-culture with MEFs or mouse-derived Matrigel extracellular matrix. But concerns about problems with xenografts have motivated development of alternative culture systems that do not require the use of animal-derived products.

Several groups have begun to use human cells as feeder layers (Table 2.1), but to be completely "xeno-free" the culture medium must be composed of defined components, not animal derived; substrata must consist of either a chemically treated growth surface or synthetic or recombinant extracellular matrix components (Table 2.2). Human growth factors and ECM have been combined in culture "systems" (Table 2.3) designed to support hESCs in the absence of animal-derived products.

Transfer of existing hESC lines to new culture conditions should be done slowly, with gradual changes of conditions over multiple passages. For example, to transfer a hESC line from MEF feeder layers to human feeder layers, try using a combination of MEF-conditioned medium and human feeder layers at first; then gradually, over multiple passages, reduce the concentration of MEF-conditioned medium until the cells grow well without it.

PITFALLS AND ADVICE

Strains of mouse used to make MEF-CM

MEFs from several mouse strains have been found to support hESC culture. However, the strains currently favored are isolated from the CF-1 strain or 129 strains. Other strains that have been used to support hESC growth in co-culture are FVB/N, B6/129 hybrids, and C57BL/6. The most critical variable seems to be the quality of the MEFs, which should be used between passage 3 and passage 6 and before the culture consists of many large multi-nucleated fibroblasts. CF-1 MEFs require a higher dose of radiation to inactivate them (60–80 Gy) than 129 or B6 MEFs (30–40 Gy).

Adapting hESC cultures to growth on BD Matrigel in MEF-CM

Transferring cells from fibroblast co-culture to Matrigel/MEF-CM system may require a couple of passages to allow the cells to adapt to culture without feeders.

TABLE 2.1 Feeder cells derived from human tissue sources that support pluripotent growth of hESCs

Human feeder cell type	hESC tested	Reference	Comments
hFS (fetal skin fibroblast) ATCC: D551/CCL-10 hFM (fetal muscle fibroblast) hAFT (adult fallopian tube epithelial cells)	HES3 HES4	Richards *et al.*, 2002, 2003	First to report the use of humanized culture system Human feeders and human serum used for culture All three hf support HES growth Derivation of new line on hFM cells hFeeders have greater proliferative potential than MEFs, but limited availability
Human foreskin fibroblast ATCC: Hs27 (CRL1634) ATCC: Hs68 (CRL1635)	TE03 TE06 WA09	Amit *et al.*, 2003	Longer proliferative potential in culture than MEFs, >42 passages Knockout D-MEM, 15% KSR, 4 ng/mL FGF2
Fetal foreskin fibroblast ATCC: CRL 2429	HS 181 HS 207	Hovatta *et al.*, 2003	Derived two new hESC lines on this feeder line starting with five blastocyts Knockout D-MEM, 20% FBS, hLIF
Human foreskin fibroblast ATCC: BJ (CRL-2522) ATCC: Hs27 (CRL-1634) ATCC: Hs68 (CRL-1635)	HES2 HES3 HES4	Choo *et al.*, 2004	Evaluated growth of HES lines in FBS and KSR on each of the commercially available hFF lines All three feeder lines supported long-term undifferentiated growth, in knockout D-MEM, 15% KSR, 4 ng/mL FGF2. None supported growth in D-MEM, 20% FBS
Adult bone marrow stromal cells	WA01	Cheng *et al.*, 2003	Supports undifferentiated growth, but these stromal cells need to be prepared frequently as they senesce with increasing time in culture as do MEFs
hUECs (uterine endometrial cells) hBPCs (breast parenchymal cells) hEFs (embryonic fibroblasts)	Miz hES1	Lee *et al.*, 2004	Noted hESC colonies are flatter and thinner on UECs and EFs than on MEFs or hBPCs D-MEM/F12, 20% KSR, 4 ng/mL FGF2
hUECs (uterine endometrial cells)	Miz hES 9, 14, 15	Lee *et al.*, 2005	hUESC "endo-1" line was used to derive new hESC lines D-MEM/F12, 20% KSR, 4 ng/mL FGF2
PLFb (human placental fibroblasts)	UC01 UC06 WA01 WA09	Genbacev *et al.*, 2005	Found human placental fibroblasts to be equivalent to MEFs (CF-1) Derived a novel line on this feeder: UCSF-1 Knockout D-MEM, 20% KSR, 4 ng/mL FGF2

(*Continued*)

TABLE 2.1 (Continued)

Human feeder cell type	hESC tested	Reference	Comments
HEF1-hTERT hESC-derived fibroblast-like feeder cells, immortalized by infection with retrovirus expressing hTERT	WA01 H7 WA09	Xu *et al.*, 2004	First report of genetically modified hESC differentiated cell type First genotypically homogeneous system used to culture hESCs
hESC-df (hESC-derived fibroblast-like feeder cells)	WA01 hES-NCL1 Novel line	Stojkovic *et al.*, 2005a	hESC-df support undifferentiated growth of hESCs when co-cultured directly and when used to condition hESC medium and cultured on Matrigel Was used to derive new hESC line Knockout D-MEM, 20% KSR, 4 ng/mL FGF2
Diff -Miz-hES6 (hESC-derived fibroblast-like feeder cells)	Miz-hES1 Miz-hES4 UC06	Yoo *et al.*, 2005	D-MEM/F12, 20% KSR, 4 ng/mL FGF2
PEL (primitive endoderm-like cells, derived from differentiated hESCs)	WA09 Novel line	Gonzalez *et al.*, unpublished	Co-culture on mitotically inactivated PEL Used to support the derivation of new hESC lines D-MEM/F12, 20% KSR, 20 ng/mL FGF2

TABLE 2.2 Feeder-free culture systems that support undifferentiated pluripotential growth of hESCs

Culture system	hESC line	Reference	Comments
Laminin and MEF-CM + Fibronectin and MEF-CM + Collagen and MEF-CM + (MEF-CM + 8 ng/mL FGF2)	WA01 WA07 WA09 WA14	Xu *et al.*, 2001	Found Matrigel to be superior to any individual ECM component tested
Matrigel MEF-CM + FGF2 (8 ng/mL)	WA01 WA07 WA09	Rosler *et al.*, 2004	Long-term evaluation of hESCs cultured using the system described by C Xu. The cells were in continuous culture for 2 years
Matrigel Fibronectin MEF-CM + FGF2 (8 ng/mL)	BG01 BG02 BG03	Brimble *et al.*, 2004	Feeder-free culture and passaged using EDTA-free trypsin Maintained normal karyotype when tested after 16 passages
Matrigel FGF2 (40 ng/mL)	WA07 WA09	Xu C, *et al.*, 2004	
Fibronectin FGF2 (4 ng/mL) TGFβ1 ± hLIF D-MEM, 20% KSR	TE03 TE06 WA09	Amit *et al.*, 2004	Human and bovine fibronectin worked equally well as substratum
Laminin Activin A (50 ng/mL) Nicotinamide (10 mM) Keratinocyte growth factor (50 ng/mL) D-MEM, 20% KSR	UC06	Beattie *et al.*, 2005	Carried for 20 passages, normal pluripotency markers Activin A secreted by MEFs TGFβ1 and activin A act through the "AR-Smad" pathway
Matrigel FGF2 (40 ng/mL) Noggin (500 ng/mL) D-MEM/F12, 20% KSR	WA01	Wang *et al.*, 2005	Noggin is a BMP antagonist. High concentrations of FGF2 provide a system that supports undifferentiated growth on Matrigel and in KSR KSR has high levels of BMP activity

(*Continued*)

TABLE 2.2 (Continued)

Culture system	hESC line	Reference	Comments
Matrigel FGF2 (40 ng/mL) Noggin (500 ng/mL) D-MEM, 20% KSR	WA01 WA09 WA14	Xu R *et al.*, 2005	
Matrigel FGF2 (40 ng/mL) D-MEM, 20% KSR	WA07 WA09	Xu C *et al.*, 2005	Tested various growth factors, including FGF2, SCF, Flt3L. Found that FGF2 alone or in combination supported long term undifferentiated growth
Substratum: FBS, coated plates CMD Activin A (10 ng/mL) FGF2 (12 ng/mL)	WA09	Vallier *et al.*, 2005	First to use a simplified chemically defined medium with low concentrations of growth factors to maintain pluripotency Chemically defined media (CDM): 50% IMDM/50% F12 NUT-Mix, ITS, 5 mg/mL BSA
Matrigel Activin A (5 ng/mL) D-MEM/F12, 20% KSR	TE06 WA01	Xiao *et al.*, 2006	Activin A stimulates expression of Oct4 and nanog Matrigel is likely to supply the TGFβ1 that works with activin to keep cells pluripotent
Matrigel N2/B27-CDM FGF2 (20 ng/mL)	WA01 UC06	Yao *et al.*, 2006	CDM: D-MEM/F12, 0.5 mg/mL BSA Since KSR is not in the medium no need to add BMP antagonists Not fully defined, but allows directed differentiation in monolayer culture
Human serum ECM matrix hES-dF CM + FGF2 (8 ng/mL) 1% ITS (insulin-transferrin-selenium)	WA01 hES-NCL1	Stojkovic *et al.*, 2005b	Culture dishes were incubated with human serum for 1 h at room temperature, serum was removed, dishes allowed to air dry for at least 1 h at room temperature before culture medium added to dish

TABLE 2.3 Defined culture systems

Culture system	hESC l	Reference	Comments
Laminin (human) X-Vivo 10 FGF2 (80 ng/mL)	WA01 UC01	Li *et al.*, 2005; Genbacev *et al.*, 2005	X-Vivo 10 (Cambrex, Wakersville, MD, USA) is purified from human plasma, mostly human albumin Does not require conditioning and contains only human and recombinant proteins
Fibronectin ± collagen Fibronectin sufficient HESCO medium: D-MEM/F12, ITS, cholesterol, albumin, April/BAFF, Wnt 3a, FGF2	WA09 BG01	Lu *et al.*, 2006	hESCs cultured in this medium do not elaborate a surrounding layer of differentiated fibroblast-like cells
TeSR1 medium Human serum FGF2 (4 ng/mL), LiCl, GABA, pipecolic acid, TGFβ	WA09 WA15 WA16	Ludwig *et al.*, 2006a	This medium is very expensive to prepare. However, cultures grow at high density and do not produce differentiated fibroblast-like cells WA09 >55 passages, with normal karyotype
mTeSR1 BSA, Matrigel, zbFGF (zebrafish FGF)	WA14	Ludwig *et al.*, 2006b	Two novel hESC lines derived from five blastocysts. Neither had normal karyotype after seven months in culture Less expensive modification of TeSR1, but not fully humanized medium

Passage to low-density feeders on BD Matrigel and conditioned medium for the first couple of passages may ease the transition to feeder-free culture.

Growth factors

It is important to keep in mind that protein growth factors are easily degraded if not treated properly. Care should be taken when diluting and aliquoting all proteins. Keep them in a solution containing low concentrations of albumin – usually 0.1% BSA (diluted from fraction V, Sigma catalog no. A7979) in PBS. When pipetting or filtering low concentration solutions one may want to coat pipettes and filters with 0.1% BSA to lower the likelihood of the growth factor sticking to the pipettes and filters.

BD Matrigel

The source of this ECM mixture is the Engelbreth-Holm-Swarm mouse tumor. Its major components are laminin, collagen IV, heparan sulfate proteoglycans, and entactin. At room temperature, BD Matrigel matrix polymerizes to produce biologically active matrix material resembling the mammalian cellular basement membrane.

While quality control measures are used to minimize the variability between production lots, lot-to-lot variations are inherent in any cell-derived product. Culture results may vary depending on the production lot, the concentration that is plated, the length of time the plates are incubated with Matrigel, and how the plates are stored prior to use.

Matrigel comes as a solution measured in weight/volume. More consistent results may be achieved by calculating milligram per milliliter concentration rather than using a strict final dilution of 1:30 regardless of the concentration of the stock lot.

An alternative to preparing Matrigel-coated plates in the laboratory is to purchase pre-coated plates, which have been coated with optimized concentration and prepared and tested for culture with hESCs (e.g. BD BioCoat™ BD Matrigel six-well plates for ES culture, BD Biosciences Catalog number 354671).

Equipment

- Tissue culture incubator: 37°C, 5% CO_2 in humidified air
- Class II biosafety cabinet
- Microscope: Phase contrast with 4×, 10×, 20× objectives
- Centrifuge: low speed 300–1000 rpm
- Access to 4°C, −20°C, −80°C, and cryogenic freezers.

Supplies and Reagents

Item	Supplier	Catalog no.	Alternative
BD Matrigel Reduced growth factor	BD Biosciences	354230	
BD Matrigel pre-coated plates (specifically for hESC culture)	BD Biosciences	354671	
Tissue culture flask 75 cm^2	Corning	430641	Nunc/Nalgene
Tissue culture flask 150 cm^2	Corning	430825	Nunc/Nalgene
250 mL 0.2 μm low protein binding filter units, PES	Corning	431096	Millipore
50 mL conical tubes, sterile	Corning	430291	Many
15 mL conical tubes, sterile	Corning	430053	Many
Dulbecco's modified Eagle's medium (D-MEM), (high glucose, L-glutamine, no pyruvate)	HyClone	SH30022	Invitrogen
Fetal bovine serum (FBS)	HyClone	SH30070	
Non-essential amino acids (100×)	HyClone	SH30838	Invitrogen
L-Glutamine (200 mM)	HyClone	SH30852	Invitrogen
Pen-strep (10 000/10 000)	HyClone	SV30010	Invitrogen
Trypsin/EDTA (0.5%, 0.53 mM EDTA)	HyClone	SH30236	Invitrogen
PBS w/o Mg^{2+}, Ca^{2+}	HyClone	SH30028	Invitrogen
D-MEM, high glucose, without glutamine	Invitrogen	11965-092	HyClone
Knockout D-MEM	Invitrogen	10829-018	
KnockOut serum replacement (KSR)	Invitrogen	108280-028	
2-Mercaptoethanol 55 mM (note this is the working concentration)	Invitrogen	21985-023	Sigma, but at 14.3 M, must be diluted before use!
Human FGF2	Invitrogen	13256-029	Chemicon, R&D Systems, Sigma

Recipes

Stock solutions

FGF2 (10 μg/mL)

Component	Amount	Stock concentration
Human FGF2	10 μg	10 μg/mL in 0.2% BSA in PBS

1. Dissolve 10 μg in 1 mL of PBS containing 0.2% BSA.
2. Aliquot in 50–100 μL samples.

3. Store frozen at −20°C or −80°C for long-term storage.
4. Thawed aliquots are stable for up to two weeks at 4°C.

Stock solutions

Collagenase IV (200 U/mL)

Component	Amount	Stock concentration
Collagenase IV	20 000 units	200 units/mL

1. Dissolve 20 000 units in 100 mL of knockout D-MEM.
2. Filter (2 μm). Aliquot and store at −20°C.
3. Alternatively, make 1 mg/mL solution, filter, aliquot, and store at −20°C.

MEF medium (for propagation of MEFs) (500 mL)

Component	Amount	Final concentration
D-MEM, high glucose	435 mL	90%
FBS	50 mL	10%
Non-essential amino acids (100×)	5 mL	1×
L-Glutamine (200 mM)	5 mL	2 mM
Pen-strep[a] (100×)	5 mL	1×

[a]Antibiotics optional.

Add all ingredients to the top of a 500mL filter unit to filter. Store at 4°C. Discard unused medium after one month.

hESC medium (for making MEF-CM) (500 mL)

Component	Amount	Final concentration
Knockout D-MEM	400 mL	80%
KnockOut serum replacement	100 mL	20%
Non-essential amino acids (100×)	5 mL	1×
L-Glutamine (200 mM)	2.5 mL	1 mM
2-Mercaptoethanol (Invitrogen 55 mM solution)	910 μL	0.1 mM
Pen-strep[a] (100×)	2.5 mL	1×
Human FGF2[b] (10 μg/mL)	20 μL	4 ng/mL

[a]Pen-strep is optional.
[b]Add FGF2 to medium after it has been filtered.

Mix all ingredients, except FGF2, in the top of a 500mL 2mm PES filter unit. Store at 4°C. Discard unused medium after two weeks.

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CHAPTER

3

Mouse Embryonic Fibroblast Feeder Cells

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Introduction

Mouse embryonic fibroblasts (MEFs) have been used reliably as feeder cells for mouse embryonic stem cells (mESCs) since the early 1980s when the first mESC lines were being derived and cultivated. The first published derivation of hESC used MEF feeder layers, and many laboratories continue to use them routinely for long-term hESC culture.

MEFs are primary cells derived from day 12.5–14.5 fetuses, and are primary cells that do not continue to proliferate indefinitely. Once the cells begin to senesce they seem to lose their capacity to support undifferentiated growth and proliferation of hESC, so they are used optimally between passage 3 and passage 6. Usually large batches are made, tested, and cryopreserved so that this process needs to be repeated only occasionally.

Overview

Batches of MEFs need to be prepared on a routine basis and each newly prepared batch should be tested for robust recovery from cryopreservation, and support of

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undifferentiated proliferation of hESC cultures. They must also be tested for mycoplasma and should be subjected to mouse antibody pathogen (MAP) testing, usually by an outside service.

It is important that MEFs are mitotically inactivated before being co-cultured with hESCs, or the MEFs will become a growing contaminant cell type that is difficult to remove. There are two common ways of inactivating MEFs: (1) irradiation and (2) mitomycin C treatment. The cells can be cryopreserved either before or after mitotic inactivation.

NOTE: Several suppliers provide prepared stock vials of MEFs from various mouse strains with or without selectable markers. Depending on the quantity of MEFs that will be required, it may be cost-effective to purchase MEFs that have passed quality control by a supplier.

PROCEDURES

Isolation of MEF feeder cells

You will need to plan well in advance in order to prepare your own batch of MEFs. It is important to follow your institutional, local, state, and federal regulations regarding the use of laboratory mice. There are usually institutional guidelines covering the use of vertebrate animals, governed by the Institutional Animal Care and Use Committee (IACUC), which reviews proposed use of vertebrate animals in research. Obtain IACUC approval, then obtain the desired mouse strain and set up matings as approved by IACUC.

Preparation

Animal facility

- Mice: female(s) 13.5 dpc (days post coitum)
- Surgical instruments: sterilized
- Ethanol: 70%
- One 50 mL conical tube containing 25 mL D-PBS.

Tissue culture laboratory

- Prepare the tissue culture hood
- Three 10 cm sterile Petri dishes containing 15 mL of sterile D-PBS per pregnant female mouse.

Isolation of MEF

The protocol below is a method that has worked well to produce high-quality MEFs from various mouse strains including, 129, C57B/6, FVB/N, and CF-1.

Day 1: Preparing and plating MEFs

1. *In the animal facility:* using approved euthanasia method, sacrifice a 13.5 dpc female mouse. At 13.5 dpc, the female will be very visibly pregnant.

2. Place the female on her back on a clean bench or inside a hood. Spray the abdomen with 70% ethanol. Using sterile forceps and scissors make a small lateral incision under the diaphragm. With two hands pull the skin back, revealing the peritoneum. Make an incision in the peritoneum and remove the uterus.
3. Using aseptic techniques, remove the uterus, containing 13.5 dpc embryos and place in the sterile conical tube containing 25 mL of sterile D-PBS.
4. *In the tissue culture lab:* Using aseptic techniques in the tissue culture hood, remove the uterus from the 50 mL conical tube and place in a dish containing 15 mL D-PBS.
5. Dissect the embryos from the uterus and carefully remove each from their yolk sac and placenta.
6. Place embryos into a fresh Petri dish containing 15 mL of D-PBS.
7. Remove the head and internal organs (dark red tissue in the abdomen) using a pair of small sharp scissors and watchmaker forceps.
8. Rinse each carcass well, by placing in a fresh Petri dish containing 15 mL of D-PBS and gently swirling the dish to remove any remaining blood.
9. Place dissected embryos in 10 cm dish containing 10 mL of 0.05% trypsin and using very sharp, fine scissors mince the tissues into fine pieces.
10. Add another 5 mL of trypsin and triturate the solution until it easily moves into and out of a 5 mL pipette.
11. Place the Petri dish into the incubator for ~5 min, just long enough to dissociate the cells, but not so long as to produce a "stringy sludge" of DNA. As soon as you notice stringy material in the plate stop reaction by adding 15 mL of feeder cell medium and place the entire solution into a 50 mL conical tube.
12. Allow the large pieces of tissue to settle to the bottom of the tube for 5 min.
13. Carefully remove the supernatant to a clean 50 mL conical and add D-MEM to final volume of 50 mL. Mix by gently inverting the tube several times.
14. Spin the tube at 1000 rpm for 3–5 min.
15. Aspirate the supernatant and discard.
16. Resuspend the cell pellet in 10 mL of feeder cell medium, put 5 mL into each of two 150 cm^2 tissue culture flasks containing 20 mL of feeder cell medium.
17. Place in the incubator overnight.

Day 2: Observe the cultures

The flask should be 60–70% covered with healthy fibroblasts.

1. Aspirate the medium.
2. Rinse once or twice with 10 mL of D-PBS to remove any debris and red cells that were carried along in the isolation process (the fibroblasts will remain firmly attached to the tissue culture flask).

3. Place 25 mL of feeder cell medium in each 150 cm flask and return to the incubator overnight.

Day 3: The cells should be confluent and ready to passage
Passage the cells using 0.05% trypsin/EDTA 1:3 and return to the incubator.

1. Rinse the flasks with 15 mL of D-PBS.
2. Add 5 mL of 0.05% trypsin/EDTA.
3. Incubate for 3–5 min at 37°C.
4. Add 10 mL of feeder cell medium and gently triturate to mix the cells.
5. Add 5 mL of cells to each of three 150 cm^2 flasks containing 20 mL of feeder cell medium.
6. Return to incubator.

Day 4–5: Observe the cultures for growth following passaging and make sure there is no contamination
By this point the cultures should have very characteristic fibroblast morphology. Harvest cells for batch freezing when confluent; this may be on day 4 or day 5. Harvest cells with trypsin/EDTA.

1. Rinse each flask with 15 mL of D-PBS.
2. Add 5 mL of 0.05% trypsin/EDTA.
3. Incubate for 3–5 min at 37°C.
4. Add an equal volume of feeder cell culture medium to inactivate trypsin.
5. Triturate the cell suspension and distribute to conical tubes for centrifugation. Centrifuge at 1000×*g* (0.2 rcf) for 5 min. Aspirate supernatant and resuspend cells in a small volume (about 1 mL for each flask).
6. Count the cells using a hemocytometer and test for viability with trypan blue. The cells should be nearly 100% viable.
7. Prepare cryovials – usually this procedure generates 30–50 vials at 3–5 × 10^6 cells/vial from each pregnant female. This is "passage 2."
8. Dilute the cell suspension to about 6 × 10^6cells/mL. Add an equal volume of 2× cryopreservation solution and distribute 1 mL to each vial.
9. Slow freeze to –80°C, using a freezing container (usually with isopropanol) or Styrofoam box. Transfer the vials to liquid nitrogen if possible.

Test MEF stocks for pathogens and growth recovery after freezing

A week or so after preparing the MEF, thaw a vial to test for viability and pathogens. Mycoplasma tests can be performed inhouse or by a service lab (see chapter on hESC culture methods). In addition, the cells should be tested to be sure that they do not

harbor mouse pathogens. Mouse antibody pathogen (MAP) testing is usually performed by a service lab such as RADIL (www.radil.org).

Thawing and culture of feeder cells

The protocol below describes the culture of actively dividing cultures. If you are using mitotically inactivated feeder cells, follow the supplier's instructions for thawing, plating, and use.

1. Prepare feeder cell medium.
2. Place 15 mL of medium into a 75 cm^2 tissue culture flask and put the flask into the incubator 15–30 min prior to thawing the cells in order to allow the medium to equilibrate.
3. Thaw the vial of cells by gently shaking it in a 37°C water bath without submerging the vial below the O-ring on the cap.
4. As soon as the contents have thawed, less than 2 min, remove the vial from the water bath and spray it with 70% ethanol. Dry the vial with a clean Kimwipe, and move to a tissue culture hood where the rest of the procedures will be performed aseptically.
5. Transfer the contents of the vial to the prepared flask and incubate the culture over night at 5% CO_2 in humidified air at 37°C.
6. The next morning, observe the culture. The flask should be 40–60% confluent with a healthy culture of feeders.
7. Replace the medium with 15 mL of fresh feeder cell medium and return to the incubator.
8. Monitor the cultures daily and passage 1:3 when the cultures are 80–90% confluent.

Passaging feeder cells

1. Aspirate the feeder cell medium.
2. Wash the flask with 5–10 mL of D-PBS.
3. Add 3–4 mL of trypsin-EDTA to the flask and incubate at 37°C for 3–5 min.

Note: Use less time for human fibroblasts, slightly more for MEFs. Gently shake the flask to make sure that the entire surface area is coated with trypsin.

4. Gently shake the flask to remove the cells, add 10 mL of culture medium, rinse the surface of the flask and transfer 5 mL to each of two new flasks.
5. Add 10 mL of medium to all three flasks and return to the incubator for further culture.
6. Monitor the culture daily and passage 1:3 when the cultures are 80–90% confluent.

Inactivating feeder cells

The feeder layer plays a complex role in helping to maintain hESCs in an undifferentiated state. The feeder layer must be healthy and rapidly dividing prior to inactivation in order to provide the best substrate for the growth of the hESCs.

Following inactivation, feeder layers remain adequate for the culture of hESCs for 5–7 days. In order to keep the feeder layer healthy, it is advisable to change the medium on the inactivated cells every 3 days. Always observe the feeder layer under the microscope prior to using them for the culture of hESCs in order to confirm that the cell layer is still intact and the cells have not begun to deteriorate. Since hESCs are usually passaged every 6–7 days, the feeder layer can start to deteriorate before the hESCs are ready to passage if "old" feeder layer dishes are used. For best results, inactivate the feeders *the day before* passaging the ESCs.

Inactivation of the feeder cells is accomplished by either irradiation or treatment with mitomycin C. Inactivated feeder cells are usually plated on gelatin (collagen I)-coated dishes to aid in their attachment.

NOTE: Although the cells are unable to undergo mitosis, they still replicate their chromosomes and can become multiploid. When karyotyping hESCs, occasionally a feeder cell is included in the cell count. Mouse cells are distinguishable by their acrocentric chromosomes.

Inactivation by gamma irradiation

1. Trypsinize the feeder layer as you would for passage.
2. Remove the cells from the flask and wash with feeder cell medium up to 10 mL.
3. Place cells in a sterile 15 mL conical tube and irradiate for a total of 30–40 Gy (3000–4000 rads).

NOTE: 3000–4000 rads is standard for irradiation of mouse embryo fibroblasts. Higher or lower levels of irradiation are sometimes suggested, but the important issue is that the cells are alive but unable to proliferate. See Pitfalls and Advice section for more detailed information.

4. Following irradiation, dilute the feeders to 3×10^5 in feeder cell medium, and re-plate the cells on the appropriate configuration of gelatinized culture dishes to meet experimental goals, and incubate overnight.
5. The next morning aspirate the feeder cell medium, rinse with D-PBS, and replenish the culture dish with hESC medium.

Inactivation by mitomycin C treatment

NOTE: Mitomycin C is a cytotoxic antitumor agent and must be handled carefully; it works by cross-linking the DNA, which blocks cell division. Follow your institution's rules for safe handling and disposal. Handlers should wear latex or nitrile protective gloves and work in a biological safety or fume hood. One effective method is to inactivate the mitomycin C with an equal volume of household bleach. Inactivation is rapid.

1. Remove the feeder cell medium.
2. Add 10 mL/75 cm^2 of mitomycin C medium.

NOTE: Make sure the entire flask is covered with mitomycin C medium so that the inactivation is complete and all cells are exposed for the entire incubation time.

3. Incubate for 3 h at 37°C in 5% CO_2.
4. Remove mitomycin C solution and inactivate it with bleach or other recommended procedure.
5. Wash inactivated feeder layer three times with 10 mL each of PBS.
6. Trypsinize the cells to remove from flask, resuspend in feeder cell medium, and re-plate the cells on the appropriate configuration of gelatin-coated (see below) culture dishes to meet experimental goals, and incubate overnight.

NOTE: at this point, inactivated feeder cells can be cryopreserved for later use. Be sure to indicate on the freezing vial that the cells are already inactivated.

7. The next morning, wash the dishes of inactivated fibroblasts with PBS and re-feed with either feeder cell medium or hESC medium in preparation for hESC culture.

Substratum support for feeder cell layers

In order to provide better support for the long culture periods required for hESC culture, inactivated feeder cells are plated on gelatinized dishes.

1. Coat culture dishes with 0.1% gelatin solution.
2. Incubate 1 h to overnight at 37°C.
3. Just prior to plating inactivated feeder cells, remove gelatin and rinse the dish with D-PBS.
4. Plate the inactivated feeders on the gelatin coated dishes and allow them to attach in the incubator for at least 4 h before culturing with ESCs.

Photomicrographs MEF feeder layers

Figure 3.1 shows the morphology of MEF feeder layers. MEFs do not form the whorls of cells that are typical of other fibroblasts used as feeder layers, such as human foreskin fibroblasts. The bottom photo shows high-density hESC (WA09 line) colonies cultured on MEF feeder layers.

ALTERNATIVE PROCEDURES

MEF cells from commercial sources

There are several commercial sources for MEFs from various mouse strains and containing various selectable drug-resistant markers. Depending on the level of use, this can be a convenient and economical alternative to the *de novo* preparation of MEF feeder layers.

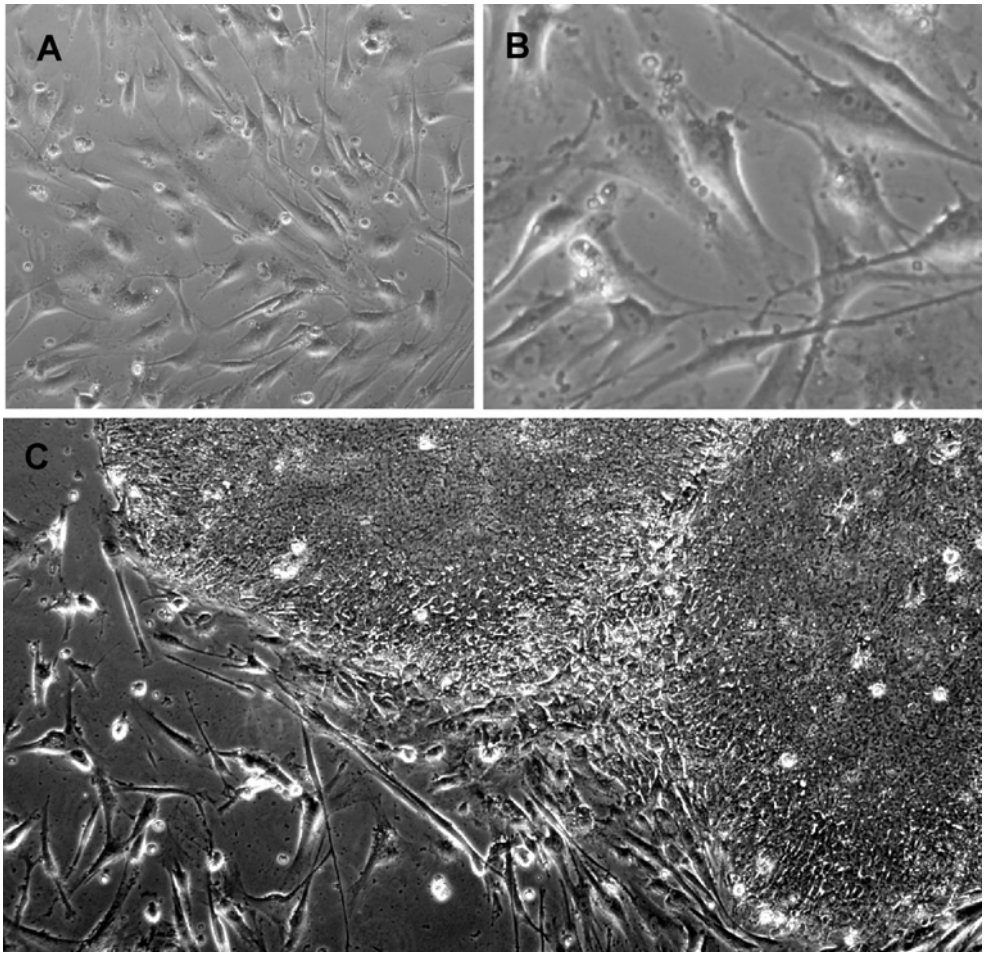

FIGURE 3.1 Low (A) and high (B) magnification photomicrographs showing morphology of MEF feeder layers. MEFs do not form the whorls of cells that are typical of other fibroblasts used as feeder layers, such as human foreskin fibroblasts. (C) High density hESC (WA09 line) colonies cultured on MEF feeder layers. Photos by Dr Maria Baracova.

- ATCC (www.atcc.org)
- Chemicon International (www.chemicon.com)
- Primogenix, Inc. (www.primogenix.com)
- Stem Cell Technologies (www.stemcell.com)
- GlobalStem, Inc (www.globalstem.com).

PITFALLS AND ADVICE

General advice

Rules of thumb:

- If the provider of the hESCs recommends a certain plating density, follow their instructions – at least initially.

- If no instructions are given, a slightly less than confluent layer seems to be optimal. The feeder layer should have healthy cell bodies spread out on the tissue culture plate. Before inactivation the MEF culture should be doubling every 24–30 h, requiring passaging at 1:3 every 3 days.
- MEF feeders should be used between passages 3 and 7. When the MEFs start to slow down in their proliferation or the cultures contain many multinucleate cells or floating debris, dispose of them and thaw a fresh vial.
- Some laboratories have a strong preference for MEFs derived from particular mouse strains. Others indicate that the strain is unimportant. As a rule, we suggest that the most important characteristic of any cells used for feeder layers is that they be rapidly growing, free of pathogens, and low passage when they are inactivated.

MEF cell plating density

The density of the feeder layer plays a role in the appearance of the hESC colonies, the rate at which the media components are depleted and the concentration of the feeder-derived culture components. However, there is as yet no predetermined optimal recommended density for the plating the MEF feeder layer. Each commercial hESC line has a slightly different recommended plating density for the MEF feeders:

- WiCell: 0.75×10^5/cm
- Bresagen: 2.4×10^5/cm
- mESCs: $2.5–3.0 \times 10^5$/cm dilute to 3×10^5/mL for plating.

Table 3.1 gives an example of a plating guide that can be adapted as the researcher determines the optimal conditions for the cell lines being cultured.

Determining timing and dose for inactivation of feeder cells

The exact time of irradiation will depend on the irradiator and the cell type used. We have found that MEFs perform better when irradiated at 30 Gy and human fibroblasts 40 Gy. If the chamber of the irradiator is large enough to accommodate the culture dishes, the cells can be irradiated after they have attached to the dishes that will be used for passage of the hESC cultures.

To determine the effective dose to mitotically inactivate the cells, test at least three exposure periods on identical tubes of cells. After irradiation, dilute the cells so that you can plate 100 cells per 10 cm plate. Plate the cells on gelatin and observe the dishes for about a week. If any cells have failed to inactivate, you will see clones, and will have to use a longer exposure.

How to rid ESC cultures of contaminating feeder cells

Having hESC cultures contaminated with mitotically active feeders should be avoidable if care is taken to inactivate them thoroughly. But should valuable cultures

TABLE 3.1 Example of a plating guide: mouse embryonic fibroblast feeder layer for hESC culture: density = 1.2×10^5 cells/cm^2

Culture container type	Container area (cm^2)	Total no. of MEFs per container	Volume of medium normally used in the container (mL)	Volume of MEF suspension at 5.0×10^5/mL (mL)[a]
IVF dish	1.0	1.2×10^5	0.5–1.0	0.25
35 mm dish	10	1.2×10^6	2–3	2.4
60 mm dish	20	2.5×10^6	5–6	4.8
100 mm dish	60	7.2×10^6	10–12	14
T-25 flask	25	3.0×10^6	5–6	6
96-well plate	0.3/well	3.6×10^4/well	0.2 per well	0.1
24-well plate	2/well	2.4×10^5/well	1–2	0.5
Six-well plate	10/well	1.2×10^6/ well	3–5	2.4
Four-chamber slide	1.8	2.1×10^5	0.8–1.5	0.5

[a] Make a stock suspension of MEF cells at 5×10^5/mL. Make sure the cells are at least 90% viable. Note that the volume of MEF suspension to use does not exactly match the amount of medium normally used for that container. For the small volume containers (96-well, IVF dish, 24-well), you must add additional medium (not cells) to avoid too much evaporation and uneven distribution of feeder cells because of the effect of the meniscus.

appear to contain living feeder cells there is a straightforward solution; if the feeder cells are primary cells, they have a limited lifespan in culture and will eventually senesce.

If the fibroblasts are an immortal line, they can be diluted by panning the more adhesive fibroblasts in dissociated cultures on a series of tissue culture dishes, or by serially passaging a small portion of the center of a hESC colony. If the feeders are a different species, "immunosurgery" (complement-mediated lysis) has been used successfully to remove them.

EQUIPMENT

- Class II biosafety cabinet: NuAire or Baker
- Tissue culture incubator: 37°C, 5% CO_2, in humidified air
- Phase contrast microscope, 4×, 10×, 20× objectives
- Low-speed centrifuge (200–1000 rpm)
- Pipettors: p2, p20, p200, p1000
- Pipette aid
- Refrigerator (4°C)
- Freezers: –20°C, –80°C, and liquid nitrogen.

Supplies and Reagents

Feeder cell culture reagents

Item	Supplier	Catalog no.	Alternative
D-MEM (Dulbecco's modified Eagle's medium), high glucose, L-glutamine, no pyruvate	HyClone	SH30022.02	Invitrogen
Fetal bovine serum (FBS)	HyClone	SH30070.03	BGS (bovine growth serum) HyClone no. SH30541.03
MEM non-essential amino acids (100×)	HyClone	SH30238.01	Invitrogen
Pen-strep	HyClone	SV30010	Invitrogen
D-PBS	HyClone	SH30028.03	Invitrogen
Trypsin/EDTA 0.05%	HyClone	SH30236.01	Invitrogen
Mitomycin C	Sigma	M0503	
DMSO	Sigma	D2650	

Tissue culture disposables

Item	Supplier	Catalog no.	Alternative
10 cm Petri dish	Corning		
75 cm^2 flask	Corning	430641	
150 cm^2 flask	Corning	430825	
15 mL conical tube	Corning	430053	
50 mL conical tube	Corning	430291	
5 mL pipettes	Corning	4487	
10 mL pipettes	Corning	4488	
25 mL pipettes	Corning	4489	
250 mL 2 μm, PES filter unit	Corning	431096	
500 mL 2 μm, PES filter unit	Corning	431097	
2.0 mL cryogenic vials (internal thread)	Corning	2028	Nunc no.
Permanent cryogenic storage labels (Cryo-babies)	Diversified Biosciences	LYC-1700	

Recipes

Stock solutions

Mitomycin C (Sigma M0503)

Component	Amount	Stock concentration
Mitomycin C	2 mg/2 mL	1 mg/mL in water

Feeder cell medium (500 mL)

Component	Amount	Final concentration
D-MEM (formulation contains L-glutamine)	440 mL	
FBS	50 mL	10%
Non-essential amino acids (100×)	5 mL	1×
Pen-strep (100×)	5 mL	10 U penicillin/ 10 μg streptomycin

Working in a tissue culture hood and using aseptic techniques: Remove 50 mL of D-MEM from a 500 mL bottle, add the other components. Replace the bottle cap and gently invert the bottle several times to mix contents. Store at 4°C.

Mitomycin C inactivation medium (200 mL)

Component	Amount	Final concentration
D-MEM (formulation contains L-glutamine)	180 mL	
FBS	20 mL	10%
Mitomycin C (1 mg/mL)	2 mL	10 μg/mL

Working in a tissue culture hood and using aseptic techniques, Mix media contents in the top of a 2 μm low protein binding filter unit. Aliquot: 20 mL/50 mL sterile conical tube. Store at −20°C. Thaw an aliquot just prior to use and bring to room temperature before applying to feeder cell culture.

0.1% Gelatin solution (250 mL)

Component	Amount	Final concentration
Gelatin	0.1 g/100 mL water	0.1%

Quality Control Methods

Pathogen testing

- Mycoplasma
- Mouse Antibody Production Test ("IMPACT" test: www.radil.org).

Reading List

Isolation and use of MEFs for culturing ESCs

Other protocols describing isolation of MEFs

Hogan B, Beddington R, Constantini F, Lacy E (1994). *Manipulating the Mouse Embryo*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Robertson EJ (1987) Embryo derived stem cell lines. In: Robertson EJ (ed.). *Teratocarcinoma and Embryonic Stem Cells: A Practical Approach*. Oxford: IRL Press.

mESC derivation

Evans MJ, Kauffman MH (1981). Establishment in culture of pluripotent cells from mouse embryos. *Nature* 292: 154–156.

Martin G (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci USA* 78: 7634–7638.

hESC derivation

Thompson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM (1998). Embryonic stem cell lines derived from human blastocysts. *Science* 282: 1145–1147.

Web resources

WiCell Research Institute, Inc. (www.wicell.org)

Geron Corp. (www.geron.com)

NIH ES Cell (www.nih.gov)

CHAPTER

4

Cryopreservation of Human Embryonic Stem Cells

Chris Stubban, Robin L. Wesselschmidt, Igor Katkov, and Jeanne F. Loring

INTRODUCTION

Cryopreservation is used to stabilize cultures at specific points in time with specific genetic characteristics. Without the ability to cryopreserve our cell lines, we are forced to continuously subculture them, during which time the cells may accumulate genetic changes and become heterogeneous. Cryopreservation allows us to produce a bank of stock vials at specific passages with specific genetic characteristics. Using validated stock vials to initiate new experiments maximizes the long-term usefulness of a cell line and minimizes experimental variation.

During cryopreservation, most of the water is removed from the interior of cells and is converted to ice. This stops metabolism and allows cells to be stored at low temperatures for long periods of time. However, recovery of hESCs from cryopreservation is sometimes very poor, and because of the slow growth rate of hESCs, the time from thawing of the vial to having cultures suitable for experimentation can be weeks to months.

Human Stem Cell Manual, edited by J. F. Loring, R. L. Wesselschmidt, and P. H. Schwartz.
ISBN: 978-0-1237-0465-8.

Overview

The methods outlined here work well in some laboratories but not others, for reasons that are not clear. This is an area of active research, to maximize the viability and maintain pluripotency of hESCs after cryopreservation. The most important issue is to make sure that the cells used for cryopreservation are in excellent condition, actively proliferating, and that the cultures have very little differentiation. Freezing the cells at high density appears to improve their viability after thawing, but since hESCs do not survive well after being dissociated into single cells, the exact densities cannot be easily quantified.

We provide two different methods in this chapter. The main method is a modification of a standard slow freezing protocol that works well for at least some hESC lines. For this protocol, we recommend that the entire population of a culture vessel be placed into 1–3 vials, and when thawed, the cells should be placed in the same size vessel.

The second method, vitrification, is offered as an alternative procedure. Vitrification is a rapid freezing technique that minimizes formation of damaging ice crystals. While vitrification requires considerable skill, for some hESC lines it gives consistent results, and for researchers who master this method it is a recommended technique for cryopreserving small numbers of cells from newly derived hESC lines.

Procedures

Slow freezing of cells

1. Prepare actively proliferating, high-density cells as you would for passaging. Change the culture medium just before harvesting the cells.
2. Label 1.8 mL cryovials with cell line name, date, and passage number.
3. Mix 2× stock cryopreservation medium (see Recipes) and keep on ice.
4. Dislodge the colonies from the plate mechanically using a sterile pipette tip. Alternatively, treat with 200 U/mL of collagenase IV for 5–10 min at 37°C. Remove collagenase and replace with ESC medium (3 mL for each well of a six-well dish).
5. For each well of a six-well dish, collect the cells in 3 mL of ESC medium and transfer to a 15 mL conical tube.
6. Centrifuge 5 min at 0.2 rcf (usually about 1000 rpm). Aspirate supernatant, leaving a small amount of medium.
7. Gently resuspend the pellet in protein-containing ESC medium (usually 1.5 mL for each well of a six-well dish or one half of the final freezing volume). Use a 5 mL pipette to gently triturate the cells.
8. Drop wise, add an equivalent volume of ice-cold 2× stock cryopreservation medium, mixing constantly by tapping the tube.
9. Place 1.0 mL of cell mixture in each cryovial.

10. Rapidly transfer the vials to a precooled (4°C) Nalgene freezing container (containing isopropanol), and place immediately in a freezer at –70 to –80°C.

NOTE: Do not leave the cells in DMSO at room temperature for long periods of time.

11. Transfer cells to liquid nitrogen for long-term storage.

Thawing cells

There is a growth lag upon thawing the cells and it may take several days in order to be able to visualize the colonies. It is advisable to observe the cultures under 4× magnification 24 h after thawing, but not exchange the medium for at least 48 h. There will be a lot of floating debris and dead cells upon thawing the cells – this is normal (Figure 4.1).

1. Gently thaw the vial of cells by shaking it gently in a 37°C water bath and remove while a sliver of ice still remains.
2. Spray the tube with 70% EtOH and dry with a Kimwipe.
3. In the biosafety hood, aseptically remove the vial contents and place into a 15 mL conical tube.
4. Slowly, with gentle tapping, add 10 mL of room temperature culture medium.
5. Spin very gently at 0.2 rcf (1000 rpm) for 5 min.
6. Remove the supernatant.
7. Tap the tube to dislodge the pellet.
8. Add 3 mL of ESC medium to the tube and transfer to one well of a six-well dish that has been prepared with an inactivated feeder layer or extracellular matrix.
9. Place plate into the incubator and allow the cells to attach to the plate.
10. Allow 3–7 days for the cells to attach. During this time replace half of the medium every other day.
11. The medium should be replaced daily starting 4–7 days after thawing the cells, or when the cells appear to be attached.

PITFALLS AND ADVICE

Centrifugation

Centrifugation can damage sensitive cells. The major factors that can be important are: relative centrifugal force (200×*g*, 400×*g*, 800×*g*), time (5, 10, and 15 min), length of the column of liquid (3 mL, 6 mL, and 12 mL in 15 mL tubes) and the type of centrifuge (fixed angle or swinging bucket).

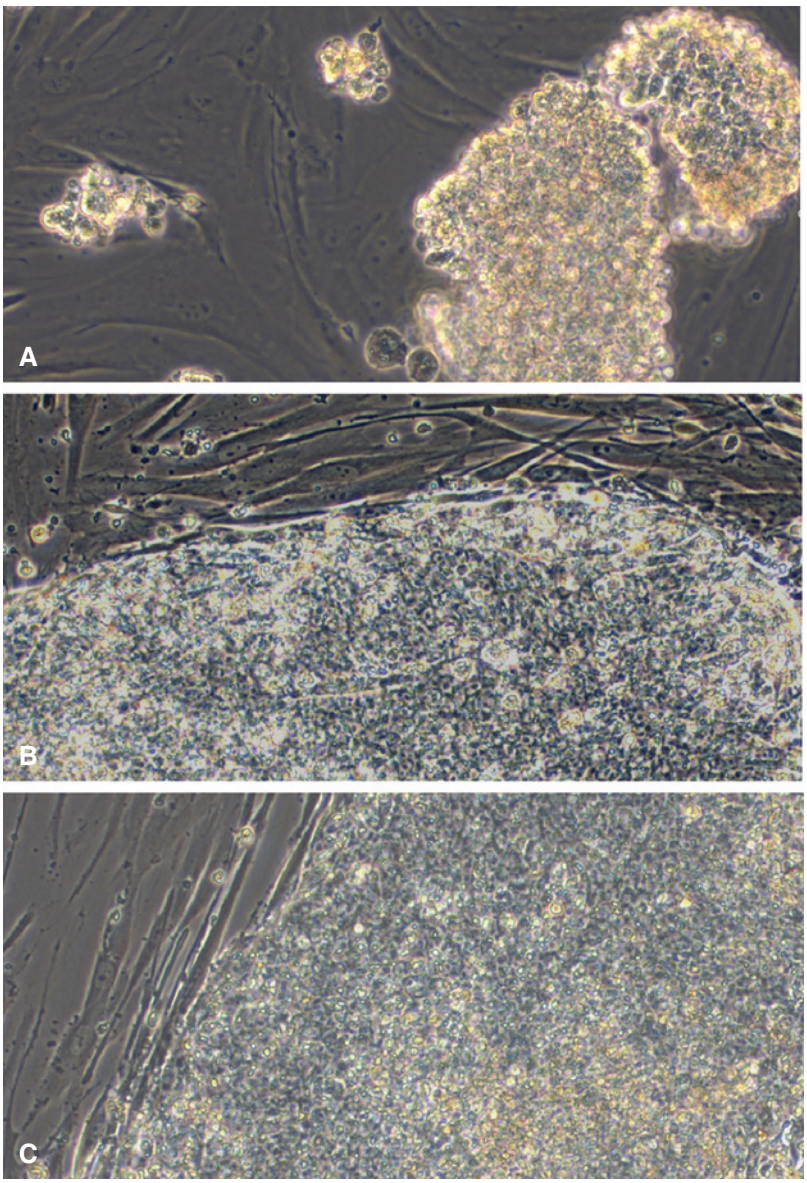

Figure 4.1 Appearance of thawed hESC cultures: (A) immediately after thawing, (B) 3 days after thawing and (C) 6 days after thawing.

Cryoprotectants

DMSO could contribute to hESC death and differentiation. First, addition and removal of DMSO causes osmotic stress that may affect the survival of delicate cells. Second, DMSO itself has been shown to be a potent inducer of apoptosis and differentiation. Alternate cryoprotectants that are being investigated are permeable agents such as ethylene glycol, propylene glycol, glycerol and erythritol and non-permeable sugars and sugar-alcohols such as D-glucose and fructose, sucrose, trehalose, mannose, raffinose, adonitol, glucitol, and sorbitol.

Equipment

- Standard tissue culture equipment
- Pipettors
- Centrifuge
- −80°C freezer
- Liquid nitrogen freezer.

Supplies and Reagents

- Pipette tips for dislodging colonies: 10 μL VWR micropipette tips (VWR catalog no. 53511-681) work well for collecting undifferentiated colonies; Neptune micropipette tips (Neptune catalog no. CLP 2142.S) tend to be stickier and are useful for removing areas of differentiation from cultures.
- Cryovials such as Nunc (VWR catalog no. 66021-996).
- Slow-freezing container such as Nalgene Labware Cryo 1°C Freezing Container, "Mr. Frosty."

Recipes

Stock solution

2× Stock cryopreservation medium (5 mL) for hESC

Component	Amount (mL)	Stock concentration (%)
Complete hESC medium with serum replacement	1	20
KnockOut™ serum replacement (Invitrogen)	3	60
DMSO	1	20

Alternative Procedures

Freezing cells by open pulled straw vitrification

Open pulled straw (OPS) vitrification is not a trivial technique. It requires preparation of three different media and careful work under a dissecting scope. In this method, hESC colonies are dissected, and 10–12 individual undifferentiated pieces of colonies are carefully collected and placed into sequential vitrification media with increasing concentrations and combinations of cryoprotectants. The cells are then placed into straws and frozen by plunging into liquid nitrogen. In spite of the extra time spent preparing and

the great care to freeze only undifferentiated clumps of cells, this method results in a very high (>90%) percentage recovery of the frozen cellular aggregates, with very low percentage of differentiation in the cultures following recovery from cryopreservation.

It is possible that since there is low percentage of cell death and rapid recovery following OPS vitrification, the selective pressures that may be at play during more traditional cryopreservation methods may not be as much of an issue with this method.

NOTE: This method was adapted from methods used at the Monash Institute of Medical Research Laboratory of Embryonic Stem Cell Biology.

Freezing cells by vitrification

For this method, hESC colonies are dissociated using a combination of dispase (10 mg/mL in serum-containing medium) and mechanical dissection into clumps of ~100–200 cells each.

The sequential incubations are performed on a 37°C heated stage of a dissecting microscope. This procedure should not take more than *3 min* from the time the cells are placed into vitrification solution 1 (VS1) until they are placed into liquid nitrogen. Work quickly.

Set-up for vitrification

Label 4.5 mL cryovials with cell line, passage number, and date. Puncture vials with an 18G needle through the top and on the side so that liquid nitrogen can fill the vial.

Use a four-well plate with three wells containing 1 mL each of holding medium (HM), VS1, VS2 vitrification solutions on a heating stage of a dissecting microscope (Figure 4.2). Transfer of the cells will be done in droplets of VS2 on the inside of the lid of the four-well dish.

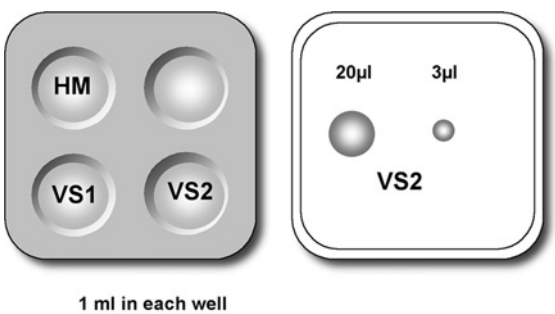

FIGURE 4.2 Four-well plate and lid set up for transfers.

Freezing hESCs

1. Place 8–10 clumps of hESCs in HM in the dish on the warming stage.
2. Collect the undifferentiated clumps of cells with a 20 µL pipettor and transfer to a well containing VS1 (10% DMSO). Start timing: it should be no more than

3 min from the time the cells are placed into VS1 until they are placed into liquid nitrogen.

3. Incubate for 1 min.
4. Move clumps to the well containing VS2 (20%) for 25 s.
5. Remove clumps in 20 μL VS2 and transfer to the lid of the four-well plate.
6. Pick up the clumps in approximately 3 μL and place the drop on the lid. Immediately touch the narrow end of the straw to the droplet and the clumps and solution will be drawn up into the straw by capillary action.
7. Hold the straw at a 45-degree angle and plunge into liquid nitrogen.
8. Using a pair of forceps, carefully load the straws into the labeled 4.5 mL cryogenic vials.
9. Store submerged in liquid nitrogen.

Solutions

Holding medium (HM)	D-MEM containing HEPES (add 1 M stock HEPES to make 10 mM final) 20% FBS
Vitrification solution 1 (VS1)	10% DMSO (Sigma catalog no. D2650) 10% Ethylene glycol (Sigma catalog no. E9129)
Vitrification solution 2 (VS2)	20% DMSO (Sigma catalog no. D2650) 20% Ethylene glycol (Sigma catalog no. E9129) 0.5 mol/L Sucrose (Sigma catalog no. S7903)

Supplies and equipment

- Dissecting microscope with heating stage, such as Leica ZM6
- Glass capillaries, 1.0 mm outer diameter for dissecting hESC colonies (Harvard Apparatus GC 100T-15)
- 4.5 cryogenic vials (Nunc catalog no. 379146)
- Four-well plates (Nunc catalog no. 176740)
- Label with cell line, passage number, and date
- Puncture with an 18G needle through the top and on the side so that liquid nitrogen can fill the vial
- Vitrification straws: French mini-straws (250 μL) gamma irradiated. LEC Instruments (www.lecinstruments.com)
- Dewar flask containing liquid nitrogen.

Thawing vitrified cells

This is a timed process with sequential removal of cryoprotectants by incubating the frozen clumps of cells in decreasing concentration of sucrose.

Set-up for thawing cells

On a 37°C heated stage of a dissecting microscope, set up a four-well plate containing 1 mL each of the following media that will be used for the indicated cell incubations (Figure 4.3):

HM 0.2 M sucrose	1 min
HM 0.1 M sucrose	5 min
HM (two wells)	5 min each well

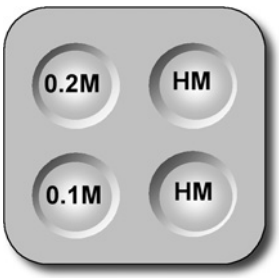

FIGURE 4.3 Four-well plate for transferring cells during thawing.

Procedure

1. Carefully remove the straw from the cryogenic vial.
2. Immediately submerge the narrow end of the straw (the end containing the cells) into the well containing HM + 0.2 M sucrose.
3. Place your forefinger on the top of the straw; as the straw warms, the contents of the straw will be expelled into the well.
4. Incubate the clumps of cells for 1 min in HM + 0.2 M sucrose.
5. Using a 20 μL pipettor, transfer the clumps of cells to a well containing HM + 0.1 M sucrose.
6. Incubate for 5 min.
7. Transfer the clumps to a well containing HM.
8. Incubate for 5 min.
9. Transfer clumps to fresh well containing HM.
10. Incubate for 5 min.
11. Pick up the clumps and place them in the prepared culture dish.
12. Place the culture dish into the CO_2 incubator.

NOTE: The clumps should attach to the feeder layer overnight and the medium should be replaced as usual for feeding the cells and they should be ready for passaging in 7 days.

Supplies and equipment

- Dewar flask containing liquid nitrogen
- Timer, stopwatch

- Prepared culture dishes containing feeder layer or extracellular matrix and equilibrated with hESC medium.

Reading List

Cryopreservation

Katkov II, Kim MS, Bajpai R, Altman YS, Mercola M, Loring JF, Terskikh AV, Snyder EY, Levine F (2006). Cryopreservation by slow cooling with DMSO diminished production of Oct-4 pluripotency marker in human embryonic stem cells. *Cryobiology* 53: 194–205.
This article describes a protocol for testing recovery from freezing using a POU5F1/OCT4-GFP cell line to track the fate of cells.

Ware CB, Nelson AM, Blau CA (2005). Controlled-rate freezing of human ES cells. *Biotechniques* 38: 879–880, 882–873.
This is a detailed method for freezing human ES cells using conventional methods.

Vitrification

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This article reports application of the embryo freezing technique to hESC.

Trounson A, Leeton J, Besanko M, Wood C, Conti A (1983). Pregnancy established in an infertile patient after transfer of a donated embryo fertilised in vitro. *Br Med J (Clin Res Educ)* 286: 835–838.
This is an early report of freezing human embryos using vitrification.

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PART

II

Characterization of Stem Cells

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CHAPTER

5

Classical Cytogenetics: Karyotyping

Robin L. Wesselschmidt and Jeanne F. Loring

INTRODUCTION

Embryonic stem cells (ESCs) are arguably the most stable normal diploid cells that can be maintained in long-term culture. However, aneuploidies and other chromosomal abnormalities have been reported to occur in both human and mouse ESCs. Aneuploid mouse ESCs do not contribute to the germline in chimeric animals. But since this ultimate test of normalcy cannot be applied to hESCs, the cultures must be routinely evaluated for chromosomal abnormalities.

When hESCs are cultured using high-quality validated reagents, and passaged using gentle techniques, the cells can maintain a normal karyotype for years of continuous culture. However, under certain conditions that are believed to stress the cells, such as enzymatic passaging or culture in the absence of feeder cells, cultures tend to acquire karyotypic abnormalities. hESCs that acquire an extra chromosome 12 and the long arm of chromosome 17 (17q) appear to have a growth advantage and can eventually dominate the cultures. While we do not know when accumulated genotypic changes tip the scales to make a hESC culture no longer useful for experimental or therapeutic applications, we do know that the higher the percentage of abnormal cells in our cultures, the more cells are drifting towards an abnormal phenotype, and the less reproducible and dependable are the results.

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There are several methods for assessing chromosomal stability, including the classical cytogenetics approaches described here, and the SKY, FISH, and SNP methods described in other chapters. These methods differ in resolution and the types of abnormalities they can detect. The best resolution obtainable by classic cytogenetic methods is estimated to be about 10 Mb, while spectral karyotyping (SKY) resolves at 1–2 Mb, and single nucleotide polymorphism (SNP) and copy number polymorphism (CNP) mapping can give 30 kb resolution. However, more resolution is not necessarily better; for example, SNP genotyping cannot be used to detect translocations or inversions, and SKY cannot detect inversions or duplications.

Cytogenetics is currently the most accessible method for detecting chromosomal abnormalities. But there are shortcomings to this technique. First, cytogenetic analysis can only be applied to metaphase-stage cells, so a rapidly dividing population is required. And second, while most hospitals have laboratories that perform karyotyping as a service, such laboratories routinely examine metaphases from 20 cells, 6 of which are analyzed and 14 are counted. This gives only a hint of the composition of the cell population, and many hESC researchers prefer analysis of 100 metaphases. For this reason, and to lower costs, some research laboratories are learning to perform their own karyotyping, at least at the gross level of counting chromosomes to detect aneuploidies.

It is relatively easy to count the chromosomes to determine the modal chromosome number and, with training, one may be able to identify chromosomes by their individual size and banding pattern. It is unlikely that an untrained eye will be able to identify the translocations or deletions that do not change the chromosome count, but may drastically modify the genome. So while we suggest that a research laboratory should routinely count chromosomes, we recommend that the detailed karyotype of the culture be obtained from a trained cytogeneticist every 10–15 passages.

Overview

The basic conventional cytogenetic method involves chromosome harvest, slide preparation, banding of the chromosomes, analysis of banding patterns, and interpretation of the results. In this chapter we will describe:

- How to prepare a culture to maximize the number of metaphase chromosomes
- How to prepare slides containing chromosome spreads
- How to stain chromosomes
- How to interpret the cytogenetic report.

Chromosome harvest consists of arresting the cell cycle at metaphase, hypotonic treatment of the cells and their fixation. After fixation, the chromosomes are spread onto glass slides, air-dried, and aged before banding. Chromosomes are stained and visualized as a continuous series of light and dark bands. A band is defined as that part of a chromosome that is clearly distinguishable from its adjacent segments by appearing darker or lighter. Slide preparation profoundly affects the quality of banding and it is one of the most challenging steps in chromosome preparation and analysis.

Different banding patterns, such as G-, Q-, R-, C-, T-, or NOR-banding, can be generated for analysis. The G-banding method (using the Giemsa dye mixture) is the most commonly used staining method, and it can generate up to 1000 bands per haploid human genome. Each band has a specific number assigned to indicate its location on the human chromosome. The nomenclature of band assignment and chromosome aberrations is sanctioned by the International System for human Cytogenetic Nomenclature (ISCN 2005).

PROCEDURES

Metaphase harvest of hESCs

A culture with actively dividing cells is the best way to obtain high-quality metaphases. Since hESCs are usually actively dividing, it is relatively easy to obtain many quality metaphase chromosomes from a culture. We suggest harvesting the cells for karyotyping roughly one day before they would normally be passaged. This strategy should yield a high number of dividing cells and therefore a sufficient number of metaphase chromosomes in order to make an accurate analysis of the culture.

This procedure describes harvesting cells from a 35 mm dish or one well of a six-well plate.

1. Add 1/100 volume of colcemid stock solution to the culture.
2. Return the culture to the incubator for 2–3 h.
3. Aspirate the medium and wash with 1 mL of PBS.
4. Trypsinize the cells with 0.3 mL trypsin, recover with 0.6 mL complete medium, and transfer into a microfuge tube.

NOTE: Good metaphase spreads require a single cell suspension.

5. Spin in a microfuge at 3000 rpm for 5 min at room temperature.
6. Aspirate the medium carefully, leaving about 50–100 μL. Resuspend the cell pellet by tapping the tube.
7. Add 1.5 mL of hypotonic solution and let stand at room temperature for 15 min.

NOTE: The timing is important; if the cells are incubated too long they may burst and the chromosomes will spill out of the cell membrane. If too short, the chromosomes may be too tightly packed to analyze.

8. Gently invert the tube several times in order to resuspend the cells and then add a few drops of fixative (3:1 methanol:glacial acetic acid). Mix by inverting the tube several times.
9. Spin in a microfuge at 3000 rpm for 5 min at room temperature.
10. Aspirate the hypotonic solution carefully, leaving about 50–100 μL. Resuspend the cell pellet well by tapping the tube.

11. Add 1 mL of fixative (3:1 methanol:glacial acetic acid); mix well.
12. Spin in a microfuge at 3000 rpm for 5 min at room temperature.
13. Aspirate the fixative carefully, leaving about 50–100 μL. Resuspend the cell pellet well.
14. Repeat steps 10 and 11: Add 1 mL fixative (3:1 methanol:glacial acetic acid), mix well, then spin in a microfuge at 3000 rpm for 5 min at room temperature.
15. Add an appropriate amount of fixative to the pellet for making slides immediately.

NOTE: Fixed cells may be stored at 4°C for up to a week before slides are made.

Slide preparation: making chromosome spreads

There are many ways to make slides. Below we describe a protocol that has been used successfully to make high-quality chromosome spreads and will provide a good starting point from which one can develop an individualized method.

Set-up for chromosome spreads

1. Prepare a Coplin jar with slides soaking in 100% methanol. It is a good idea to use single frosted slides, so the slides can be easily marked.
2. Make fresh fixative (3:1 methanol:glacial acetic acid).
3. Prepare a slide-making area with 2–3 sheets of paper towels, a folded Kimwipe for slide cleaning, and a pencil for marking the slides. Place the Coplin jar with slides soaking in 100% methanol and a beaker of deionized or distilled water on one side and fixative with a Pasteur pipette on the other side (if you are right-handed, the slides and water should be on the left and the fixative should be on the right). The chromosome harvest can be placed either in the middle or on the same side as the fixative, with a Pasteur pipette alongside.
4. Remove a slide from the Coplin jar of methanol and use a folded Kimwipe to polish the surface of the side to be used for the cells. It is important to keep track of the polished side of the slide so that the chromosome harvest is dropped on the polished side; frosted end slides are useful for this purpose.

Chromosome spreads

If you are right-handed, hold the slide with your left hand so that you can hold a pipette in your right hand. The descriptions below are for a right-handed person.

NOTE: The following steps will be performed at nearly the same time, so you will need to coordinate left and right hands. We recommend that you practice these steps several times before using cell samples that may be limited.

1. *Left hand:* Dip the slide back into the methanol jar briefly, remove and swirl the slide in a beaker of deionized or distilled water. The rinse should be just long enough for a uniform film of water to coat the polished side of the slide. You will be able to observe this easily as you lift the slide out of the water.

FIGURE 5.1 The two steps for making slides of chromosome spreads. Apply chromosome harvest in three drops, starting at the far end of the slide. Immediately flow the fixative across the top of the slide.

2. *Right hand:* Gently but thoroughly mix the prepared cells with a glass Pasteur pipette.
3. *Right hand:* Draw the cell mixture into the Pasteur pipette and allow the suspension to sit in the pipette, so that it is ready for dropping onto the slides. At the same time, proceed with the next step with the other hand.
4. *Left hand:* Hold the frosted end of the slide between fingers and thumb (index and middle fingers on the polished side and thumb on the back side) and keep the polished side up as you lift the slide out of the beaker of water. Keep the long edge of the slide in contact with the paper towel and tilt the top edge of the slide forward quickly to drain off the excess water. Then tilt the top edge backwards until your thumb is rested on the paper towels (approximately 30 degrees between the back side of the slide and the paper towels).
5. *Right hand:* Hold the Pasteur pipette horizontally about 2–7 cm above the slide. Drop **three** drops of chromosome harvest, evenly spaced, along the slide, starting from the free end of the slide and moving toward the end you are holding. The drops should land on the slide slightly above the midline of the length of the slide – about one-third of the slide width from the top of the slide (Figure 5.1A).

NOTE: The number of drops per slide can be adjusted according to the density of the chromosome harvest after the test slide is evaluated. If it requires more than four drops of chromosome suspension, spin down the suspension in the microfuge and reduce the amount of fixative accordingly.

6. *Right hand:* Fill a Pasteur pipette with fresh fixative and flow it across the top of the slide immediately after dropping the chromosome harvest (Figure 5.1B).
7. *Left hand:* Tilt the slide forward and tap gently on the paper towels to drain off the fixative.
8. *Right hand:* Wipe off the back and the long edges quickly, and mark the slide with a pencil on the frosted end.

Preparation for staining and banding

- Drying: The drying process can affect how the chromosomes spread and the banding quality. In general, it is sufficient to dry the slide without much manipulation when the humidity is about 50%. If necessary, the humidity can be manipulated by using a damp paper towel or hot plate as drying surface as

needed. The morphology of the chromosomes should be evaluated with a phase contrast microscope.

- Prior to staining, bake the slides at 90°C for 30 min.

G-Banding of chromosomes

Chromosomes can be stained with dyes that result in a specific banding pattern on each chromosome. This banding pattern is used to determine the identity and integrity of individual chromosomes and the karyotype of a cell. Giemsa ("G") is the dye mixture that is most commonly used to stain chromosomes. G-banding allows the specific identification of individual chromosomes as well as segments of each individual chromosome.

The "bands" are differently stained regions (and subregions) that are recognizable in chromosomes, and are given numerical designations, from proximal to distal on the chromosome arms. The short and long arms of chromosomes are designated as p and q, respectively.

A cytogeneticist can identify deletions, translocations, inversions, and duplications of chromosomes by analyzing G-banded chromosome spreads. G-bands are provided in reference materials that describe genes (for example, NIH's NCBI Entrez Gene: www.ncbi.nlm.nih.gov/entrez/query.fcgi?DB=gene, and the European Bioinformatics Institute: http://www.ebi.ac.uk/), and the Online Mendelian Inheritance in Man database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?DB=omim) and provide disease information linked to banding data. The nomenclature of band assignment and chromosome aberrations is provided by the International System for human Cytogenetic Nomenclature (ISCN 2005).

Set-up for chromosome staining

Set up five Coplin jars and label them:

No. 1 Coplin jar	0.05% Trypsin/EDTA
No. 2 Coplin jar	0.9% NaCl
No. 3 Copin jar	0.9% NaCl
No. 4 Coplin jar	Giemsa stain (freshly prepared: 2.5 mL in 47.5 mL Gurr's buffer)
No. 5 Coplin jar	dH_2O

Chromosome staining

1. Dip the dried slides into no. 1 Coplin jar, trypsin solution; timing varies from 3 to 10 s depending on the cell type.

NOTE: Too long in the trypsin and the chromosomes become "ragged," too short a time and the bands will not be visible.

2. Rinse quickly twice in no. 2 and no. 3 Coplin jars containing 0.9% NaCl.
3. Stain in Giemsa solution (no. 4 Coplin jar) for up to 10 min.
4. Rinse the slide in dH_2O water (no. 5 Coplin jar) and dry the slide with lens paper while resting it on a flat dry surface.

5. Air dry.
6. Cover with mounting medium and a coverslip.
7. Observe chromosomes using a bright field 100× oil immersion lens.

Interpreting results

Professional cytogeneticists examine at least 20 metaphases. Generally, six metaphases are analyzed and the other 14 are counted. However, if an abnormal chromosome is observed, the cytogeneticist will search the slide for this and other abnormalities and may end up evaluating more than 20 spreads to determine whether this particular abnormality represents the clonal expansion of a cell within the culture or a random change that does not represent a significant shift to a neuploidy.

The International System for human Cytogenetic Nomenclature (ISCN, 2005) establishes the rules for identifying and naming individual chromosomes and chromosomal abnormalities that are used by cytogeneticists to determine the karyotype of a cell. A book containing this information is updated and published periodically.

Karyotype

The karyotype, by convention, provides the following information: modal number, sex chromosome, abnormal abbreviation (1st chrom; 2nd chrom) (arm band number; arm band number):

- Modal number: total count of number of chromosomes in each cell of a given cell line
- Sex chromosomes: complement of X and Y chromosomes
- Band number: numerical description of the location of a band on a chromosome arm, in order, from the centromere to the end of the chromosome. These numbers are a standard determined by the ISCN, revised in 2005.

Example	Interpretation
47, XY, +12	Male with an extra copy of chromosome 12
46, XX, del(5p15.2)	Female with a deletion of band 15.2 in the p-arm of chromosome 5 (cri du chat syndrome)

Karyogram

A karyogram is made by taking a photograph of a G-banded metaphase. Then the individual chromosomes are cut out of the photograph (originally, the "cuts" were made by scissors, but now software is usually used) and arranged in a standardized template by size, specific banding pattern, and centromere location. By convention, the short (p) arm is at the top of the chromosome image (Figure 5.2).

Resolution

One of the variables in classical karyotyping by G-banding is the "resolution." The resolution of the karyotype is related to the number of bands that are visible and therefore

the smallest segment of the genome that can be detected using this method. The most common method to determine the resolution of banded chromosomes is to count the number of bands visible on chromosome 10 and then estimate the resolution from the chart in Table 5.1.

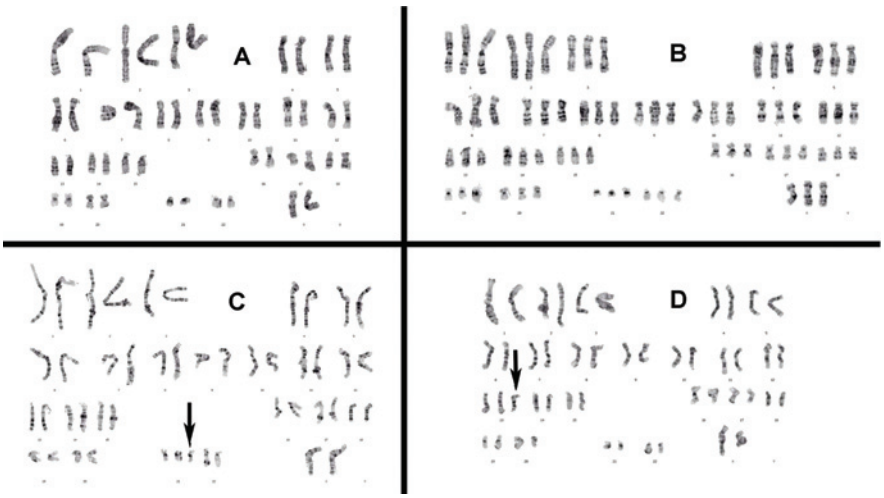

FIGURE 5.2 Karyograms: (A) Normal female: 46, XX. (B) Trisomy of all chromosomes: 69, XXX, +1–23. (C) Trisomy 21: 47, XX, +21. (D) Trisomy 13: 47, XX, +13.

TABLE 5.1 The resolution of a karyotype is determined by counting the bands found on chromosome 10

Average number of bands in chromosome 10	Estimate of the total number of bands in one haploid set (= resolution)
12	375
13–14	400
15–16	425
17–18	450
19–21	475
22–23	500
24–25	525
26–28	550
29	575
30	600
31	625
32	650
33	675
34	700
35	725
36	750
37	775
38	800
39	825
40–41	850

Alternative Procedures

DAPI staining

This is a simple method used to stain DNA and count chromosomes, but it will not allow identification of individual chromosomes.

1. Add a drop of mounting medium containing DAPI to the slide.
2. Seal the coverslip.
3. Count chromosomes under UV light using 100× oil immersion lens.

Pitfalls and Advice

Cultures

The cultures should be subconfluent and actively dividing for best results.

One effective method of obtaining enough metaphase chromosomes is to harvest hESCs for karyotyping the day before they would normally be passaged.

During the metaphase of mitosis, the chromosomes reach their highest level of condensation and become identifiable under the microscope. The chromosomes are less condensed at early metaphase and become more condensed as the cell progresses towards the end of metaphase. Since the goal of harvesting the cells is to obtain as many quality metaphase chromosomes as possible in order to make an accurate analysis of the culture, colcemid is added to the cultures as it blocks the cells in metaphase.

Longer treatment with colcemid will increase the mitotic index, but prolonged treatment will lead to higher fraction of cells with condensed, short chromosomes, and the resolution of G-bands will be low. In order to obtain both a good mitotic index and good chromosome length, the optimum length of time the cells are incubated with colcemid can be determined empirically.

Chromosome spreads

Unlike mouse chromosomes, human chromosomes generally have distinct arms visible on both sides of the centromere. However, chromosomes often overlap in the metaphase spread, so it can be difficult for the untrained eye to identify individual chromosomes; this is especially difficult for the smaller chromosomes, 21, 22, and Y.

Hypotonic solution is used to swell the cells and allow the chromosomes to separate. If the cells are left too long in the hypotonic solution the cells will burst and it becomes impossible to determine which chromosomes belong together.

Drying the slides immediately after the cells are dropped to make the spreads seems to be the most critical variable in making good slides. Best results are obtained when drying in an atmosphere of 50% humidity at 22°C. This can be achieved by monitoring the humidity and temperature in the working area using a portable hygrometer/thermometer

and adjusting humidity by adding or removing wet paper towels in the immediate slide-making area.

Even after one becomes proficient in making slides, variables such as temperature and humidity may be difficult to control. In general, a "test" slide is made to determine whether the density of the cell suspension is adequate and whether the condition of slide drying is appropriate for the specific harvest and for the given day.

Equipment

- Tissue culture incubator, 37°C, 5% CO_2 in humidified air
- Tissue culture hood, class II
- Microcentrifuge
- Microscope with 100× oil immersion objective and 40× phase contrast objective
- Camera for photographing spreads
- Coplin jars with lids
- Forceps
- Water bath 60–65°C
- Oven heated to 90°C
- Portable hygrometer/thermometer for monitoring the humidity and temperature in the working area.

Supplies and Reagents

Reagents

Item	Supplier	Catalog no.	Alternative
Colcemid (10 μg/mL)	Invitrogen	15212-012	Many
Potassium chloride (KCl)	Sigma Aldrich	P4504	Invitrogen
Sodium chloride (NaCl)	Sigma Aldrich	S9625	0.9% solution
Acetic acid, glacial (99.8%) 17.4 M	Sigma Aldrich	A9967	Many
Methanol, anhydrous 100% (reagent grade)	Sigma Aldrich	322415	Many
Ethyl alcohol (99.5%, 200 proof)	Sigma Aldrich	459836	Many
Trypsin/EDTA 0.05%, 1 mM EDTA	HyClone	SH30236.01	Many
Giemsa stain (stock solution)	Invitrogen	10092-013	Many
Gurr's buffer pH 6.8 (tablets)	Invitrogen	10582-013	Many
Mounting medium	VWR	48212-290	Many
Mounting medium with DAPI	Vector Laboratory	H-1200	

Supplies

Item	Supplier	Catalog no.	Alternative
Slides	Corning	2949-75 × 25	Fisher, super frost
Coverslips no. 1	VWR	48393-081	Many
Pasture pipettes 5″ glass	VWR	14672-608	Many
Coplin jars	VWR	25460-000	Many
Pipettes: 5 mL, 10 mL	Corning	4487,4488	Many
15 mL conical tubes	Corning	430766	Many
1.5 mL microcentrifuge tubes	VWR	20170-355	Many

Recipes

Stock solutions

Component	Amount	Stock concentration
Colcemid	5 mL	10 μg/mL
Potassium chloride	100 mL	0.75 M
Fixative	40 mL	3:1 Methanol:acetic acid
Gurr's buffer solution pH 6.8	1000 mL	1 tablet/1000 mL dH_2O
NaCl	100 mL	0.9%

Fixative: methanol:acetic acid 3:1 (40 mL)

Component	Amount	Final concentration
Methanol	30 mL	75% by volume
Acetic acid	10 mL	25% by volume

Make fresh at time of use.

Hypotonic solution (0.075 M KCl, 100 mL)

Component	Amount	Final concentration
KCl	0.559 g	0.075 M
MilliQ water	100 mL	N/A

Alternatively, this solution can be purchased pre-made and ready to use.

0.9% NaCl (100 mL)

Component	Amount	Final concentration
NaCl	0.9 g	0.9%
MilliQ water	100 mL	N/A

Gurr's buffer stock solution (100 mL)

Component	Amount	Final concentration
Gurr's buffer tablet	1 tablet	
MilliQ water	1000 mL	N/A

Gurr's buffer/Giemsa stain solution (50 mL)

Component	Amount	Final concentration
Gurr's buffer stock solution	47.5 mL	95% by volume
Giemsa stain	2.5 mL	5% by volume

Acknowledgment

The authors and editors thank Dr Chih-Lin Hsieh, University of Southern California and Dr Touran Zadeh, The Genetics Center, Orange CA, for helpful discussions and comments.

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CHAPTER

6

Spectral Karyotyping and Fluorescent in situ Hybridization

Suzanne Peterson, Stevens Rehen, Willem Westra, Yun Yung, and Jerold Chun

INTRODUCTION

Spectral karyotyping (SKY) is a hybridization-based diagnostic technique originally developed to diagnose chromosomal aberrations associated with cancer and genetic disease. SKY can be used to detect specific inter- and intra-chromosomal genomic rearrangements, and unambiguously determine both the total number and individual identity of all chromosomes in a metaphase nucleus.

Fluorescence in situ hybridization (FISH) is a similar technology, but it can be used on non-dividing cells at interphase, while SKY requires metaphase chromosomes. FISH is used for preimplantation genetic diagnosis of single blastomeres, and is very useful when studying ESC differentiation, as the terminally differentiated cell populations are typically post-mitotic and thus cannot be karyotyped using SKY. However, FISH also has certain drawbacks compared to SKY: it does not allow enumeration of every chromosome in a single experiment because each chromosome must be assessed separately with a different FISH probe and it is difficult to detect genomic rearrangements.

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The advantages of SKY and FISH for hESCs is their ability to generate information at the single cell level. Often in the case of complex processes such as cellular differentiation and disease progression, valuable data from single cells may be obscured by the heterogeneity of the cell population, a limitation which both SKY and FISH have the potential to overcome.

Overview

The underlying procedure behind metaphase cell analysis using SKY is straightforward. SkyPaint® (Applied Spectral Imaging) is hybridized to metaphase chromosome spreads from the cells of interest. SkyPaint is a mixture of probes specific to single chromosomes, each of which contains a spectrally unique combination of fluorescent nucleotides thus allowing the user to "paint" each chromosome a different color. After acquiring a metaphase spread image using a microscope equipped with an interferometer that reads emissions across the entire visible spectrum, individual chromosomes are assigned using SkyView® software (Applied Spectral Imaging). SkyView analyzes the spectral image in two dimensions and displays each chromosome with a distinct classification color from which it creates a karyotype table (Figure 6.1).

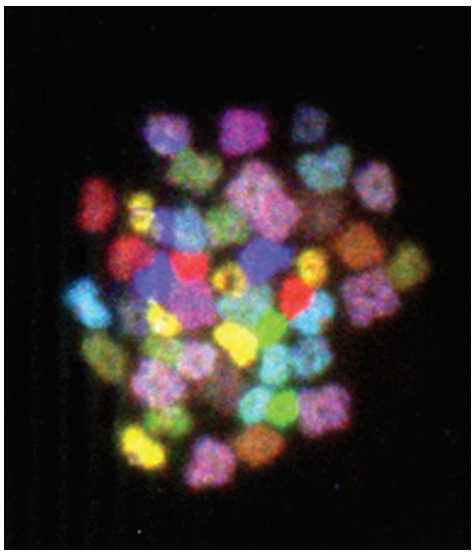
FISH is a technique used to identify the presence of a single nucleic acid sequence (often specific to a particular chromosome) through hybridization of fluorescently labeled DNA probes to denatured chromosomal DNA in cytological material. Interphase nuclei are hybridized with the FISH probe, though metaphase spreads can be used as well. FISH probes can be purchased commercially (www.vysis.com, www.openbiosystems.com), or made by the user (see http://info.med.yale.edu/genetics/ward/tavi/FISH.html). The hybridized nuclei can then be viewed using a fluorescent microscope (Figure 6.2).

Because probes for both SKY and FISH are generated using a direct labeling technique (the fluorophore is covalently attached to the nucleotide) they can be stripped from the template DNA by heat denaturation, and new probes hybridized to the same nuclei afterwards.

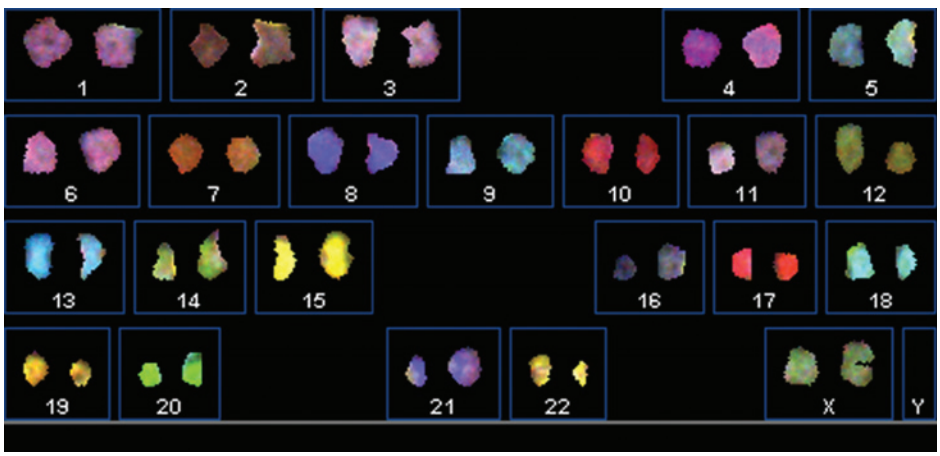
The following protocol describes basic techniques that can be used for both FISH and SKY analyses. Places where the protocols differ are noted in the text.

Procedures

To obtain a sufficient number of metaphase spreads for SKY from hESCs it is often necessary to harvest the cells to be karyotyped 1–2 days after splitting them. When cells are harvested later, there may not be enough dividing cells to obtain an adequate number of metaphase spreads. In terms of cell density, it is possible to do karyotyping with a few thousand cells but it is always better to have more. We recommend taking two confluent wells of a six-well plate of hESCs and splitting them 1:2 to make four wells. One or two days later harvest all four wells for karyotyping. The following procedure can be used for generating material for FISH as well, though it is not crucial to have dividing cells.



A



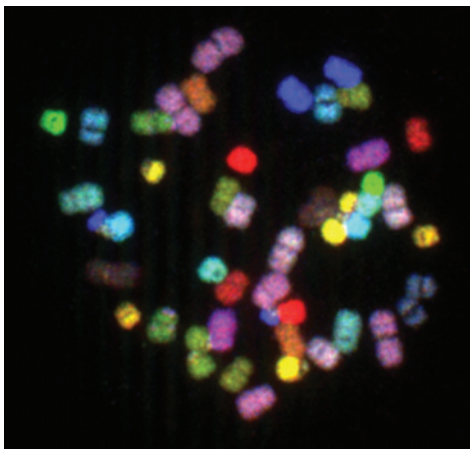
B

B. WA09 p68

85% 46, XX: no trends in chromosome loss or gain

spread#	Karyotype	spread#	Karyotype
2	46, XX	16	46, XX
3	46, XX	17	46, XX
4	46, XX	19	46, XX
5	46, XX	21	46, XX
9	45, XX, −1	23	46, XX
10	46, XX	24	46, XX
11	46, XX	25	44, XX, −14, −21
12	46, XX, −8, +19	26	46, XX
14	46, XX	28	46, XX
15	46, XX	29	46, XX

FIGURE 6.1 Spectral karyotyping of hESCs. (A) SKY image and karyotype table for late passage WA09 cells. (B) Karyotyping results for late passage WA09. 85% of the cells are 46, XX.



C

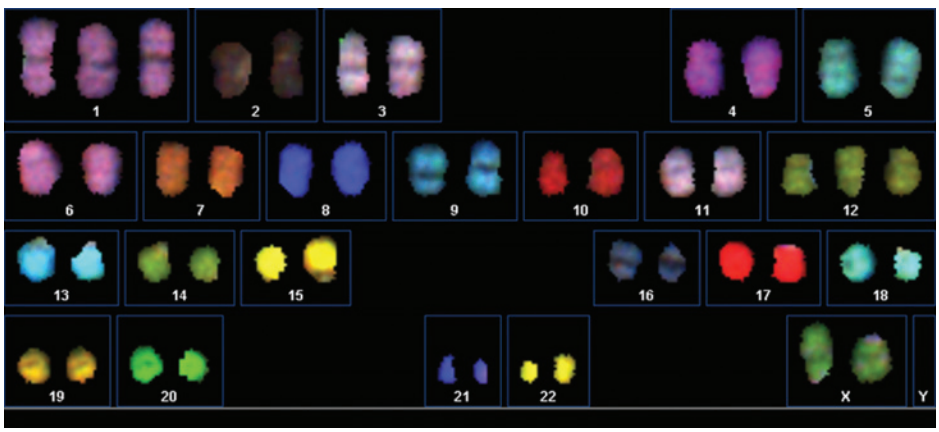


FIGURE 6.1 (Continued) (C) SKY image and karyotype table for late passage WA07 cells. This spread is trisomic for chromosomes 1 and 12.

Day 1: Cell preparation and harvest

1. Add colcemid to the cells at a final concentration of 0.1 μg/mL and return cells to the incubator for 5–6 h (necessary for SKY, optional for FISH).

NOTE: Colcemid is added to the cells to arrest them in mitosis. Because they have an extended cell cycle, longer colcemid incubations (5–6 h) are needed for hESCs compared with other cell types.

2. Trypsinize cells using 0.05% trypsin/EDTA to obtain a single cell suspension; some remaining clumps are fine.

3. Wash cells with 10 mL of PBS and aspirate the supernatant. Flick the pellet so it is easy to resuspend.

4. Add 10 mL of 0.075 M KCl to the tube, making sure that the cells are well suspended. Incubate cells in a water bath at 37°C for 15 min.

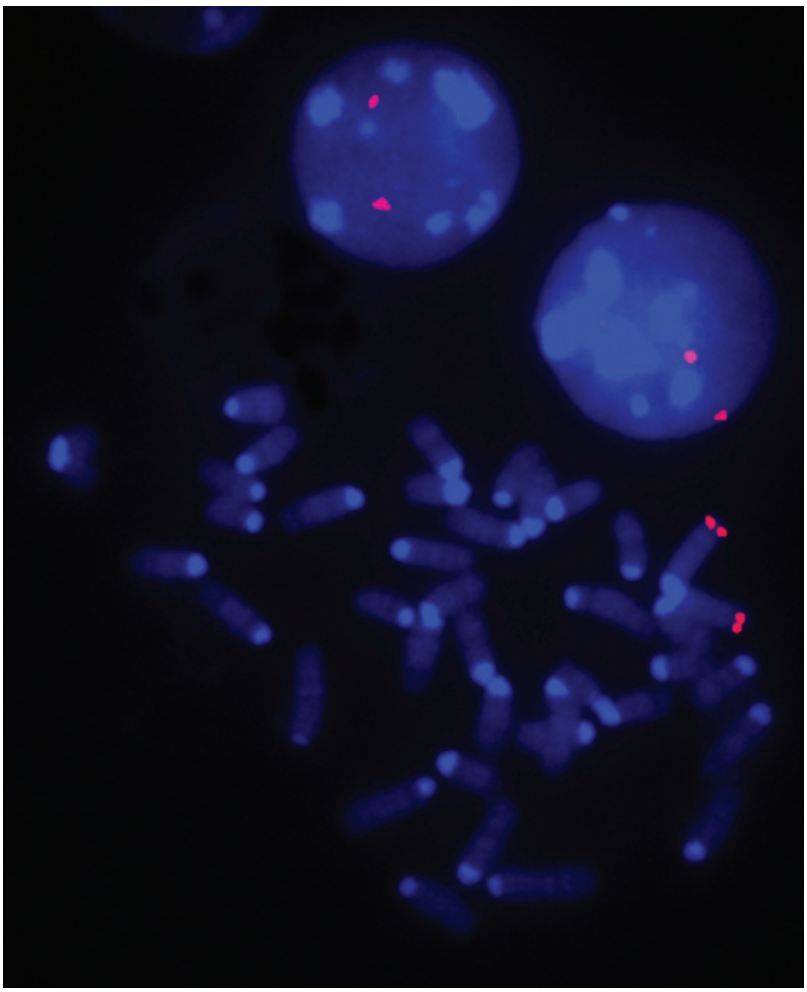


FIGURE 6.2 Chromosome X FISH. Cells from a female mouse were hybridized with an AlexaFluor 555-labeled FISH probe recognizing the X chromosome (red signals). Note that there are two interphase nuclei, both of which are disomic for the X chromosome as indicated by the presence of two well-separated dots on each nucleus. Below the nuclei there is a metaphase spread where individual chromosomes can be seen. In the metaphase spread the FISH signals appear as two apposed dots at the end of each X chromosome (red arrows). This is a consequence of DNA replication which produces two sister chromatids for each chromosome.

NOTE: Exposure of the cells to this hypotonic solution causes them to swell. This way they will break open when dropped onto a slide (see Making chromosome spreads). This step is not necessary for FISH.

5. After the 15 min incubation, add three drops of fixative dropwise with a transfer pipette to the cells while flicking the tube between drops.
6. Spin cells at 1000 rpm for 5 min at room temperature.
7. Aspirate most of the supernatant off and then flick the tube to resuspend the pellet.

8. Add 5 mL of fixative dropwise while slowly vortexing the tube. It is VERY important not to make too many cell clumps at this point.
9. Incubate at 4°C overnight.

Day 2: Making chromosome spreads

Making chromosome spreads is not necessary for FISH but this technique can be used to adhere the nuclei to the slide.

1. Let the cells warm up to room temperature and wash twice with fixative. Resuspend cells in 1 mL of fixative.

NOTE: It may be necessary to spin the cells down later and resuspend them in a smaller or larger volume of **fixative**, depending on how many cells you have. Try 1 mL to start and if the spreads on the slide look too sparse or too close together (see step 6), adjust the volume appropriately and repeat the slide-making process.

2. Open the lid on the 80°C water bath and let some of the steam dissipate. Make sure the heating plate is positioned close to the water level (1 cm) in the water bath. See Figure 6.3 for a schematic illustration of the water bath and heating plate set-up.
3. Flick cells to resuspend then take 20 μL of cell suspension and pipette it onto the slide. Hold the slide level for about 15–20 s – you should see the center of the slide become granular as the fixative evaporates.
4. Quickly flip the slide over (cell side down) and briefly hold it to the steam coming from the water bath (about 5 cm above the water level in the bath).
5. Immediately place the slide, cell side up, on the metal heating plate in the water bath until the liquid on the slide beads up and is mostly evaporated. Immediately remove the slide and look at it under a microscope.
6. Check the spreads for two things: (1) spread density and (2) chromosome color/contrast.

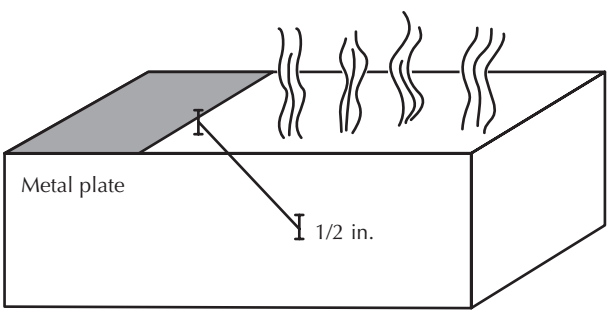


FIGURE 6.3 Diagram of water bath and heating plate. Place a thin metal plate (gray rectangle in figure) in an 80°C water bath. Position the metal plate so that it is about ½ in (1 cm) from the water level in the bath.

NOTE: In terms of density, you want a sufficient number of spreads without having them too close to one another. In Figure 6.4A the spreads are too close together to tell which chromosome belongs to which spread. In Figure 6.4B there is a good distance between spreads. In terms of chromosome color/contrast, you want the chromosomes in your spreads to look light gray in color (see Figure 6.4C). You do not want chromosomes that appear "bright," this is caused by too much steam (Figure 6.4D). You also do not want chromosomes that are too dark (black) as these have been left on the heating plate too long. Bright and dark chromosomes do not hybridize well.

7. Make 5–10 good slides/sample. Put them in a slide box and store them at room temperature for 1–7 days. This "aging" time will improve the results.

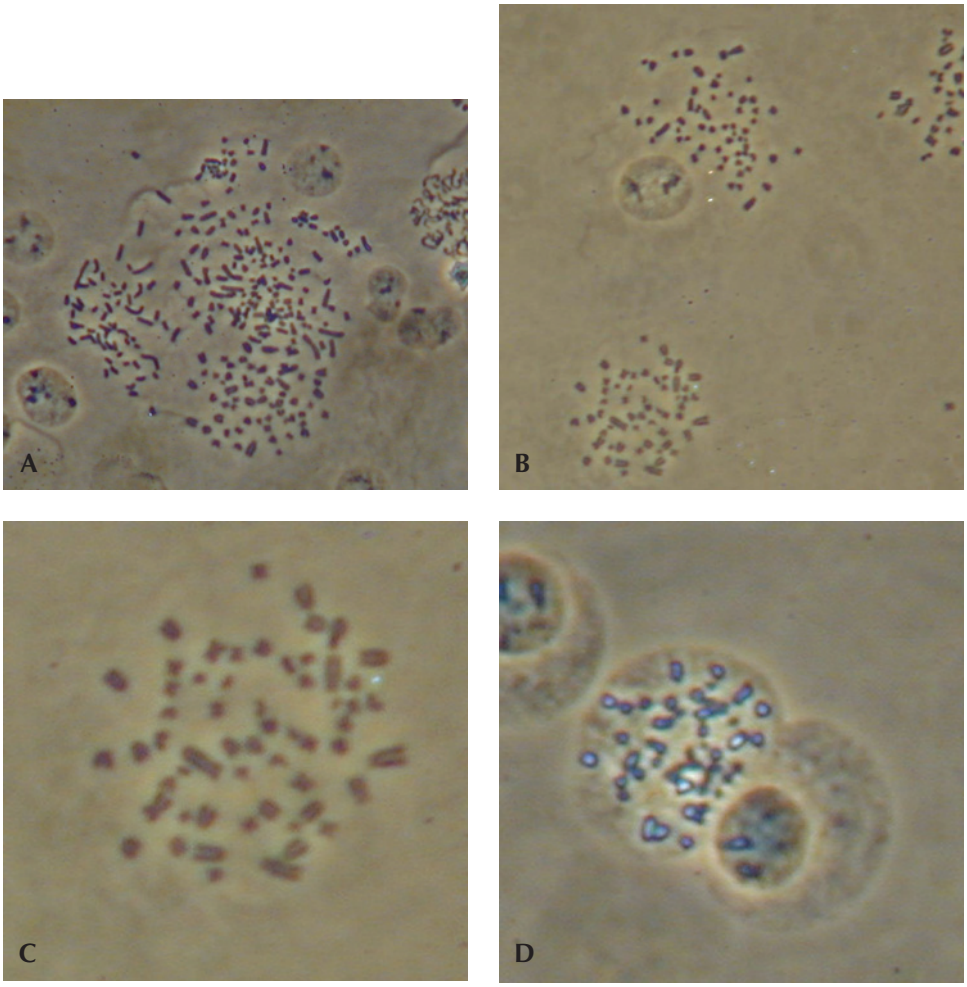


FIGURE 6.4 Examples of good and bad chromosome spreads. (A) The picture shows three or four spreads mixed together. Cell density is too high – dilute the cell suspension. (B) Spreads are well separated from each other. This is a good cell density. (C) Example of a good metaphase spread. Note the light gray color of the chromosomes and how the spread is contained in a well-defined area. (D) Example of a spread with bright chromosomes. The chromosomes contain too much water and will not hybridize well.

8. Fill the tube containing the remaining cell suspension to 5 mL with fixative and store at 4°C. Cells can be stored like this for at least one year.

Day 3: Slide pretreatment

Slides should be aged at room temperature in the dark for 1–7 days.

1. Wash slides in 2 × SSC at room temperature for 5 min.
2. Add 25 μL of 100 mg/mL pepsin to 50 mL of 0.01 M HCl solution that has been pre-warmed to 37°C. This gives a final pepsin concentration of 50 μg/mL. Make sure the pepsin is thoroughly mixed into the solution.
3. Incubate slides in the pepsin solution for 5 min at 37°C.

NOTE: Chromosome spreads and interphase nuclei from hESCs often need longer pepsin pretreatments to remove cytoplasmic debris compared with other cells. Pepsin concentration and incubation time should be determined empirically depending on the cell type. Be careful not to expose the slides to the pepsin solution for too long as this will denature the chromosomes and make them difficult to hybridize.

4. Wash slides twice with PBS for 5 min at room temperature.
5. Incubate slides for 5 min at room temperature in PBS with 50 mM $MgCl_2$.
6. Incubate slides in 50 mM $MgCl_2$ in PBS containing 1% formaldehyde for 10 min at room temperature.
7. Wash slides in PBS for 5 min at room temperature.
8. Dehydrate slide in 70%, 80%, 100% EtOH sequence, 1 min each.
9. Air dry slides.

Slides can be hybridized immediately or stored for at least a year in a dessicator at –20°C.

Paint and probe preparation

1. Place 10 μL of SkyPaint or the manufacturer's suggested amount of FISH probe in a small microfuge tube at 37°C. Vortex tube every 3–5 min for 30 min. Protect paint and probe from light!
2. Denature SkyPaint or probe for 10 min in a water bath (or thermocycler) at 80°C, then leave for 60 min at 37°C.

NOTE: The vortexing and centrifugation ensure all of the SkyPaint or probe is in solution and well-mixed. The paint and probe must be denatured into a single-stranded conformation in order for it to successfully hybridize to the target sequences.

Slide preparation

1. Dehydrate slide in 70%, 80%, 100% EtOH sequence, 1 min each.
2. Air dry slide.

3. Denature slide in denaturation solution at 73°C for 1.5 min.
4. Immediately dehydrate slide in 70%, 80%, 100% EtOH sequence, 1 min each.
5. Air dry slide.

NOTE: Denaturation separates the homologous chromosomes of the target genome and rapid dehydration holds them in the single-stranded state prior to hybridization.

6. Place the slide on a 37°C slide warmer for 5 min prior to addition of the SkyPaint.

Hybridization

1. Apply SkyPaint or probe (from Paint and probe preparation section) to a coverslip (24 × 24 mm). Apply coverslip to slide immediately after adding paint or probe, and seal the edges with rubber cement.
2. Place slide in a pre-warmed humidified box and allow hybridization to proceed overnight in a 37°C incubator. Two day hybridizations are fine as well.

NOTE: Sealing the slide with rubber cement and the use of a humidified hybridization chamber are both done to prevent the probe mix from evaporating. Hybridization is done at 37°C because this temperature is low enough to promote the binding of complementary sequences but high enough to deter the binding of mismatched sequences.

3. Make up the wash solutions for the next day and preheat to 45°C.

Day 4: Washes

1. Carefully remove the rubber cement seal using forceps.
2. Place slide in 2 × SSC until the coverslip falls off.
3. Wash slide three times in formamide solution at 45°C for 5 min.
4. Wash slide three times in 1 × SSC at 45°C for 5 min.
5. Wash slide once in 4 × SSC + 0.1% Tween-20 for 5 min.
6. Make up staining reagent: combine 10 μL of reagent 3; 5 μL of reagent 4 (both from the Concentrated Antibody Detection (CAD) kit from Applied Spectral Imaging); and 1 mL of 4 × SSC. Vortex solution for 10 s and spin in a microfuge for 2 min to pellet fluorescent aggregates.

NOTE: This step is not necessary for FISH. Proceed to step 9 of this section.

7. Remove as much water as possible from the slide without letting it dry out then add 100 μL of staining solution to the slide. Cover the slide with a coverslip.
8. Place slide in a humidified chamber at 37°C for 30 min in the dark.
9. Remove coverslip and wash slide three times in 4 × SSC + 0.1% Tween-20 at 45°C for 5 min.
10. Incubate slide in 4 × SSC + 0.5 μg/mL DAPI for 5 min at room temperature.

11. Immediately dehydrate slide in 70%, 80% and 100% EtOH sequence, 1 min each.
12. Air dry slide in the dark.

NOTE: Washes are done to remove unbound and weakly hybridized paint or probe from the slide. High stringency washes may reduce background but could also remove specifically bound paint or probe. Reduced stringency washes may increase background fluorescence. Stringency can be increased or decreased by changing the temperature, formamide concentration, and salt concentration.

Interpretation

1. Apply Vectashield (antifade) and add coverslip (24 × 50 mm).
2. View slide using a microscope equipped with an interferometer and SKY software for SKY analysis. Only a fluorescent microscope is needed for FISH analysis.

Storage

Store slide at –20°C in the dark.

RECIPES

Denaturing solution (70% formamide/2 × SSC solution)

For 70 mL: add 49 mL of formamide and 14 mL of water to 7 mL of 20 × SSC. Important: Adjust pH to 7.0. Between periods of use, store at 4°C. Use each batch of denaturant for 7 days and then discard.

Formamide solution (50% formamide/2 × SSC)

For 150 mL: add 75 mL of formamide to 75 mL of 4 × SSC and adjust pH to 7.0. Between periods of use, store at 4°C. Use each batch for 7 days and then discard.

Ethanol sequence solutions

For final concentrations of 70%, 80%, and 100%, prepare v/v dilutions of 100% ethanol with H_2O. Use dilution for up to 7 days and then discard. If solution evaporates or becomes diluted, replace with fresh solution. Between periods of use, store at room temperature.

4 × SSC + DAPI

For 50 mL: add 5.0 µL of DAPI (5 mg/mL stock) to 50 mL 4 × SSC.

4 × SSC + 0.1% Tween-20

For 500 mL: add 100 mL of 20 × SSC, 400 mL of H_2O and 0.5 mL of Tween-20. Mix well.

20 × SSC

For 1 L: dissolve 175.3 g of sodium chloride and 88.2 g of sodium citrate in 1 L of water. pH to 7.0.

Fixative

For 20 mL add 15 mL of methanol to 5 mL of glacial acetic acid. Make fixative solution up fresh EACH DAY.

Supplies and Reagents

- SkyPaint available from Applied Spectral Imaging
- FISH probe available commercially from Vysis, Cambio or other companies
- Vectashield Antifade Mounting Medium (Vector Labs catalog no. H1000)
- Coverslips (24 × 24 mm and 24 × 50 mm)
- Slides (Fisher catalog no. 12-544-7)
- Rubber cement
- Pair of forceps
- Corning Falcon Tubes (50 mL)
- Concentrated Antibody Detection Kit (CAD Kit) from Applied Spectral Imaging
- Formamide (Sigma catalog no. F7503)
- DAPI (Sigma catalog no. 32670)
- Colcemid (Invitrogen catalog no. 15212-012)
- Pepsin (Sigma catalog no. P7000)
- Methanol (Fisher catalog no. A454-1)
- Glacial acetic acid (Fisher catalog no. A38-212)
- Formaldehyde (Sigma catalog no. F1268)
- 0.05% Trypsin/EDTA solution (Invitrogen catalog no. 25300-054)
- Tween-20 (Sigma catalog no. T8787).

Equipment

- Slide warmer
- Micropipettors (10, 20, 200 μL)
- Microcentrifuge
- Water baths with temperature control (two or more)

- Vortex mixer
- Heated block or PCR machine
- Fluorescent microscope with interferometer and SKY software available from Applied Spectral Imaging
- –20°C freezer and 4°C refrigerator
- 80°C water bath with metal plate (see Figure 6.3).

Pitfalls and Advice

Feeder cells in hESC cultures

For SKY it is not necessary to separate the hESCs from feeder layer cells because the feeder cells are not dividing and thus will not contribute to the metaphase population. When analyzing cells via FISH the feeder layer cells will be included in the analysis, but they are usually karyotypically grossly abnormal because of the methods used for mitotic inactivation. If feeder cells appear to be confusing the results, feeder cells stably expressing green fluorescent protein (GFP) can be used and then sorted out before cells are harvested. Alternatively, individual ESC colonies can be picked off the feeder layer prior to trypsinization to generate a cell population with very few feeder cells. It is also possible to culture the cells to be karyotyped under feeder-free conditions.

Chromosome spreads

Making chromosome spreads is an art. Getting a good spread depends on many variables (e.g. humidity), which cannot be easily controlled. Here are some things to try if you are not getting good spreads:

- Wash cell suspension with fixative again.
- Slow down the evaporation process by not placing the slide on the heated metal plate – just allow it to dry slowly.
- To decrease the water content in the chromosomes, do not expose the slide to steam.
- Try using a different fixative. We have had some luck with 1:1 glacial acetic acid to methanol.
- Repeat the procedure on a different day when (presumably) atmospheric conditions are different.

Spectral karyotyping

Background staining can be divided into two general categories, large chunks of paint that are scattered across the slide and fluorescent haze that coats the slide evenly.

Chunks of paint

This type of background typically occurs when the SkyPaint is not fully solubilized before hybridization. Placing the tube of SkyPaint at 37°C and vortexing periodically for 30–60 min will clear this up.

Fluorescent haze

This type of background is typically due to improper slide preparation or pretreatment, which can leave residual cytoplasmic debris on the slide that can non-specifically bind labeled SkyPaint. A longer pepsin pretreatment can decrease this type of background.

Fluorescent in situ hybridization

Background fluorescence

This can be divided into two general categories, depending on where the background staining is originating:

- Originating from the chromosomes: This type of background typically comes from non-specific hybridization of probe DNA to the target genome. Addition of Cot-1 carrier DNA, hybridization at a higher temperature, additional washes at a higher stringency, and the use of less probe during the hybridization can decrease this background.
- Originating among and around the chromosomes: This type of background is typically due to improper slide preparation or pretreatment, which can leave residual cytoplasmic debris on the slide which can non-specifically bind labeled probe. A longer pepsin pretreatment or preparation of a new cell suspension and can decrease this type of background.

Weak staining

- Wash was too stringent – decrease wash temperature and increase salt concentration.
- DNA was not adequately denatured prior to hybridization – repeat the slide and probe denaturation.
- Increase the signal using tyramide amplification (available from Molecular Probes and Perkin Elmer).

Intense staining

- Wash more, or at a higher stringency (increased temperature, increased formamide concentration, decreased salt concentration).
- Use less probe – sometimes if there are not very many metaphase spreads and/or nuclei on the slide use half the recommended amount of probe.

Reading List

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CHAPTER

7

Genotype and Epigenotype by Single Nucleotide Polymorphism (SNP) Analysis

Kevin Gunderson, David L. Barker, Marina Bibikova, and Jian-Bing Fan

INTRODUCTION

Determining the DNA sequence of the human genome was the first step on the long road to a molecular understanding of complex human diseases with a genetic component. Knowing a reference DNA sequence led to the formation of an international consortium dedicated to developing a catalog of common human genomic variation. The International Haplotype Mapping Consortium set as its initial goal the identification of all common single nucleotide polymorphisms (SNPs), defined as SNPs with minor allele frequency >5%, every 5 kilobases (kb) across the genome. This highly successful project (International HapMap Consortium, 2005) not only provided a resource for studying genomic variations that contribute susceptibility to common disease, but also stimulated the development of molecular tools for highly multiplexed examination of genetic abnormalities, in high resolution, across the entire genome.

Human Stem Cell Manual, edited by J. F. Loring, R. L. Wesselschmidt, and P. H. Schwartz.
ISBN: 978-0-1237-0465-8.

In this chapter, we describe how SNP genotyping can be used to provide unambiguous identification of ESC lines, and, more importantly, to monitor genomic integrity by detecting variations that frequently occur in culture, including genomic duplications and deletions, and loss of heterozygosity (LOH). We also describe a new method that adapts SNP genotyping to the determination of DNA methylation of specific CpG sites in the human genome. Recent evidence indicates that human ESCs all share a unique pattern of DNA methylation that may be linked to their properties of self-renewal and pluripotence. Monitoring this pattern is likely to become a powerful method for characterizing ESCs and their differentiated products *in vitro*.

Overview

Illumina SNP genotyping assays

Illumina developed and commercialized two SNP genotyping assays. The GoldenGate assay (Fan *et al.*, 2003; Shen *et al.*, 2005) multiplexes from 96 to 1536 SNPs determined on each sample, with data read out from hybridization of an assay mixture to a universal array. The Infinium® assay uses a method of sample preparation that makes it possible to read out any number of SNPs from one sample, limited only by the number of elements present on the microarray. Illumina provides Infinium BeadChip arrays of varying multiplex level ranging from 10 000 SNPs across 12 samples to over 500 000 SNPs for a single sample. Illumina's microarrays use 3 μm silica beads as the array elements. Each bead has ~700 000 full-length oligos of the same sequence covalently attached to it. Each bead type is defined by the oligo sequence it carries, and this sequence is about 23 bases in length for the universal arrays used in the GoldenGate assay, and about 80 bases in length for the SNP-specific arrays used with the Infinium assay (Gunderson *et al.*, 2004, 2005a, 2005b). On every array, each bead type is present an average of 15–30 times. This redundancy produces exquisite accuracy in calling of genotypes.

The GoldenGate assay

Figure 7.1 shows the workflow for the GoldenGate assay. Three oligos are designed to target each SNP, two allele-specific oligos to provide SNP discrimination, and one locus-specific oligo. Three oligos for each of 1536 SNP sites are pooled together and hybridized to genomic DNA that has been bound to a solid support. Two enzymatic reactions, polymerase extension of the correct allele-specific oligo and ligation of the extended oligo to the locus-specific oligo, are used to create a fused oligo at each site that becomes a substrate for PCR using universal primers. During PCR, amplicons are labeled with a fluorescent tag. The pool of amplicons is hybridized to a universal array that contains sequences complementary to a tag sequence on each locus-specific oligo. When the array is scanned with a BeadArray Reader, the fluorescent color of each bead type identifies the allele at each SNP site.

The GoldenGate assay has the flexibility to address any set of 1536 SNPs that support functional assays. Since the read-out is on an array of universal address sequences, a new set of 1536 SNP assays can be created simply by designing and synthesizing a new pool of oligos. This method is very useful for studies of specific biochemical pathways, or genomic regions that are known to be susceptible to aberrations.

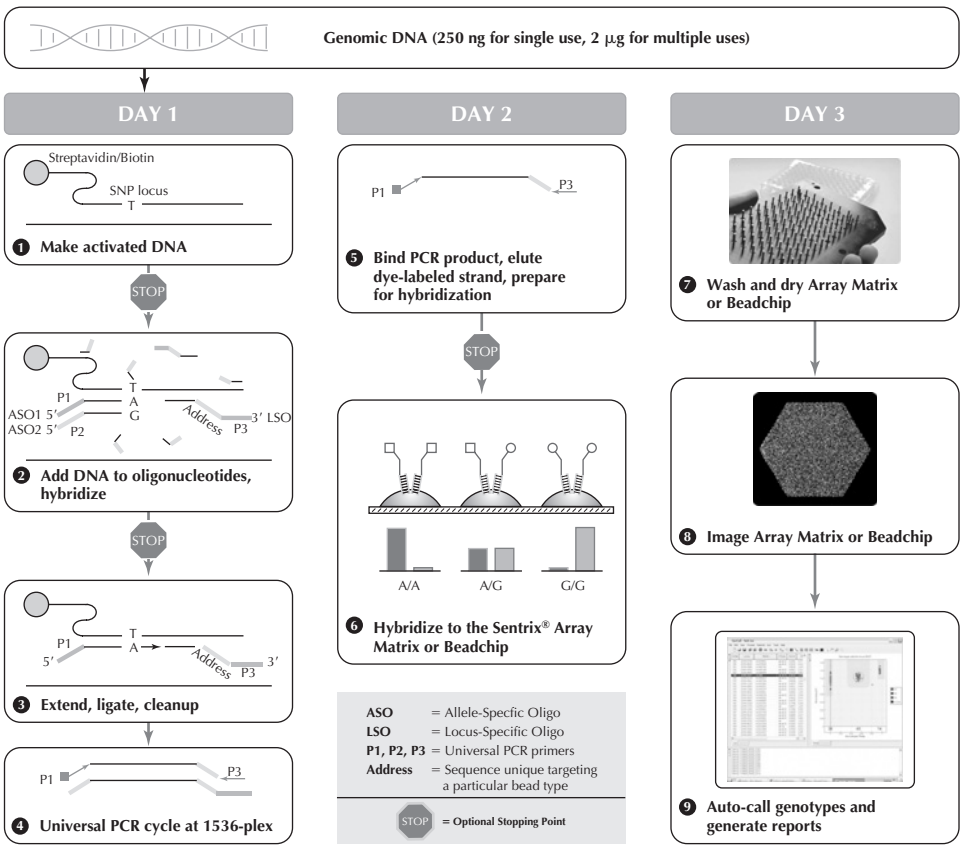

FIGURE 7.1 Workflow for the GoldenGate SNP genotyping assay.

The Infinium assay

Figure 7.2 shows the workflow for the Infinium assay. Genomic DNA is whole genome amplified about 1000-fold, fragmented, and hybridized to a BeadChip. The oligos attached to each bead type are complementary to a 50-base region adjacent to a SNP site. A primer extension reaction extends the oligo attached to the bead and in the process identifies the allele present at the SNP site. Two forms of the assay are used (Figure 7.3). For the Infinium 1 assay, allele-specific primer extension (ASPE) is used to score the SNP site, requiring two beads for each SNP (Gunderson *et al.*, 2005b). For the Infinium 2 assay, one bead type is used, and the allele is scored by single base extension (SBE) reaction using labeled terminators (Steemers *et al.*, 2006). The advantage of the Infinium assay is that it allows almost unrestricted SNP choice and that it scales to any number of SNPs in a single assay, limited only by the complexity of the array. However, each new set of SNPs requires manufacture of a new array, so the Infinium assay is used for standard SNP sets, or for custom sets that will be used for thousands of samples.

Identity of cell cultures

Traditionally, genomic identity for forensic or parental determination applications has been carried out using di- or trinucleotide repeats that vary in number in different

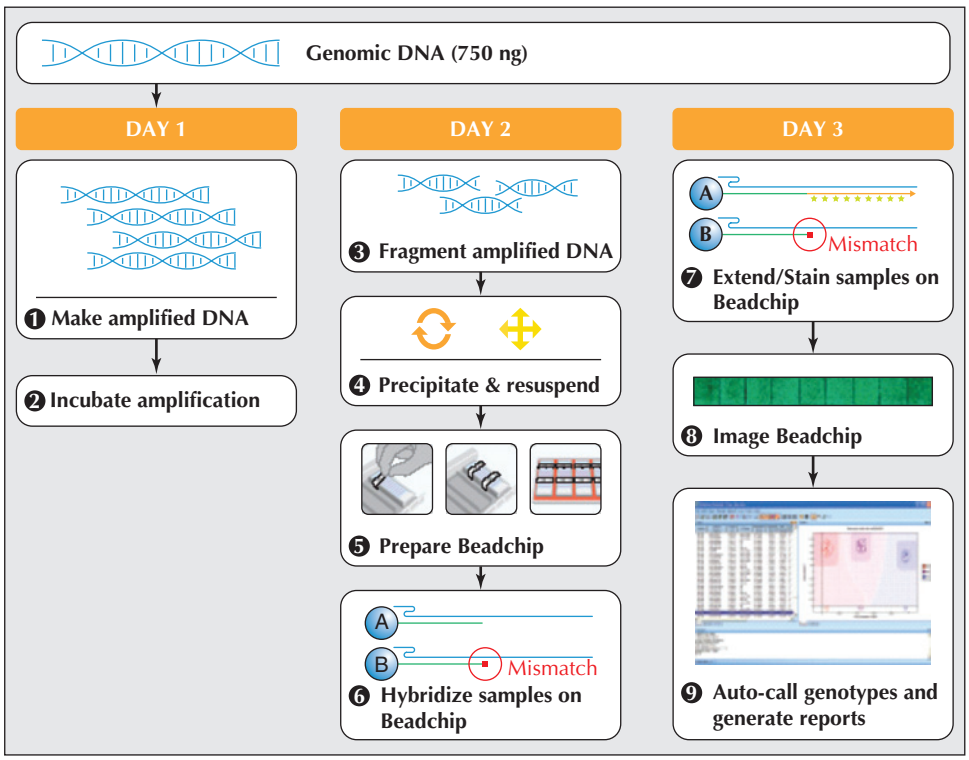

FIGURE 7.2 Workflow for Infinium Assay.

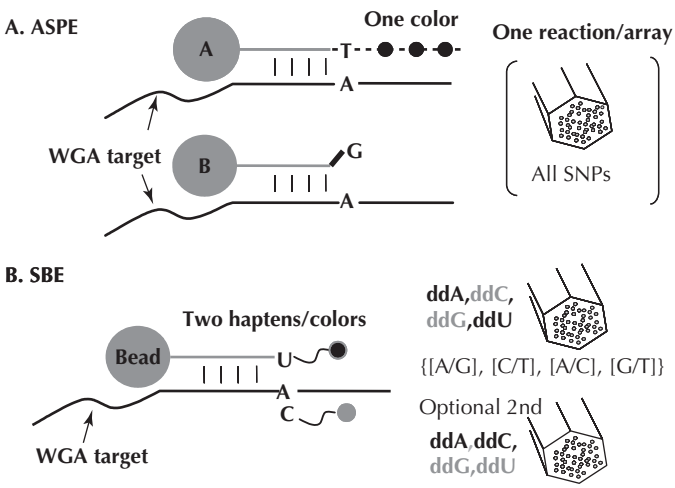

FIGURE 7.3 Two forms of the Infinium Assay.

genomes. Since identity can be unambiguously determined using less than 20 such markers, low-throughput methods such as PCR followed by gel electrophoresis separation were adequate. Since the high-throughput methods described below monitor hundreds to hundreds of thousands of SNP variations, identification of the genome under investigation is an automatic byproduct of these highly multiplexed genotyping assays.

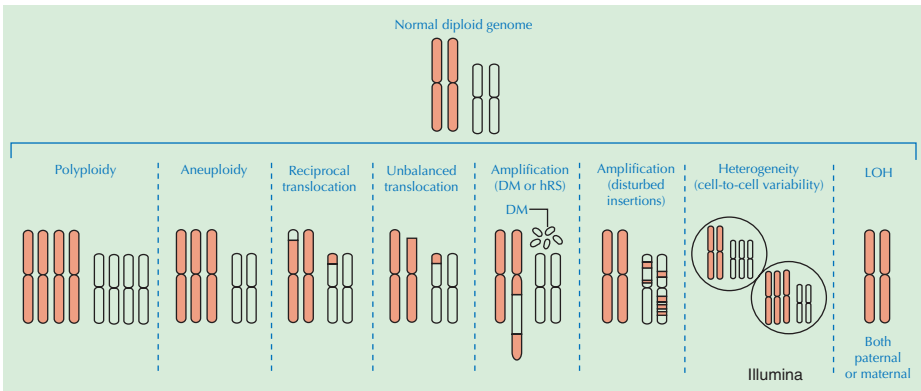

FIGURE 7.4 Types of chromosomal abnormalities. From Speicher and Carter (2005) Modified from Albertson *et al.* (2003).

Genomic abnormalities

Other chapters describe traditional karyotyping and more recent fluorescent methods for detecting genomic abnormalities. Recently, fluorescent hybridization methods for monitoring genomic integrity have been applied to high-density microarrays, which produce thousands of data points in one experiment and have the potential to dramatically increase resolution within the genome. Initially, methods were adapted from the cytological technique comparative genome hybridization (CGH) (Pinkel *et al.*, 1986, 1998; Barrett *et al.*, 2004). With the development of highly multiplexed SNP genotyping, it became possible to achieve higher resolution and also gain extra information. Figure 7.4 illustrates common types of chromosomal abnormalities. Both array CGH and SNP genotyping are able to detect polyploidy, aneuploidy, and amplifications and deletions, while neither detects reciprocal translocations. SNP genotyping, however, also detects regions where heterozygotes are absent, which can result from loss of all or a part of one chromosome with replacement by the other chromosome (Figure 7.4, far right). Duplications are a common occurrence in cancer cells and in primary cells kept for long periods in culture. Figure 7.5 shows an example in a sample of hESCs, first described by Maitra and colleagues (2005) using Affymetrix arrays, and replicated here on the same sample using Illumina arrays.

LOH is also common, and an example is shown in Figure 7.6, where the red line marks a position where heterozygotes are absent (as seen by the allele frequency, lower graph) but there is no change in copy number, as measured by the intensity of the SNP signals (upper graph). For this reason, we favor SNP genotyping over array CGH to detect genomic abnormalities. Illumina offers genotyping methods at multiplex levels of from 96 SNPs per sample up to more than 500 000 SNPs per sample. The method of choice will depend on the particular application and resolution required (see Procedures).

In addition to measuring genomic abnormalities, high-density SNP arrays provide comprehensive information regarding the genetic profile of each stem cell analyzed. Eventually, genotyping information in all coding and promoter regions could be used

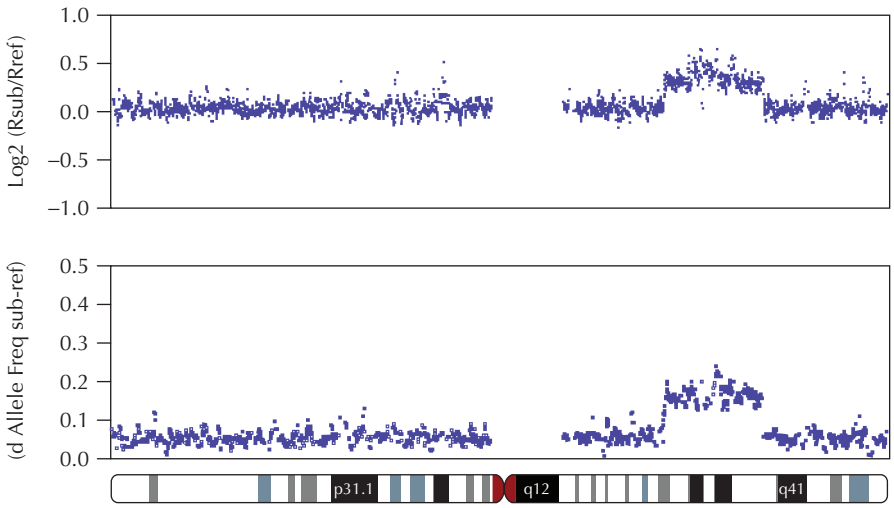

FIGURE 7.5 32 Mb duplication in q31.1 on Chr1 of hESC WA09 passage 79 cell line detected with the Human 1 BeadChip containing 109K SNP assays. The top chart plots the $\log_2$ intensity ratio of hESC WA09 passage 79 vs. passage 33 using a 500 kb moving average across the genome. The bottom chart plots the allele frequency difference between the heterozygous SNPs between passage 79 and passage 33. A duplication should generate a theoretical allele frequency difference of 0.17 (0.67–0.5 or 0.5–0.33).

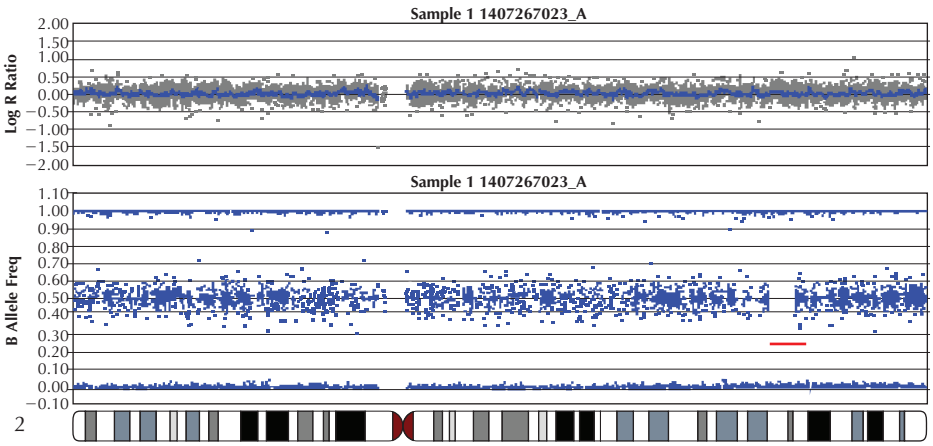

FIGURE 7.6 Loss of heterozygosity (LOH) in the absence of a copy number change.

not only for understanding the basic biological properties of stem cells, but also to assess each individual cell line's therapeutic utility.

DNA methylation analysis

DNA methylation plays a critical role in the regulation of gene expression during differentiation and development, and loss of normal methylation patterns is implicated in several disease states, particularly cancer (Robertson, 2005). In the human genome,

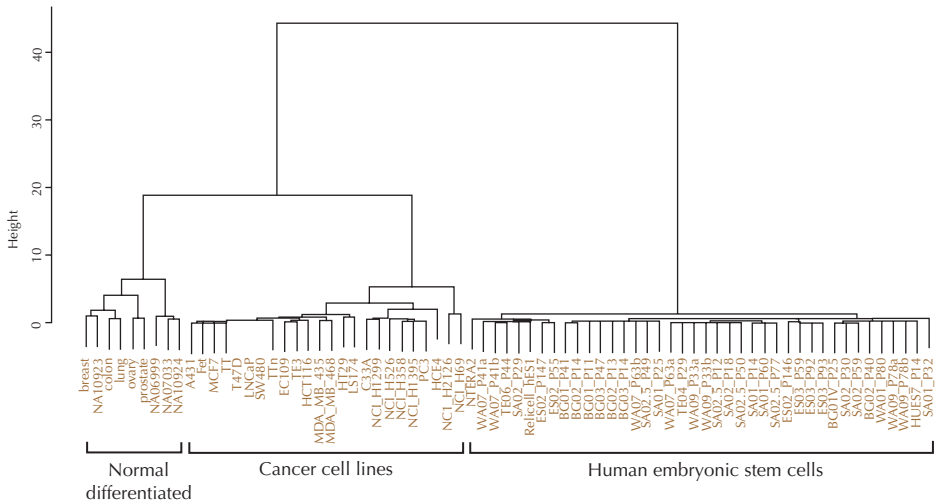

FIGURE 7.7 Cluster diagram based on 23 CpG sites that separate hESCs from other cell types.

DNA methylation occurs almost exclusively on cytosines adjacent to guanine residues. CpG islands can occur in or near promoter regions of genes, and methylation can result in gene silencing through the interaction of methyl cytosine binding proteins with other structural elements of chromatin, making promoters inaccessible to transcription factors.

At Illumina, we developed a method for analyzing the methylation state of 1536 CpG sites simultaneously and applied this method to studies of cancer cells and human embryonic stem cells (Bibikova *et al.*, 2006a, 2006b). The method takes advantage of the fact that treatment of DNA with bisulfite under carefully controlled conditions will quantitatively convert cytosine to uracil, while methyl cytosine remains unchanged (Wang *et al.*, 1980). Once the conversion is complete, the treated DNA can be "genotyped" to determine whether a particular cytosine has been converted to uracil (which acts like thymine) or remains methyl cytosine. With the help of collaborators, we applied this method to 36 different human ESC samples, including 14 independently isolated cell lines and up to three passages of each line. We compared the DNA methylation pattern of ESCs to normal adult cells, somatic stem cells, and cancer cell lines, and found that ESCs have a unique pattern shared by lines derived throughout the world, and unlike the pattern of other cell types (Bibikova *et al.*, 2006a).

Figure 7.7 shows a cluster diagram based on the methylation pattern of 23 CpG sites that shows remarkable similarity of all ESCs, regardless of origin or time in culture. Normal adult cells, somatic stem cells and lymphoblastoid cells are well separated from human ESCs by the pattern of methylation of these CpG sites. The unique methylation pattern of ESC DNA is likely to be related to the special properties of ESCs. Therefore, monitoring of methylation in ESCs should be valuable for tracking the undifferentiated state and for characterizing pathways of differentiation.

PROCEDURES

GoldenGate genotyping

The GoldenGate genotyping assay protocol, illustrated in Figure 7.1, allows for a high degree of locus multiplexing in a single reaction through highly specific extension and amplification steps. It is conducted according to the instructions in the *GoldenGate User's Manual* (Illumina, Inc.). Briefly, 250 ng of genomic DNA is first biotinylated for binding to paramagnetic particles (step no. 1). Assay oligonucleotides, hybridization buffer, and paramagnetic particles are then combined with the activated genomic DNA (step no. 2), in which the query oligonucleotides hybridize to the genomic DNA bound to paramagnetic particles. Two allele-specific oligos (ASOs) and one locus-specific oligo (LSO) are designed for each SNP. All three oligonucleotides contain sequences for genomic complementarity and universal PCR primer sites; the LSO also contains a unique address sequence complementary to a particular bead type on an Illumina BeadArray.

After oligonucleotide hybridization to activated genomic DNA, several wash steps are performed to remove excess and mis-hybridized oligonucleotides. A DNA polymerase with high specificity for 3′ match and no strand displacement or exonuclease activity is added to extend the ASO(s) that perfectly match the target sequence at the SNP site and fills the gap between the ASO and LSO (step no. 3). A DNA ligase is added to seal the nick between the extended ASO and the LSO to form PCR templates that can be amplified with three universal PCR primers P1, P2, and P3 (step no. 4). Universal PCR primers P1 and P2 are Cy3- and Cy5-labeled, respectively, while primer P3 is biotin-labeled. High locus specificity is achieved by the requirement that both the ASO and LSO oligos hybridize to the same target site; extension of the appropriate ASO and ligation of the extended product to the adjacent LSO joins information about the genotype present at the SNP site to the address sequence on LSO.

The PCR products are bound to paramagnetic particles. The dye-labeled single-stranded products are eluted by denaturation (step no. 5) and hybridized to their complement bead type through their unique address sequences (step no. 6). The array hybridization is conducted under a temperature gradient program. Hybridization of the assay products onto the Array Matrix or BeadChip allows for the separation of the assay products in solution onto a solid surface for individual SNP genotype readout (step no. 7). After the hybridization, the BeadArray Reader is used to detect fluorescence signal on the Array Matrix or BeadChip (step no. 8), which is then analyzed using software for automated genotype clustering and calling (step no. 9).

Infinium genotyping

Infinium genotyping is conducted according to the *Infinium User's Manual*. First, whole-genome amplification is used to amplify 750 ng of genomic DNA by a factor of ~1000–2000× in a relatively unbiased manner. The Infinum assay uses a preformulated WGA amplification kit (MP1 and AMM) for compatibility with downstream fragmentation and processing. Amplification proceeds at 37°C for ~20 h. After amplification, a fragmentation protocol is used to reduce the fragment size to 200–300 bp. This fragmentation improves both resuspension and hybridization

efficiency. After fragmentation, the reaction is stopped and the DNA precipitated by addition of a precipitation reagent (PA1) and one volume of isopropanol. This step reduces the carry-over of dNTPs, and concentrates the DNA sample. The DNA pellet is resuspended in a formamide-containing hybridization buffer (RA1) by incubating at 48°C for 1 h and vortexing for about 1 min as specified in the user manual. The average yield from the WGA reaction is ~1.5 μg/μL and the final hybridization concentration is 5–6 μg/μL. After resuspension, the sample is denatured at 95°C for 20 min, allowed to cool on the bench for 5–10 min and applied to the BeadChip array.

After hybridization, the BeadChips are washed, primer extended, and stained on a Tecan Genesis/Evo robot using a GenePaint slide processing system. This "XStain" process involves pipetting of various reagents to the Te-Flow Through Chambers placed on the GenePaint Te-Flow Chamber Rack, equilibrated to 44°C. These reagents are used according to the *Infinium User's Manual*. Basically, the BeadChips are washed with hybridization buffer (RA1), blocked with protein (XB1), primer extended with a polymerase and labeled nucleotide mix (EMM), and stained with repeated application of LMM (staining reagent) and ASM (anti-staining reagent). After staining is complete, the slides are washed with low salt wash buffer (PB1), dried down, and imaged on Illumina's BeadArray Reader.

Bisulfite conversion for methylation analysis

The EZ DNA methylation kit (Zymo Research, Orange, CA, USA) is used for bisulfite conversion of the genomic DNA, according to the manufacturer's recommendations. Briefly, genomic DNA is mixed with a bisulfite-containing conversion reagent and incubated at 50°C for 12–16 h. The reaction is then loaded into a Zymo-Spin column, followed by centrifugation, repeated washes, de-sulfonation treatment, repeated washes, and elution. One microgram of genomic DNA is used for each conversion. Bisulfite-converted genomic DNA from one conversion can be used for up to five array experiments. After bisulfite treatment, the remaining assay steps are identical to the GoldenGate genotyping assay.

Alternative Procedures

Copy number polymorphisms, without genotyping information, may be detected on direct hybridization arrays offered by Agilent and through a service offered by Nimblegen. SNP genotyping arrays manufacturing by Affymetrix may also be used (Slater *et al.*, 2005), as can the ROMA method, developed by Michael Wigler and colleagues (Sebat *et al.*, 2004). Both ROMA and the assay method used by Affymetrix involve a reduced representation of the genome, which limits the potential resolution of the analysis.

Pitfalls and Advice

The most important factor to keep in mind for successful SNP genotyping is to be certain that adequate DNA is included in the assay. For this reason, accurate quantitation of the DNA sample, using a reliable assay such as PicoGreen (Invitrogen), is essential.

The GoldenGate assay is tolerant of a certain amount of degradation of the genomic DNA used. As a result, samples extracted from formalin-fixed, paraffin-embedded tissue can be successfully genotyped with this method, as can samples that have been whole-genome amplified.

The Infinium assay begins with a whole genome amplification step. Accurate representation of all parts of the genome during whole genome amplification requires high-quality, intact DNA for best results.

Equipment and Supplies

All equipment and supplies for carrying out the GoldenGate and Infinium assays are obtained from Illumina, Inc. (www.illumina.com). Illumina also provides genotyping services for large projects. Many university core facilities also provide Illumina genotyping services, as does the Centers for Inherited Disease Research (www.cidr.jhmi.edu).

Quality Control Methods

Both the GoldenGate and Infinium assays include internal controls that monitor each step of the assay. These may be used for troubleshooting failed assays. As mentioned above, the most important factor for guaranteeing success is quantifying the DNA input to the assay with an accurate measure such as PicoGreen.

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CHAPTER

8

Flow Cytometric Analysis of Human Embryonic Stem Cells

Andrew L. Laslett, Andrew Fryga, and Martin F. Pera

INTRODUCTION

Flow cytometric techniques are used to quantify and separate subpopulations of cells contained within complex cell mixtures. This is accomplished by exploiting the differences in the basic optical properties of the cells, in their specific immunoreactivity to different antibodies or combinations of antibodies, or in their physiological or biochemical properties.

In a flow cytometer, cells in suspension are made to flow one at a time by a light source, typically a laser, with the resulting light scatter (Figure 8.1) yielding information about their size (forward scatter, FSC) and cellular complexity (side scatter, SSC). Cells can be labeled with flurophore-tagged antibodies or loaded with multiple dyes, with the emitted fluorescence estimating cellular parameters such as nucleic acid content, membrane potential and density of membrane-bound antigens (Figures 8.2 and 8.3).

Flow cytometry's power is its ability to analyze multiple parameters on a cell-by-cell basis at very high speed. Modern analyzers with their multiple lasers (up to four) can

Human Stem Cell Manual, edited by J. F. Loring, R. L. Wesselschmidt, and P. H. Schwartz.
ISBN: 978-0-1237-0465-8.

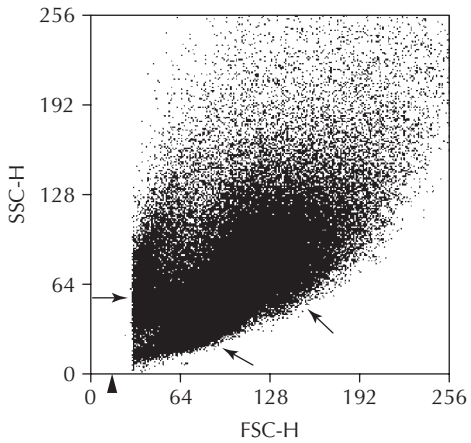

FIGURE 8.1 Dot plot of the light scatter characteristics of a human stem cell population. Three main clusters of cell subpopulations can be observed (arrows). Typically, the data for region of lowest forward scatter (arrowhead) is not collected as it contains predominately, but not solely, cell debris.

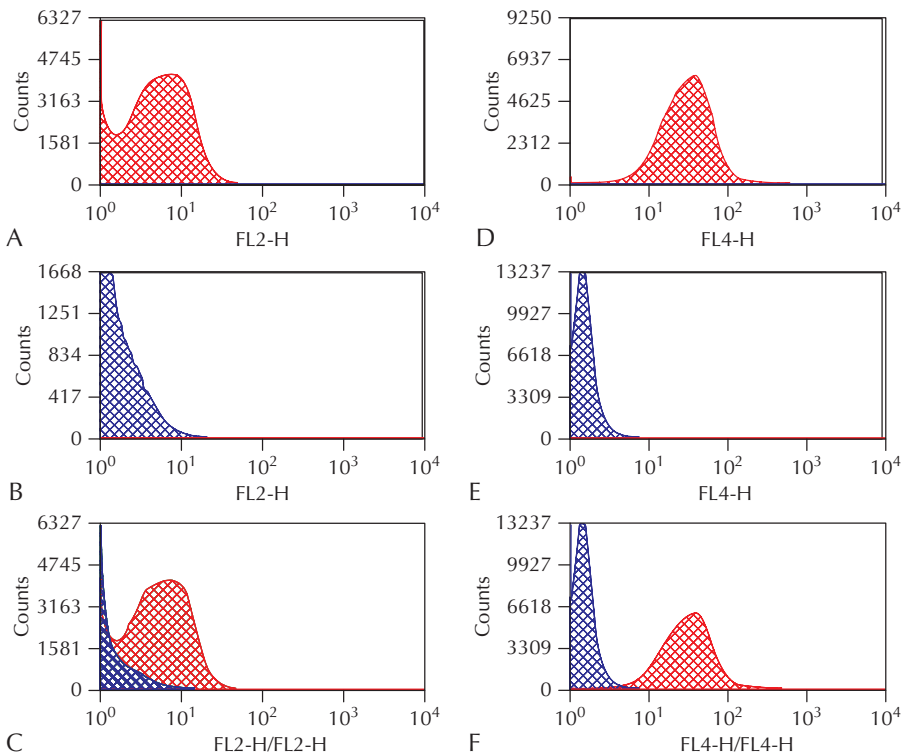

FIGURE 8.2 An example of two-antibody staining of a stem cell population and corresponding appropriate isotype controls. The antibody in (A) is labeled with the fluorophore phycoerythrin (PE) and the signal is seen by photomultiplier tube FL2. (B) shows its corresponding isotype control and demonstrates that there is both positive and negative staining in A. Using the DakoCytomation software Summit 3.1, an overlay plot

measure up to 18 fluorescent and two scatter parameters per cell at speeds over 20 000 cells per second, generating one million event data sets in under 1 min.

The other use of flow cytometry is cell separation. Fluorescence activated cell sorters (FACS) have the ability to deposit cells of a predetermined phenotype, characterized by their scatter and/or fluorescence, into collection tubes (from one to four tubes simultaneously) or individual wells of a tissue culture plate (most common formats are 6-, 24-, 96-well dishes). Because of their high speed, sorters can enrich for subpopulations that are very small, or eliminate contaminating subpopulations from a common subpopulation. Sorters also have the option of sorting for purity, achieving purities >99.5%.

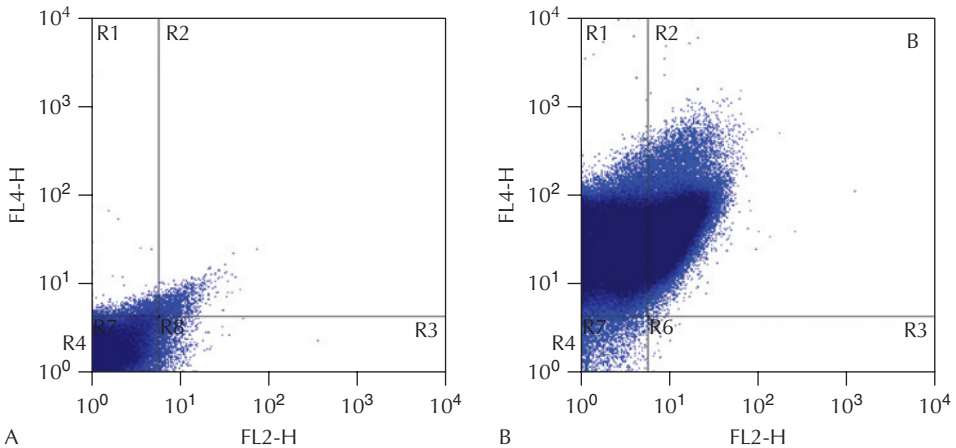

FIGURE 8.3 PE (FL2) versus APC (FL4) staining of isotype control (A) and antibodies (B). This is the same data set as Figure 8.2. In this example, gates are set using the isotype such that approximately 0.5% of the total cell population falls into each of the three positively stained regions: R1 – positive for APC only; R2 – positive for both PE and APC; R3 – positive for PE only; and R4 – negative for both PE and APC. Once the gates are set the antibody data are displayed (B) and it is easy to see, in this example, that there are significant subpopulations that are APC-positive/PE-negative (R1) and APC-positive/PE-positive (R2) but only a small number of cells are negative for both (R4) and virtually no cell is PE-positive/APC-negative (R3). Using DakoCytomation Summit software, numerical values for the number of cells in each gate are automatically calculated.

FIGURE 8.2 (Continued) (C) of A + B is created, allowing direct comparison of antibody and isotype control staining. In this plot, it is easy to see that both positive and negative staining are present. This plot also demonstrates that simple gating, using the data of the isotype control may underestimate the level of positive staining as it is clear that much of the area under the curve in A still falls within the isotype control region. In this situation, one can use a subtraction method for a better estimate or one can use a brighter fluorophore to better separate positive and negative staining. D, E, and F show staining with the fluorophore allophycocyanin (APC) and demonstrate that virtually all cells stain positively, the curve of D being well separated from the isotype control curve, (E). The overlay, F, confirms this.

Overview

This chapter will describe single and multiple-color flow cytometric analyses on both live and fixed cell preparations. The former is used if the antigens of interest present on the cell surface while the latter is used for intracellular antigens; it is necessary to fix and permeabilize cell membranes in order for antibodies to have access to intracellular antigens. Flow cytometry is usually carried out using fluorescently labeled monoclonal antibodies.

Procedures

Antibodies for detection of undifferentiated hESCs

Antibodies reactive with human embryonic stem cells (hESCs) are important tools that enable the identification, isolation and characterization of specific cell types whether differentiated or undifferentiated. Markers originally developed for human embryonal carcininoma (EC) cell lines also recognize hESCs, and other markers have been added in the last several years (Table 8.1). Antibodies that detect epitopes expressed on the surface of hESCs allow the characterization and comparison of different hESC lines and enable isolation of defined populations of live cells. Additionally, these reagents provide a simple means for evaluating different culture regimes for maintenance of pluripotency (e.g. by looking at percentages of cells expressing specific markers or combinations of markers), and loss of marker expression may be used to monitor early stages of differentiation. It is important to note that not all of the markers listed in Table 8.1 are entirely specific for undifferentiated hESCs (reactivity is often seen in embryonic tissues or more mature cell types; e.g. POU5F1/OCT4 is also expressed in germ cells), so these markers are only useful within a discrete context of stem cell commitment and differentiation. In heterogeneous cultures of cells (arguably all cultures involving hESCs or differentiated cell types), it is essential to employ combinations of markers to assure valid identification of cell types. This is especially true for the identification of cells differentiated from hESCs.

Antibodies that detect hESCs may be directed against cell surface epitopes or transcription factors with a known role in the maintenance of pluripotency (Table 8.1). Many of the epitopes detected by antibodies directed against the cell surface of hESCs are not fully characterized in molecular terms and in many cases the protein products responsible for the epitopes and their corresponding genes are not known. Indeed, many of the epitopes are not proteins at all, but complex carbohydrate and lipid moieties. Nevertheless, these antibodies are useful tools for the characterization of hESC lines.

Although there is a group of antibody markers that is characteristic of the hESC culture population, it is important to keep in mind that within that population there are subsets of cells that express these markers to different degrees.

TABLE 8.1 Cell surface and transcription factor hESC markers

Antibody	Type	Antigen	Reference	Source
Cell surface hESC markers				
20-202S	IgG2a	Heat shock 70 kDa protein 8 HSPA8	Shin *et al.*, 2003; Son *et al.*, 2005	–
GCTM-2	IgM	Keratan sulfate proteoglycan KSPG – protein core	Laslett *et al.*, 2003; Pera *et al.*, 2003	–
P1/33/2	IgG1	CD9	Laslett *et al.*, 2003; Pera *et al.*, 2003	Santa Cruz Biotechnology sc-20048
PHM-5	IgG1	Podocalyxin (PODXL): CD34 family member	Kerjaschki *et al.*, 1986	–
SSEA-3	IgM	Globoseries glycolipid	Kannagi *et al.*, 1983a	Developmental Studies Hybridoma Bank www.uiowa.edu/~dshb
SSEA-4	IgG3	Globoseries glycolipid	Kannagi *et al.*, 1983b	Developmental Studies Hybridoma Bank www.uiowa.edu/~dshb
TG343	IgM	Keratan sulfate proteoglycan KSPG – protein core	Cooper *et al.*, 2002	–
TRA-1-60	IgM	Keratan sulfate proteoglycan KSPG – carbohydrate side chain	Andrews *et al.*, 1984; Cooper *et al.*, 2002	Developmental Studies Hybridoma Bank www.uiowa.edu/~dshb
TRA-1-81	IgM	Keratan sulfate proteoglycan KSPG – carbohydrate side chain	Andrews *et al.*, 1984; Cooper *et al.*. 2002	Developmental Studies Hybridoma Bank www.uiowa.edu/~dshb
Transcription factor hESC markers				
OCT4 (C-10)	IgG2b	OCT4/POU5F1	Laslett *et al.*, 2003; Pera *et al.*, 2003	Santa Cruz Biotechnology sc-5279
NANOG	IgG	NANOG	Hyslop *et al.*, 2005	R&D Systems AF1997

The antibodies fall into four main types:

1. The stage-specific embryonic antigens (SSEA) 1, 3 and 4 are globoseries glycolipid cell surface markers recognized by monoclonal antibodies originally raised to distinguish early stages of mouse development. hESCs express SSEA-3 and SSEA-4 but express SSEA-1 only upon differentiation.
2. The second group of antibodies used to detect undifferentiated hESCs mark a set of antigens associated with a pericellular matrix keratan sulfate/chondroitin sulfate proteoglycan found on the surface of these cells. These antibodies were raised to human EC cell antigens (TRA-1-60, TRA-1-81, GCTM-2, and TG-343) and do not bind to mouse cells.
3. Prior to differentiation hESCs also express the tetraspanin molecule CD9, podocalyxin, and heat shock 70-kDa protein 8 Isoform 1.
4. Finally, specific transcription factors are associated with the maintenance of pluripotency in hESCs: POU5F1/OCT4 and NANOG.

Antibodies to differentiation markers

Possibly the most exciting attribute of an hESC is the ability to differentiate into any of the cell types that comprise the human body. The assessment of hESC differentiation requires the analysis of gene expression at the RNA and protein level and, ultimately, functional studies for complete validation. A number of antibodies have been used thus far to provide evidence that hESCs can differentiate into many different cell types. A complete listing of antibodies used to identify cell types differentiated from hESCs is beyond the scope of this chapter. However for comprehensive review of the subject see Pera and Trounson (2004) and Hoffmann and Carpenter (2005).

Single-color analyses using flow cytometric analysis and cell sorting

Staining of hESCs with GCTM-2 or SSEA3 for flow cytometry

1. Carefully harvest hESCs using a non-enzymatic dissociation buffer (Gibco, Sigma) and dissociate into a single-cell suspension by trituration:
   (a) Wash the cells twice with PBS and then incubate for 5 min with non-enzymatic dissociation buffer. Gently lift hESCs and triturate in the dissociation buffer using a 1 mL pipette tip.
   (b) Centrifuge gently (500× ***g*** for 2 min), remove supernatant and wash in a similar fashion 2× with PBS, 0.01% BSA (wash buffer).
2. Resuspend in 300 μL of mouse anti-human GCTM-2 (IgM) or SSEA3 (IgM) antibody supernatant (typically diluted 100-fold) or isotype (class-matched control antibody) for 30 min on ice.
3. Pellet for 2 min at 500× ***g*** and wash twice with 1 mL of wash buffer.
4. After washing, incubate for 30 min in the dark on ice in 100 μL of rabbit anti-mouse IgM-FITC (Dako Corporation or similar fluorescently tagged secondary antibodies) diluted 1:40 in wash buffer.

5. Wash twice as described above and resuspend in 400 μL of wash buffer.
6. Add propidium iodide (PI) to a final concentration of 1.0 μg/mL and incubate for 10 min at room temperature to discriminate dead cells.
7. Filter through a 100 μm mesh prior to running on an appropriate flow cytometer (see Equipment).

Staining of hESCs with POU5F1 (OCT4) for flow cytometry (permeabilization required)

1. Carefully harvest hESCs using a non-enzymatic dissociation buffer and dissociate into a single-cell suspension by trituration:
   (a) Wash the cells twice with PBS and then incubate for 5 min with non-enzymatic dissociation buffer. Gently lift hESCs and triturate in the dissociation buffer using a 1 mL pipette tip.
   (b) Centrifuge gently (500× ***g*** for 2 min), remove supernatant and wash in a similar fashion 2× with PBS, 0.01% BSA (wash buffer).
2. Resuspend pellet in 50 μL of wash buffer and carefully pipette slowly into 5 mL of ice-cold methanol in a 15 mL centrifuge tube.
3. Incubate on ice for 30 min, then centrifuge and wash as above.
4. After washing, resuspend in 300 μL of mouse anti-human POU5F1/OCT-4 antibody, typically diluted 100-fold, (Santa Cruz catalog no. sc-5279) or isotype control for 30 min on ice.
5. Pellet for 2 min at 500× ***g*** and wash twice with 1 mL of wash buffer.
6. After washing, incubate for 30 min in the dark, on ice in 100 μL of rabbit anti-mouse IgG-FITC (Dako Corporation catalog no. F0261 or similar fluorescently tagged secondary antibodies), diluted 1:40 in wash buffer.
7. Wash twice as described above and resuspend in 400 μL of wash buffer.
8. Add PI to a final concentration of 1.0 μg/mL and incubate for 10 min at room temperature to discriminate dead cells.
9. Filter through a 100 μm mesh prior to running on an appropriate flow cytometer (see Equipment).

Detection and analysis of GCTM, SSEA3, or POU5F1 (OCT4) immunoreactivity

- As the cells are running through the cytometer, the forward (FSC) and side (SSC) light scatter are the first parameters to be established (Figure 8.1). Center the population in the dot plot by adjusting the FSC and SSC gains.
- Run the unstained control next (this tube may contain PI), adjusting the FL1 PMT voltage until the background fluorescence is situated in the first decade (if PI is also in this tube, adjust the FL3 PMT voltages until the PI negative cells are located in the first decade of the FL3 plot).

- Run the stained cells next, collecting enough cells to analyze later (minimum 1000 positive cells).
- To analyze the data, cells should be initially gated according to forward (FSC) and side (SSC) light scatter, excluding the debris in the bottom left hand corner and the cell clumps on the right hand boundary (Figure 8.1).
- Viable cells are then selected by gating the PI negative events prior to collection of appropriate fluorescence signals (in this instance FITC at 530 nm).
- The unstained control is analyzed first. It is used to assess the amount of non-specific staining of the isotype control (Figure 8.2).
- Gates to assign positive or negative status to cells should be set using the appropriate isotype controls (see Controls section for all necessary controls).
- The boundary of the gate should be set so the background of the isotype is approximately 0.5%. Run the stained file through these gates and read off the percentage positive.

Multiple-color analyses using flow cytometric analysis and cell sorting

Essentially, multiple-color analysis is carried out as for the single-color techniques described above. Incubations with the different primary antibodies are done simultaneously. Incubation with the different secondary antibodies is also done simultaneously.

Multiple-color analysis takes advantage of the different antibody classes (IgG, IgM, etc.) or of antibodies derived from different species (rabbit, mouse, goat, etc.) that can be specifically detected by secondary antibodies conjugated to spectrally distinct fluorochromes (Figure 8.4). That is, one could use a combination of a mouse IgG with a mouse IgM primary antibody and then secondary antibodies specific to mouse IgG and mouse IgM, respectively. Alternatively, one could use a combination of a rabbit IgG and a mouse IgG primary antibody and then secondary antibodies specific to rabbit and mouse IgGs, respectively.

Alternatively, primary antibodies can be directly conjugated to fluorochromes (either purchased preconjugated or labeled using a conjugation kit such as Alexa Fluor 488 Monoclonal Antibody Labeling Kit, Molecular Probes catalog no. A-20181) that allows multiple-color analysis using antibodies of the same isotype (Figure 8.3).

Multiple conjugated primary antibodies are added simultaneously, or the antibodies can be added and washed sequentially. If primary antibodies are added simultaneously it is essential to compare to single-color controls to make sure that there is no cross-reactivity.

Fluorochromes are chosen to minimize spectral overlap (Figure 8.4).

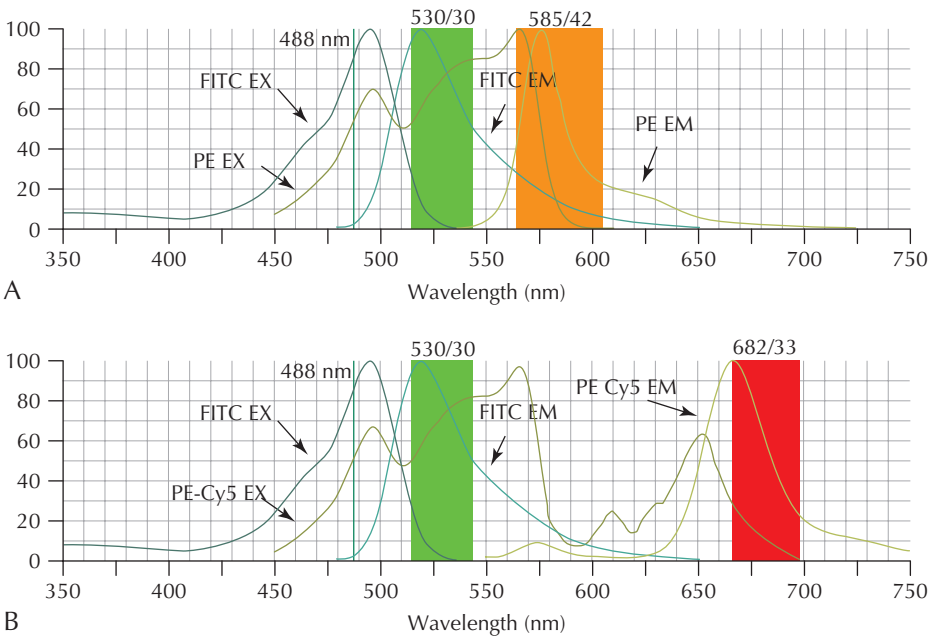

FIGURE 8.4 Choice of fluorophores has a significant impact on the complexity of data analysis of multicolor staining. (A) The choice of FITC and PE, historically one of the most common staining pairs used, shows spectral overlap: The FITC emission spectrum (FITC EM) overlaps the PE emission spectrum (PE EM) to such an extent that no appropriate filter can be chosen to eliminate the overlap. (The 530/30 and 585/42 emission filter sets and FITC and PE, respectively, are the most common ones used.) The advantage of using FITC and PE is that the same laser excitation source, 488 nm, may be used to excite both fluorophores simultaneously (see FITC EX and PE EX for their respective excitation spectra). (B) One solution to the problem. By using the PE-Cy5 fluorophore, which has the same excitation spectrum (PE-Cy5 EX) as PE but an emission spectrum (PE-Cy5 EM) well outside that of FITC, one can still use 488 nm to simultaneously excite both fluorophores but also use a different filter set, 682/33, that will pick up PE-Cy5 emission but not FITC emission. Both panels are screen prints of data generated at the BD Biosciences website, an extremely useful site for visualizing spectra characteristics of many different fluorophores.

PITFALLS AND ADVICE

Background staining

High staining of the isotype control is due to the antibody binding non-specifically and can be more pronounced with intracellular staining (e.g. POU5F1/OCT4). Here are some possible remedies for resolving the problem.

- Spin the antibodies in a microfuge and carefully remove supernatant to avoid precipitates in aliquots. Alternatively, antibodies may be filtered.
- Use fresh antibodies. Over time antibodies will degrade and increase the incidence of background and non-specific staining.
- Lower the primary antibody and/or secondary antibody concentrations.

- Increase the concentration of BSA in the wash buffer.
- Change the type of blocking serum (e.g. serum from secondary antibody host species).
- Try a different clone of the antibody for the same antigen.

Weak staining

- Test the antibodies first on known positive and known negative controls to determine if the antibody is active and specific.
- Try another antibody or batch of antibody to the same antigen.
- Increase the concentration of primary and/or secondary antibodies.
- Increase staining time.
- Change the fluorochrome to a brighter one (e.g. AlexaFluor 488 to replace FITC).
- Increase the PMT voltage for the fluorescence detector so the unstained cells sit outside the first decade. This can have the effect of achieving the cytometer's best resolution sensitivity. If this is the case you should see an increase between the ratio of the positive and negative cells compared with the lower settings.

Too much staining

- Reduce primary or secondary antibody concentration.
- Reduce staining time.
- Decrease the PMT voltage for the fluorescence detector to put the brightest cells on scale.

Controls and compensation

- The unstained control sample is the first reference. The cells in this tube are processed the same way as the stained samples (all the washes and incubation conditions) but without any antibodies. This control is used to set the level of background fluorescence on the cytometer and is used to determine if there is any non-specific staining by the isotype control.
- The isotype control is used to determine the amount of non-specific staining due to the class of antibody used.
- Compensation: When using the dyes FITC and PI, a FL1 vs FL3 dot plot should be used to monitor the amount of FITC spillover into the PI detector. If the level of FITC fluorescence is high it will bleed though to the PI detector, making the cells look PI positive (therefore dead). A small amount of FL3–%FL1 will bring the FITC-high events back into the viable gate.
- When performing multiple-color analysis, each fluorochrome used must have a control tube. While each one is run, compensation is applied to the neighboring channels to remove any spillover.
- Choose fluorochromes to minimize or eliminate spectral overlap, if possible.

Equipment

Flow cytometer

Minimum requirement is a single three-color laser machine with the following filters:

- 530 nm for FITC
- 580 nm for phycoerythrin (PE)
- red filter >610 nm for PI.

If there is too much spectral overlap between FITC and PE a multi-laser cytometer may be required. The most common alternative is a machine with an additional 633 nm laser to detect fluorochromes such as APC and AF633.

Supplies and Reagents

- Aliquots of primary antibodies (various vendors; preparation as suggested by manufacturer)
- Aliquots of secondary, fluorescently tagged antibodies (various vendors; preparation as suggested by manufacturer).

Item	Supplier	Catalog no.	Alternative
Cell Dissociation Buffer	Invitrogen	13151-014	
Phosphate Buffered Saline w/o Mg++, Ca++	Invitrogen	14190-144	HyClone SH30028
Cell Strainer, Nylon 100 μm	BD Falcon	352360	
Cell Strainer, Nylon 70 μm	BD Falcon	352350	
Cell Strainer, Nylon 40 μm	BD Falcon	352340	
Tube with Cell Strainer Cap	BD Falcon	352235	
Round bottom tube with cap	BD Falcon	352063	

Reading List

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Web resources

BD Biosciences (www.bdbiosciences.com/spectra)

Dako (www.dakocytomation.com/)

Salk Institute (flowcyt.salk.edu)

Purdue University (www.cyto.purdue.edu)

International Institute for Analytical Cytology (www.isac-net.org)

CHAPTER

9

Immunocytochemical Analysis of Stem Cells

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INTRODUCTION

Immunocytochemistry is the best way to determine whether a population of cells is homogeneous or heterogeneous with regard to a particular molecular marker. Immunocytochemistry allows for the visualization of individual cells within a colony or culture and thus provides an overall assessment of the distribution of expression of a particular marker throughout the culture under specific culture conditions. For this reason it is a valuable tool to complement biochemical assays that cannot discriminate individual cells in a population (such as immunoblot, PCR, and microarray). Immunocytochemistry also reveals the subcellular localization of the antigen. The success of immunocytochemistry is dependent on the quality of the antibodies used, which can recognize antigens that are protein, glycolipid (such as the SSEA-4 epitope), carbohydrate, small molecule, or DNA.

Specimens are often described as "weakly positive" or "strongly positive." When using a new antibody or testing a new sample, it is essential to confirm the presence of the antigen using another method, such as RT-PCR if the antigen is a protein. In general, "weakly positive" samples always must be verified. If both protein and transcript are present in your cells, there will be little doubt that the antigen you are examining is present. Other methods used for confirmation of antibody staining include the use of a second antibody that recognizes another epitope on the same molecule, and

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immunoblots (Western blots), in which molecular weight information adds confirmation of the identity of the antigen.

Immunocytochemistry for cultured cells uses an amplification technique to make submicroscopic molecules visible. Ideally, every experiment includes negative controls (such as no primary antibody) and positive controls (such as a cell type known to express the antigen) in order to assess the efficacy of staining.

Overview

This chapter will describe the most popular immunocytochemical method in the stem cell field – using fluorescently tagged secondary antibodies to detect the primary antibody that is bound to an epitope on the molecule of interest. Secondary antibodies recognize the heavy chain of the primary antibody's isotype. Generally, these methods employ a long incubation period for the primary antibody, a series of washes to remove unbound antibody, and a shorter incubation for the secondary antibody, followed by washes and preparation for microscopy.

Primary antibodies vary widely in their binding affinities and specificities and must be tested to determine whether they recognize the antigen when the specimen is prepared for immunocytochemistry. Antibodies bind to specific epitopes, which may be short stretches of amino acids in a protein, conformational characteristics like an exposed alpha helix, or structural elements of a small molecule. Polyclonal antibodies contain multiple antibodies that usually recognize several different epitopes on a molecule. In contrast, monoclonal antibodies are of a single antibody type and recognize a single epitope.

Key considerations

- Primary antibody: Epitope(s) recognized
- Secondary antibody: Match to the isotype of the primary antibody
- Fixation method: Affects the accessibility of the primary antibody to the epitope
- Permeabilization: If the epitope of interest is located inside the cell the membrane must be permeabilized to allow antibody entry. If the epitope is on the cell surface, permeabilization may interfere with its detection.

Procedures

The protocol described below is easy, routinely gives publication-quality photos, and can be done by devoting only a short time each day. If rapid analysis is desired, the alternative protocol can be used, with timing indicated at the end of each section.

Choosing the right antibodies

Most fluorescence microscopes have the ability to excite and discern several unique fluorochromes using various optical filters. In designing a plan for staining for more

TABLE 9.1 Example staining plan: One eight-well slide for 14 antigens, DAPI, and controls

Well	Blue (AMCA)	Green (Cy2)	Red (Cy3)	Far Red (RRX)
1	Tubulin-Mouse IgG	GFAP-Guinea-pig	Nestin-Rabbit	SOX 2-Goat
2	Secondary only: Anti-mouse IgG	Secondary only: Anti-guinea-pig	Secondary only: Anti-rabbit	Secondary only: Anti-goat
3	GFAP-Guinea-pig	Synapsin-Rabbit	MAP2-Mouse IgG	DCX-Goat
4	Secondary only: Anti-guinea-pig	Secondary only: Anti-rabbit	Secondary only: Anti-mouse IgG	Secondary only: Anti-goat
5	Nestin-Rabbit	POU5F1 (OCT4)-Goat	A2B5-Mouse IgM	Ki67-Mouse IgG
6	Secondary only: Anti-rabbit	Secondary only: Anti-goat	Secondary only: Anti-mouse IgM	Secondary only: Anti-mouse IgG
7	DAPI (DNA staining)	SSEA4-Mouse IgG	O4-Rabbit	CD15-Mouse IgM
8	No antibody	Secondary only: Anti-mouse IgG	Secondary only: Anti-rabbit	Secondary only: Anti-mouse IgM

than one antigen, it is important to select primary antibodies of unique species or subtypes (i.e. mouse IgG, mouse IgM, rabbit IgG, goat IgG, chicken IgG, guinea-pig IgG, rat IgG). If the primary antibodies for different antigens are from the same species and subtype, secondary antibodies will indiscriminately bind to both markers.

Table 9.1 shows how combinations of antibodies and fluorochromes can be combined to assay for 14 different antigens with all necessary controls in a single eight-well chamber slide.

Preparation of samples

Growth on glass surface

Several days prior to staining, passage the cells to chamber slides or sterile glass coverslips with the appropriate substrata (extracellular matrix such as laminin or feeder layer of cells) so that the cells will adhere to the surface and not wash off during the staining process.

NOTE: Plastic dishes scatter light and are often autofluorescent, so fluorescent antibody staining on plastic culture dishes is not advised.

BrdU labeling

For experiments using BrdU labeling of cells, BrdU (10 μM final concentration) should be incubated with the cells for 2–24 h prior to fixation. In some cases it will be desirable to remove the BrdU-containing media and culture the cells in regular medium for a few days before fixation.

NOTE: BrdU-labeled cells should be treated with HCl (1 N HCl for 20–30 min at 37°C) after fixation but prior to blocking and antibody incubation. Wash well with PBS after HCl incubation.

Immunostaining procedure

Day 0: Fixation of the cells

1. On the day of staining, carefully aspirate the growth medium and rinse cells once with PBS. Importantly, the cells should never be allowed to dry out, so you should not completely aspirate all the liquid from the well and you should always have the next solution at hand to add immediately after aspiration.
2. Fix cells for 10 min at room temperature with 4% paraformaldehyde in PBS (see Recipes). Dispense the solution down the side of the well so that it slowly floods the well without disturbing the cell surface. Use this same technique any time adding solution to the wells.
3. Wash cells twice with PBS (approximately 5 min each wash).
4. For best results, stain fixed cells within 24 h of fixation. Alternatively, store fixed cells at 4°C in PBS + 0.05% (w/v) sodium azide.

Day 1: Set up primary antibody incubation

1. Design a plan for each sample well. See Table 9.1 for an example of combining multiple antibodies. Make certain that antibody isotypes do not overlap within a given well.
2. Antibody concentration: Most manufacturers provide recommendations for antibody concentrations for specific applications (for example for immunocytochemistry (ICC) or immunohistochemistry (IHC)).
3. When using an antibody for the first time, it is a good idea to try a range of concentrations around that provided by the manufacturer. For example, if the recommended concentration is 1:100, try a range from 1:10 to 1:1000. We recommend 1:100 for most antibodies.
4. If no recommended concentration is given, start with 1 μg/100 μL.
5. Aliquot antibody dilution buffer (ADB) into 0.65 mL microcentrifuge tubes for each well for dilution of primary antibody.
6. If using eight-well culture slides, you will need a final volume of 250 μL per well. For four-well culture slides, use 400 μL per well (adjust volume per well accordingly for wells that are other sizes).
7. Add appropriate volume of primary antibody (or antibodies) to each tube with ADB and mix gently.

NOTE: Secondary-only control wells should be incubated in ADB alone (no primary antibody) or with a control Ig diluted in ADB.

8. Remove protein precipitates from the primary antibody solution by spinning at 11 000 rpm for 5 minutes in a microcentrifuge.

9. Gently remove primary antibodies to new tubes, leaving a small amount of liquid at the bottom where the sediment remains. Keep diluted antibodies on ice until added to cells.
10. Wash cells gently with PBS.

NOTE: Incubate any BrdU-treated wells with HCl then rinse with PBS (see notes on BrdU above).

11. Remove PBS and add approximately 250 μL of blocking buffer to each well. Incubate for 15 min to 1 h at ambient temperature.
12. Wash cells gently with PBS.
13. Remove PBS and add the diluted primary antibodies to the wells.
14. Remove the covers from the eight-well slides and place slides into a humidity-controlled bin (i.e. Tupperware with damp napkin). Condensation on the eight-well slide cover increases the probability of cross-contamination across the wells.
15. *Recommended method:* Incubate slides overnight at 4°C. *Alternate method:* Incubate slides 1–2 h at room temperature.

Day 2: Secondary antibody incubation

1. Dilute secondary antibody (or antibodies) in ADB using concentration as recommended by vendor or determined empirically to give the best results. We usually dilute secondary antibodies 1:250.
2. Remove the primary antibody from each well.

NOTE: Place a disposable pipette tip on the end of the aspirator pipette and replace tips for each aspiration. A used aspirator tip increases the likelihood of cross-contaminating adjacent wells.

3. Wash cells with twice with PBS. Replace aspirator tips after each use.
4. Spin secondary antibodies at 11 000 rpm for 5 min to remove protein precipitates.
5. Carefully add secondary antibodies to aspirated wells.
6. *Recommended method:* Incubate slides overnight at 4°C in a humidity controlled bin. *Alternate method:* Incubate for 1 h at room temperature.

Day 3: Visualizing immunofluorescence

1. Wash wells three times with PBS for 5 min each wash.
2. If desired, incubate cells with DAPI or Hoechst reagent to counterstain nuclei (Hoechst 33342 (Molecular Probes/Invitrogen), 1 mg/mL in DMSO, stored at 4°C, dilute 1:500 in PBS, incubate on cells for 1–5 min at room temperature, wash cells with PBS).

NOTE: It is often useful to have a cellular counterstain if it does not interfere with another antibody being detected by a fluorophore in the blue channel (such as AMCA). A nuclear counterstain is helpful when evaluating the nuclear localization of an antigen (particularly in stem cells that have a high nucleus-to-cytoplasm ratio).

TABLE 9.2 Common fluorophores

Fluorophore	Absorption peak (nm)	Emission peak (nm)
Blue: AMCA, Hoechst, DAPI	~350	~450
Green: FITC, Cy2, Alexa488	~492	~520
Red: TRITC, Cy3, Alexa555	~550	~570
Far red: Cy5	~650	~670

3. Prepare mounting media (used to minimize photobleaching of fluorescence). Examples of mounting media are: Vectashield (Vector Labs), Slow Fade (Invitrogen/Molecular Probes), and Prolong Antifade Reagent (Invitrogen/Molecular Probes).
4. Aspirate wells.
5. If using chamber slides:
   - Snap off plastic wells.
   - Carefully use a razor on one of the short ends of the gasket. Using fine tweezers peel back the gasket slowly.
   - Pipette a bead of the mounting media along the long end of the slide. Be careful not to allow bubbles to form on the bead. Gently lower a rectangular coverslip at a 45° angle on the slide. Allow the mounting medium to spread.
6. If using coverslips:
   - Carefully remove coverslip. This is best done with forceps bent for the purpose.
   - Lower the coverslip at a 45° angle onto a small drop of mounting media on a glass slide.
7. Using two fingers very gently squeeze out the extra mounting medium and/or trapped air bubbles over a disposable paper towel. Pressing too hard will displace and/or damage cultures. Aspirate the extra medium off the slide.
8. Allow the slide to dry at room temperature in a dark place overnight. *Alternate method:* Allow samples to dry briefly then proceed to the steps in Day 4 below. Note that the coverslips will still be able to move around and should be handled with care.

Day 4: Observation

1. Remove excess mounting medium by gently wiping the slide with 70% ethanol (use Kimwipes or cotton swab).
2. Seal slide with nail polish (i.e. "top coat"). Allow to dry.
3. View slides on fluorescence microscope. Afterward, store slides at –20°C (with desiccant for best preservation). Storage at –20°C can preserve the signal for months (depending on the sample, antibody, etc.).

Using the microscope

1. Seat slide on microscope stage with the coverslip facing the objective lens.

NOTE: If you are using an inverted microscope this means that the slide has to be flipped so that the coverslipped side is down.

2. Make sure the microscope shutter is closed. Turn on fluorescent light source and lamplight.
3. Using a phase contrast 20× objective, bring the sample into the focal plane.
4. Turn off phase contrast white light and use low frequency light to first evaluate staining. Bleaching of fluorochromes is accelerated during exposure to higher frequency light. We prefer an excitation of ~570 nm (RRX channel).
5. Open shutter and view cultures through the microscope's binocular lenses.
6. Scan through areas of interest while cycling through the various filters.
7. Remember to limit the exposure of the slide to fluorescent light. Close shutter when not analyzing samples.

Summary of immunostaining procedure

1. Remove media from cells, wash with PBS if desired.
2. Add fixative, 10 min, room temperature.
3. Wash PBS, 2 × 5 min
4. Add HCl if BrdU-treated cells, 20–30 min, 37°C, wash PBS, 2 × 5 min.
5. Add blocking buffer, 15 min, room temperature, remove.
6. Add diluted primary antibodies, overnight, 4°C.
7. Wash PBS, 2 × 5 min.
8. Add diluted secondary antibodies, 1 h, room temperature or overnight, 4°C.
9. Wash PBS, 2 × 5 min.
10. Add Hoechst (1:500 in PBS) or DAPI (1×), 1–5 min, room temperature.
11. Wash PBS, 1 × 5 min
12. Mount and coverslip, seal with nail polish if desired.
13. View on microscope.

PITFALLS AND ADVICE

Background staining

A sample may have a high level of background fluorescence or fluorescent debris. Here are some possible remedies for resolving this common problem and further discussion of a few specific causes of background staining that are particularly useful for tissue staining.

- Spin the antibodies to remove precipitates before adding the antibody to the sample.

- Use fresh antibodies. Over time antibodies will degrade and increase the incidence of background and non-specific staining.
- Reduce primary antibody and/or secondary antibody concentrations.
- Do not let cells dry out during rinses, fixation or antibody incubation.
- Increase PBS rinsing time or number of washes.
- Use correct blocking serum or longer blocking time. Can also try blocking with IgG-free BSA rather than animal serum (use 5% w/v in PBS for blocking buffer and 1% w/v in PBS for antibody dilution buffer).
- Refine growing conditions to avoid stressing cell cultures during growth.
- Attempt to use a different antibody for the antigen (try to choose an antibody that recognizes a different epitope on the molecule).

Species mismatch

- *Problem:* Same-species antibodies yield high background. For example, when mouse primary antibodies are used on mouse tissues, detection with anti-mouse secondary antibodies will detect all mouse immunoglobulins that are native to the mouse tissue.
- *Solution:* Use species-mismatched primary antibodies or block the endogenous antibodies by pre-incubating with an unconjugated secondary antibody. If blocking, it is necessary to use Fab fragments and important to use a Fab preparation that matches the conjugated secondary antibody that will be used for detection. Vendors often sell unconjugated Fab preparations that match the detecting secondary antibody for this purpose.

NOTE: Why use Fab fragments for blocking endogenous Ig? Whole Ig is multivalent and a block with a multivalent antibody will leave many Fab ends unbound. Subsequent treatment with the primary antibody will simply bind these exposed ends and aggravate the background problems.

Fc receptors in sample

- *Problem:* Fc receptors expressed by cells non-specifically bind primary and secondary antibodies. Particularly problematic for tissues that have been damaged and contain activated immune cells.
- *Solution:* Use Fab preparations for detection rather than whole antibodies or block using unconjugated Fc fractions that match both primary and secondary antibody preparations.

NOTE: When using Fab fragments for detection, the secondary antibody must be one that recognizes a Fab fragment. Typically, the secondary antibody used with recognize light-chain rather than heavy chain and one must take care to determine the class of light chain present in the Fab fragment (i.e. either kappa or lambda light chain).

Endogenous enzymatic activity

- *Problem:* Tissues and cells express peroxidases, galactosidases, and phosphatases that will create non-specific staining when using enzymatic methods to detect

bound antibody (for example, HRP (horseradish peroxidase)-conjugated secondary antibodies).

- Red blood cells contain high peroxidase activity. Vascular cells express high levels of phosphatase and macrophage/monocytes express high levels of all three types of enzymes.
- *Solution:* Most kits include instructions for minimizing or inactivating endogenous enzyme activity. In general, the kit protocols work extremely well and can be used without modification.

NOTE: Endogenous peroxidase activity is ablated by pre-incubating tissues with high concentrations of H_2O_2. If the H_2O_2 used is old, then this step will not work well. Tissues should visibly bubble when pre-incubated. If not, the H_2O_2 is probably old. Use a higher concentration or use a new stock.

NOTE: Controls for enzymatic detection should include:

- No primary or secondary antibody
- Secondary antibody alone. Incubation of the control samples with enzyme substrate can be used to confirm specificity of staining and check for endogenous enzyme background staining.

Generalized background

- *Problem:* Very high overall background.
- *Solution:* Titrate antibodies (both primary and secondary) for optimum signal to noise ratio. Primary or secondary antibody may recognize non-specific antigens. To determine if the problem is with the primary or secondary antibody, prepare one sample that is treated with secondary antibody alone. If background is low, then problem is with primary antibody. If background is present in samples treated with secondary antibody alone, then problem is with secondary antibody. In both cases, an alternate antibody should be tried (if available) or more aggressive means to improve specificity should be explored.

NOTE: Secondary antibody background can be reduced if the vendor provides unconjugated pre-immune serum from the same species (ideally collected from the same animal prior to immunization). This is used in the initial blocking step to bind all non-specific sites prior to the final detection using the conjugated secondary antibody preps.

Weak staining

- Test the antibodies on known positive and negative controls.
- Try another antibody to the same antigen.
- Fixation: Check the literature for papers that have used the antibody (and have nice images of immunostained cells) and follow the protocol verbatim. Most antibodies are sensitive to the type of fixation used or the amount of fixation (it is possible to over-fix).
- Increase the concentration of primary and/or secondary antibody.

- Increase the time of the primary antibody incubation.
- If positively staining slides have faded over time, be certain the nail polish sealant on slides is intact and that the slides are being stored in a desiccated environment.

Too much staining

- Reduce primary antibody concentration.
- Reduce primary or secondary antibody incubation period.
- Attempt to use different clone of antibody for the same antigen.
- See notes on blocking in "Background staining" section above.

Multiple antibody staining

The basic method as described above is also used for staining with more than one antibody simultaneously on the same sample. Staining for more than one antigen involves use of multiple primary antibodies, each of a unique class or animal species, followed by use of multiple secondary antibodies, each specific for one of the primary antibodies and each carrying a unique enzyme or fluorochrome/fluorophore marker.

If using enzyme detection, sequential reaction in each substrate will be necessary. Order of application may be important (i.e. H_2O_2 used in the HRP reaction can oxidize other enzymes and reduce activity. Consider doing DAB last).

NOTE: Care should be taken to use secondary antibodies that are highly specific for the class and species of primary antibody that needs to be detected. Some vendors provide secondary antibody reagents that are validated to have minimal cross-reactivity to a wide spectrum of antibody classes and species (Jackson Immunoresearch is a reliable source).

NOTE: Histochemical reaction products are frequently opaque. If attempting to detect co-labeling in a given cell, care must be used in choosing the specific detection method. For example, if co-labeling for BrdU in the nucleus along with a cytoplasmic marker to identify cell phenotype, use HRP-DAB for the BrdU to generate an opaque black nucleus. Use a more translucent marker for the cytoplasmic epitope (e.g. AP-vectorRed or AP-vectorBlue from Vector Labs).

Digital images of fluorescent cells

After immunostaining, cells are usually viewed on a fluorescence microscope and images of the stained cells captured with a digital camera. There are a variety of cameras and image capturing software packages available, therefore we will not go over the specific details of a particular program here (details about one program, Image-Pro, can be found in Appendix 9.1).

Many scientists bring the captured images into Adobe Photoshop, to create output for publications. In the next section, therefore, we will describe how to use several features in Photoshop and briefly introduce a program available for image quantification (NIH Image, also available as ImageJ).

Adobe Photoshop

Photoshop can open a wide variety of image files captured from a microscope-mounted camera, including ".tiff" and ".jpg" formats, and provides a variety of means to manipulate images. Here we will briefly describe how to set the color mode, alter the image size, create scale bars for an image, adjust the image brightness and contrast, and create color overlays of images.

Setting the color mode

Color digital images can either use RGB (red, green, blue) or CMYK (cyan, magenta, yellow, black) for color encoding. RGB images are more compatible for computer monitors or projectors, since they use an additive light system and printers rely on a subtractive light system.

Bright greens, reds, and blues cannot be reproduced in print as they can on a monitor, so prints of an RGB image may not convey the bright colors or fine detail visible on the computer monitor. For print purposes (and therefore for most journal submissions), it is best to convert an RGB image to CMYK. To convert to CMYK for printing, go to "Image" → "Mode" and select CMYK.

Adjusting the image size

Images captured by image acquisition software programs can come in a variety of sizes and resolutions. To find the size of your image, go to "Image" → "Image size."

Images often are captured at 72 pixels/inch and are of fairly large dimensions (in terms of inches). Journals usually request photos at a resolution of 300 pixels/inch. The easiest way to do this is to change the resolution but not change the overall size of the file so that the dimensions (in inches) of the image are more suitable for printing or incorporating into a figure.

To do this, make sure that the checkbox next to "Resample image" is unchecked (as in Figure 9.1B) then adjust the resolution (see the examples below and note that the overall pixel dimensions (1.83 M, 1600 × 1200 pixels) are the same for both while the document sizes (width, height, resolution) are different).

Scale bars

Journals usually require scale bars for all microscopic images. One way to generate scale bars for your images and to make size/length determinations is to use a *scale micrometer*. A scale micrometer is a microscope slide that has lines etched a particular distance apart from each other. The micrometer can be placed on the microscope stage and an image taken using each of the microscope objectives. Because the images will be captured at the same width (in terms of pixels) as your photographs of cells or tissues, you can determine a conversion factor that will allow you to measure real distances on your images.

As an example, if an image taken with a camera on a particular microscope using a 20× objective has a total width of 580 μm (from the scale micrometer) and 1600 pixels; this means that 100 μm would equal ~276 pixels on that image.

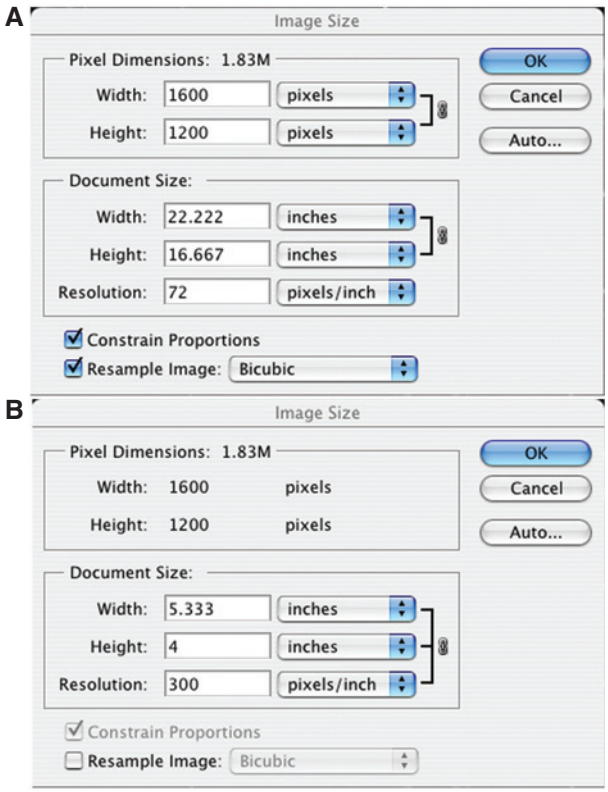

FIGURE 9.1 Adobe Photoshop "Image Size" window. (A) Before size adjustment. (B) After size adjustment.

NOTE: These measurements will be specific to the objective, microscope, and camera used, so attention must be paid to the conditions under which a particular image was captured in order to appropriately determine the scale.

For a scale bar on your photomicrograph, you can draw a line of a particular length (in pixels) in Photoshop by using the line tool (on the tool bar, which also contains the move tool, text tool, etc.) and watching the pixel location in the Navigator window ("Window" → "Navigator"; click on the "Info" tab in the Navigator window). The X and Y coordinates of the cursor location will be in pixels as long as the rulers for the image are set to "pixels" ("Preferences" → "Units and rulers").

Adjusting the brightness/contrast of an image

There are multiple ways to adjust images in Photoshop, and most are found under "Image" → "Adjustments."

One straightforward way to adjust the brightness/contrast is to use the "Levels" option ("Image" → "Adjustments" → "Levels") and adjust the sliders under the histogram (Figure 9.2). The advantage of this option is that by viewing the histogram, you can more accurately adjust the intensity of the image without altering the data.

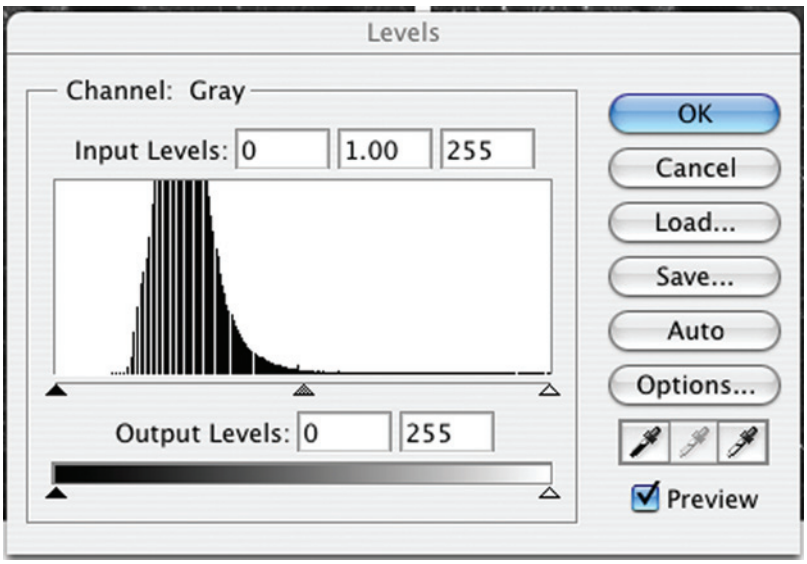

FIGURE 9.2 Adobe Photoshop "Levels" window.

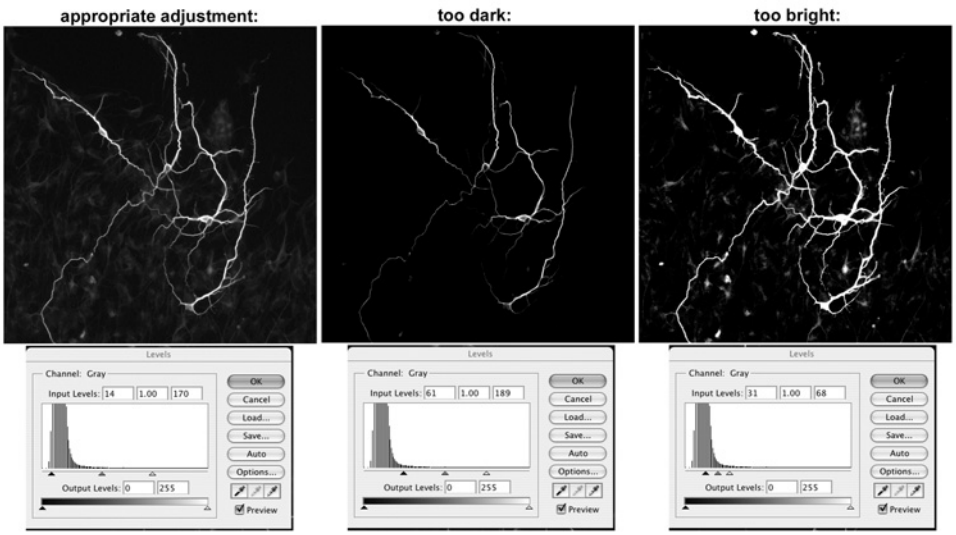

FIGURE 9.3 Adobe Photoshop "Levels" window and corresponding image changes. Moving the sliders on the "Levels" window can result in appropriate (left) image adjustment or images that are too dark (middle) or too bright (right).

It is *imperative* when using any image adjustment for data images to be extremely careful to not alter the data with the adjustment. For example, decreasing the brightness should not remove signal and increasing the brightness should not create signal or expand its zone. See Figure 9.3 for images that have been appropriately and inappropriately adjusted.

Changing grayscale images to color and overlaying color images

Cells or tissues are often double- or triple-labeled with different fluorescent molecules to allow visualization of multiple signals. Photoshop can be used to convert captured grayscale images to color and overlay the color images so that all fluorescent signals can be visualized simultaneously.

In order to create a color overlay, the images of the different fluorescent channels are brought together into a single file. The separate images are maintained on individual layers and then assigned a different color.

1. To begin, select all of the image ("Select" → "All") and copy ("Edit" → "Copy").
2. Make a new file ("File" → "New") and the size, resolution, etc. will be identical to what you just copied.
3. In the window that opens and describes the new file, switch from "Grayscale" to "RGB" (or "CMYK" if the image is solely for print media).
4. Once the new file is created, paste in the copied image ("Edit" → "Paste"). Select all and copy the other images to be overlaid, then paste them into the new file.
5. Each image will automatically be pasted into a different layer ("Window" → "Layers").
6. To change the color of an image in a layer, open the "Levels" option ("Image" → "Adjustments" → "Levels") and use the tab marked "RGB" to select either the Red, Green, or Blue channel.
7. Use the "Output levels" to alter the color: for a Red image, make the Green and Blue output levels 0 (change the number in the box on the right from 255 to 0), for a Green image, make the Red and Blue output levels 0, and for a Blue image, make the Red and Green output levels 0.
8. These steps can be repeated for different layers within the same document to create layers that are of different colors.
9. To overlay differently colored layers, position one colored layer directly above the other colored layer (in the "Layers" window) then change the button under the "Layers" tab from "Normal" to "Screen." You should now see both layers overlaid.

NIH Image (ImageJ)

NIH Image (or ImageJ) is a free program available for download that can be used to quantify a wide variety of parameters in an image. In addition to the basic features of ImageJ, there are macros that others have created (or you can write yourself) that expand the functionality of the program. For details and downloads see: http://rsb.info.nih.gov/nih–image/Default.html.

Measurements in ImageJ

1. In order to measure in ImageJ, you must first know the scale of your picture in real dimensions (see Scale bar section above). An easy way to convert this information to a scale in ImageJ is to draw a line across the entire width of your image (use the straight line tool on the toolbar).

2. Once you have drawn the line, go to "Analyze" → "Set scale" and set the known distance to the numerical value and unit of length for your image width (for example, the width of the image described in "scale bars" above would be 580 μm).
3. Keep the Pixel Aspect Ratio as 1 and use "um" for "μm."
4. If you are analyzing multiple images that were taken under the same conditions and thus have the same scale, you can check "Global" in the "Set scale" window and the scale will be automatically applied to all the images. After setting the scale, the length of any line drawn and measured will be given in the desired units.
5. To measure an element in your image, you can draw a line (straight, segmented, or freehand) and then click on "Analyze" → "Measure." "Analyze" → "Set measurements" allows you to decide what parameters will be measured.

NOTE: You can also choose other types of shapes (other than a line) and measure parameters such as area.

EQUIPMENT

- Fume hood for working with paraformaldehyde
- Hot plate in fume hood
- Inverted or upright microscope equipped for fluorescence
- Objectives: 10×, 20×, 40×, and perhaps 60× or 100× objectives
- Filter cubes appropriate for secondary antibody fluorophores. It is important to make sure that the cubes will give maximal signal for one fluorophore but not allow bleedthrough excitation of another fluorophore.

SUPPLIES AND REAGENTS

Item	Suppliers
Primary antibodies	Various commercial vendors (see product data sheets); colleagues
Secondary antibodies	Jackson Immunoresearch, Invitrogen (Molecular Probes)
Mounting media	Vector Labs, Invitrogen (Molecular Probes)
Chamber Culture slides	Lab-Tek II, Nalgene, Nunc
Cover slips, no. 1 thickness range for high mag objectives	Fisher, Carolina Biological Supply
Microfuge tubes, 1.0 and 0.65 mL	Fisher, VWR
Pipette tips	Various
Conical tubes 15 mL, 50 mL	Corning
Nail polish "clear" top coat	Drug store
Pipettes, 1, 5, 10, 25 mL	Corning
24-well tissue culture plate	Corning
Triton X-100	Sigma
Sodium azide (NaN_3)	Sigma

Recipes

Paraformaldehyde 4% (1000 mL)

Component	Amount	Final concentration
Paraformaldehyde	40 g	4%
H_2O	500 mL	
0.2 M Phosphate buffer pH 7.4	500 mL	0.1 M, pH 7.4

In fume hood:

1. Add 40 g of paraformaldehyde to 500 mL of dH_2O.
2. Heat to 60°C (do not exceed this temperature).
3. Stir until dissolved.
4. Add a few drops of 1 N NaOH until solution is clear. (Will not completely dissolve or clear without the addition of NaOH.)
5. Filter (0.2 or 0.45 μm) and add 500 mL of 0.2 M phosphate buffer, pH to 7.4 (recheck pH and adjust if necessary).
6. Store at 4°C up to one week (alternatively store aliquots at –20°C).

Important notes about fixative preparation and storage

Paraformaldehyde

- We recommend that paraformaldehyde be prepared fresh whenever possible. It will make a noticeable difference in the quality of immunofluorescence.
- Solubilization of paraformaldehyde powder is often accomplished with heat and strong base. Take care not to heat above 55–60°C and add just enough base to dissolve the paraformaldehyde. Under high heat or high pH, paraformaldehyde will iso-convert to formaldehyde which degrades rapidly to formic acid and water.
- If the solution temperature goes over 65°C during preparation, do not use it as it will produce a strong autofluorescence in cells or tissues.

Formaldehyde 37%

- Storage of 37% formaldehyde over several months results in degradation to formic acid and water.
- Old formaldehyde stocks should be disposed of every 12–24 months.

Buffered formalin 10%

- 10% "buffered formalin": pH will drift due to degradation of fomaldehyde to formic acid.
- Do not use if below pH 6.5.

Blocking buffer (50 mL)

Component	Amount	Related stock solutions
PBS-Triton	48.5 mL	PBS-Triton: PBS + 0.3% (v/v) Triton X-100
Serum from secondary antibody host species: rat, mouse, goat, donkey, etc.	1.5 mL	
Final concentration: 3%		

Antibody dilution buffer (50 mL)

Component	Amount	Related stock solutions
PBS-Triton	49.5 mL	
Serum from secondary antibody host species: rat, mouse, goat, donkey, etc.		
Final concentration: 1%	0.5 mL	

Reading List

Research articles with antibody staining of human stem cells

Reubinoff BE, Itsykson P, Turetsky T, Pera MF, Reinhartz E, Itzik A, Ben-Hur T (2001). Neural progenitors from human embryonic stem cells. *Nat Biotechnol* 19: 1134–1140.
One of the first papers on using immunohistochemistry to identify specific derivatives of hESCs.

Schwartz PH, Bryant PJ, Fuja TJ, Su H, O'Dowd DK, Klassen H (2003). Isolation and characterization of neural progenitor cells from post-mortem human cortex. *J Neurosci Res* 74: 838–851.
This paper has several examples of staining of individual cells with multiple markers.

Antibody laboratory manuals

Javois LC (1999). *Immunocytochemical Methods and Protocols. Methods in Molecular Biology*, Volume 115. Totowa, NJ: Humana Press.

Harlow EW, Lane D (1999). *Using Antibodies, A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Informational websites

Antibody Cross-reactivity resource (http://www.keithbahjat.com/abcxr/)

Chemicon Intro to Antibodies (http://www.chemicon.com/resource/ANT101/atoc.asp)

Laboratory of Experimental Pathology, NIH (http://dir.niehs.nih.gov/dirlep/immuno.html)

Molecular Probes (http://www.molecularprobes.com)

Pharmingen (http://www.bdbiosciences.com/pharmingen/protocols/)

Protocol Online (http://www.protocol-online.org/prot/Immunology/)

Stem Cell Markers and Attributes (http://stemcells.nih.gov/stemcell/scireport.asp)

The Antibody Resource (http://www.antibodyresource.com/educational.html)

Vector Laboratories (http://www.vectorlabs.com/)

Appendix 9.1 Image-Pro 4.0 and AFA Plug-in

Using Image-Pro

The following section will describe using MediaCybernetics' Image-Pro to photograph snapshots of a field of interest using a digital camera.

1. Turn on digital camera.
2. Open Image-Pro.
3. Under "Acquire" select "Video/Digital Capture." The window (Figure 9.4) will allow you to preview and snap pictures directly from the camera.
4. Click "Start preview."

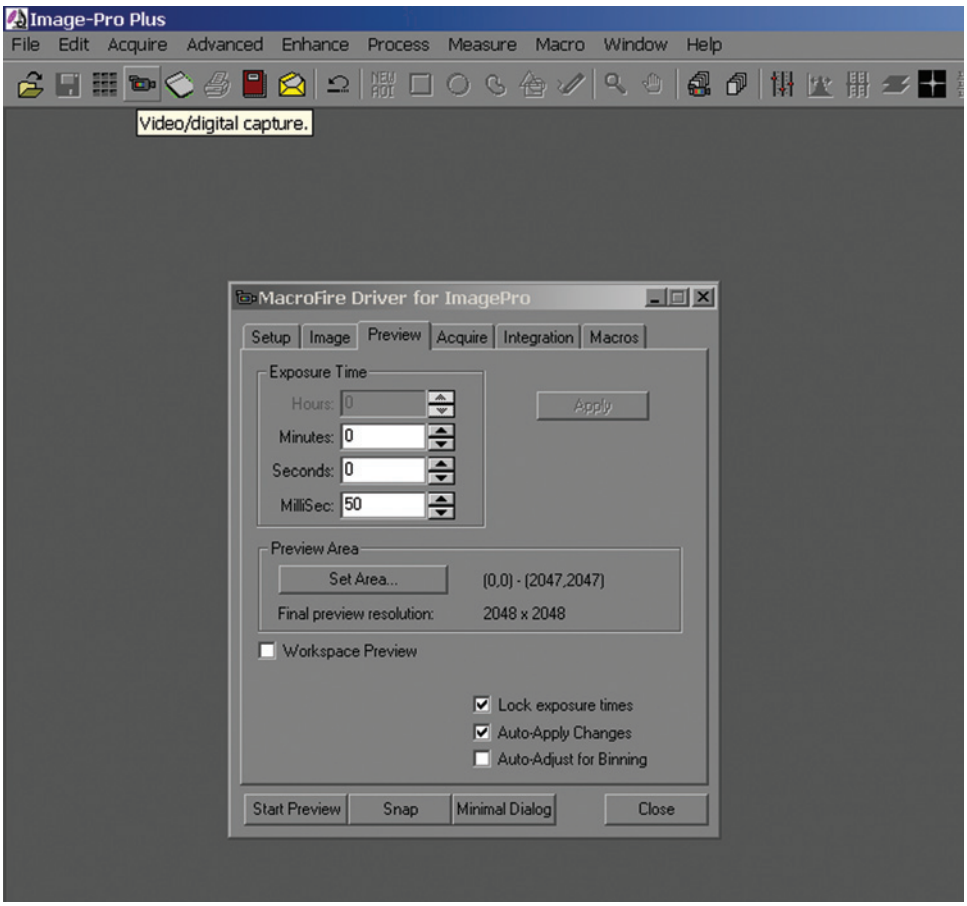

Figure 9.4 Image-Pro Plus camera settings window. The exposure times for each image acquisition can be set in this window. It is also possible to "lock" the viewed image exposure times with the taken image exposure times by checking the "lock exposure times" box.

5. Adjust the exposure time to brighten image without oversaturating digital feed (most digital camera drivers have a configuration setting to provide live saturation warnings).
6. Snap the image when you are satisfied with the previewed image.
7. Save image (a ".tiff" file format is recommended for preserving image detail).

Using Image-Pro AFA Plugin

The Image-Pro AFA Plugin is a useful tool for organizing and managing multiple channels from a field of interest. Exposures may be optimized for each channel before imaging the field of interest as a set. After a set of images is obtained, the color composite tool may be used to pseudo-color and merge channels.

1. Open the "Advanced acquisition" window (Figure 9.5).
2. Click on preview (note: the exposure times for the preview are set for the first channel).
3. Adjust exposure times for each channel.
4. Check the boxes for the channels you want to photograph.
5. Click "Acquire set."
6. If the microscope used is fully automated it will automatically rotate the filter cubes and photograph the samples. If it is not, a prompt will ask you to manually turn the wheel between pictures.
7. Once the set has been acquired, you can save it using the "Set Manager."

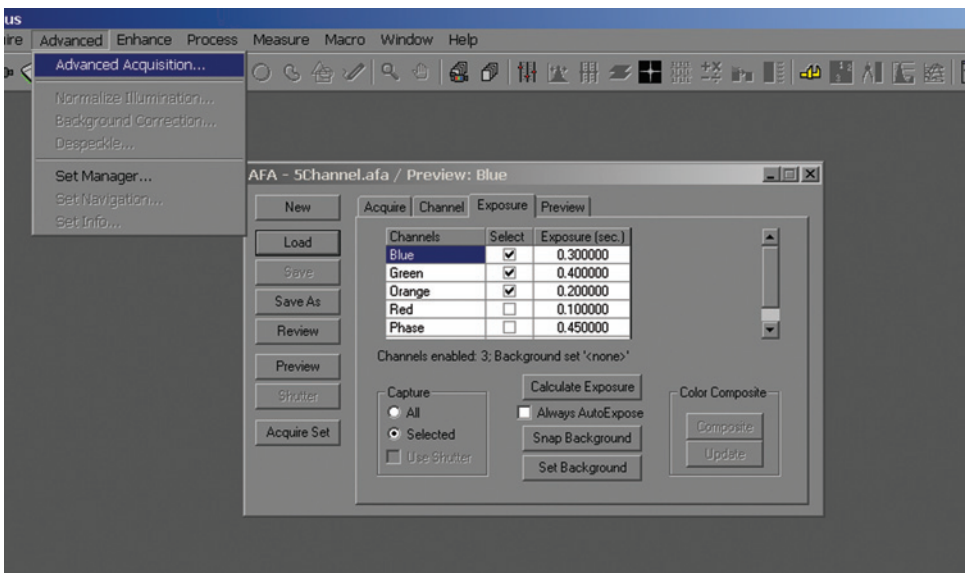

FIGURE 9.5 Image-Pro Plus image processing window. During advanced/automatic acquisitions it is desirable to set the exposure times for each channel prior to imaging.

CHAPTER

10

Characterization of Stem Cells Using Reverse Transcriptase Polymerase Chain Reaction

Shengwen Li, Ivan Kirov Jr., Henry J. Klassen, and Philip H. Schwartz

Introduction

Rapid, sensitive, and quantitative methods for the detection of developmental stages of human embryonic stem cells (hESCs) are essential to any laboratory working with these cells. Current methods include monitoring of morphological changes by phase contrast microscopy, examination of molecular markers by immunocytochemistry and flow cytometry, reverse transcriptase polymerase chain reaction (RT-PCR), and determination of physiological events. RT-PCR is commonly used because it is a relative simple, sensitive, and rapid assay.

Overview

RT-PCR is a quick and dependable technique for monitoring the gene expression profile of stem cells. It is often used in conjunction with immunocytochemistry (tissue

Human Stem Cell Manual, edited by J. F. Loring, R. L. Wesselschmidt, and P. H. Schwartz.
ISBN: 978-0-1237-0465-8.

distribution) and/or immunoblot analysis (specific molecular size), which complement the results of RT-PCR. A common practice is to use RT-PCR as a screening assay to determine whether or not to check the protein expression by immunocytochemistry or immunoblot analysis.

PROCEDURES

Typical RT-PCR for stem cell samples includes: RNA isolation, generation of cDNA by reverse transcription, and amplification of cDNA by PCR. RT-PCR can be classified by one-step protocol (described later) and two-step protocol (Figure 10.1).

RNA extraction

RNA extraction can be carried out with a kit such as Gentra System's PURESCRIPT® RNA Purification Kit. The following is adopted from the manufacturer's protocols.

The amounts of reagents used in each step are determined by total cell number. The ranges are 1–2 million cells, 3–5 million cells, and 6–9 million cells.

Cell lysis

1. Add cells in balanced salt solution or culture medium to a 1.5 mL microfuge tube on ice.
2. Centrifuge at 13 000–16 000×*g* for 5 seconds to pellet cells. Remove supernatant with a pipette, leaving behind visible cell pellet and 10–20 μL of residual liquid.
3. Vortex the tube vigorously to resuspend the cells in the residual supernatant; invert tube to check that the cell pellet has disappeared completely. This greatly facilitates cell lysis in step 4 below.

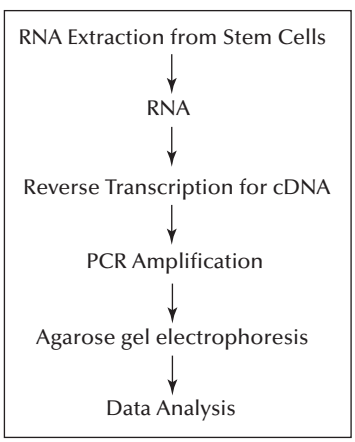

FIGURE 10.1 Two-step PCR work flow protocol.

4. Add cell lysis solution (300 μL if 1–2 million cells, 600 μL if 3–5 million cells, or 750 μL if 6–9 million cells) to the resuspended cells and pipette up and down no more than three times to lyze the cells. *Alternatively*, cultured cells that adhere to culture plates or flasks may be lysed directly on the plate or flask by first removing culture medium and then adding corresponding amount of cell lysis solution. Swirl to coverplate or flask and draw lysed cells up and down in the pipette three times before removing to a 1.5 mL microfuge tube.

Protein–DNA precipitation

1. Add protein–DNA precipitation solution (100 μL if 1–2 million cells, 200 μL if 3–5 million cells, or 250 μL if 6–9 million cells) to the cell lysate.
2. Invert tube gently 10 times and place tube into an ice bath for 5 min.
3. Centrifuge at 13 000–16 000×***g*** for 3 min. The precipitated proteins and DNA will form a tight white pellet. If the pellet is not tight, repeat centrifugation.

RNA precipitation

1. Pour the supernatant containing the RNA (leaving behind the precipitated protein–DNA pellet) into a clean 1.5 mL microfuge tube containing 100% isopropanol (2-propanol) (300 μL if 1–2 million cells, 600 μL if 3–5 million cells, or 750 μL if 6–9 million cells).
2. Mix the sample by inverting gently 50 times.
3. Centrifuge at 13 000–16 000×***g*** for 3 min; the RNA will be visible as a small, translucent pellet.
4. Pour off the supernatant and drain tube briefly on clean absorbent paper. Add 70% ethanol (300 μL if 1–2 million cells, 600 μL if 3–5 million cells, or 750 μL if 6–9 million cells) and invert the tube several times to wash the RNA pellet.
5. Centrifuge at 13 000–16 000×***g*** for 1 min. Carefully pour off the ethanol.
6. Invert and drain the tube on clean absorbent paper and allow sample to air dry 15 min.

RNA hydration

1. Add RNA hydration solution (50 μL if 1–2 million cells, or 100 μL if 3–5 million cells or 6–9 million cells).
2. Allow RNA to rehydrate for at least 30 min on ice. Alternatively, store RNA sample at –70°C to –80°C until use.
3. Before use, vortex sample vigorously for 5 s and pulse spin. Pipette sample up and down several times to ensure adequate mixing.
4. Store purified RNA sample at –70°C to –80°C.
5. Expected RNA yield ranges: 1–2 million cells (5–20 μg), 3–5 million cells (15–50 μg), and 6–9 million cells (30–90 μg).

DNase treatment and removal

The presence of genomic DNA in the PCR reaction is highly undesirable as it can interfere with primer specificity or cause a false positive signal. A kit such as Ambion's DNA-free is used to remove contaminating DNA from purified RNA to a level undetectable by RT-PCR. The following protocol is adopted from Ambion's DNA-free kit:

DNase treatment

1. Add 0.1 volume 10× DNase I buffer and 1 μL DNase I to the RNA solution.
2. Mix gently and incubate at 37°C for 20–30 min in a water bath.

DNase removal

1. Resuspend the DNase inactivation reagent by flicking or vortexing the tube. It is important to use an aliquot that is mostly white, without a significant amount of clear fluid.
2. Add 0.1 volume or 5 μL, whichever is greater, of the slurry to the sample.
3. Mix well.
4. Incubate the tube for 2 min at room temperature.
5. Centrifuge the tube at 10 000×*g* for 1 min to pellet the DNase inactivation reagent.
6. For short-term RNA storage, it is not necessary to remove the RNA solution from the pelleted DNase inactivation reagent. It can be left undisturbed when removing aliquots of RNA.

Reverse transcription

Reverse transcription generates a RNA:cDNA heteroduplex, which will be heat denatured in the subsequent PCR reaction, to allow the cDNA strand to be used as a template for polymerization.

The following protocol is adapted from Amersham's First-strand cDNA Synthesis Kit.

RNA dilution and denaturation

1. Take 1–5 μg of total RNA contained within a 20 μL volume in a 0.2 mL PCR tube.
2. Dilute RNA according to the number of cells used in the RNA extraction step: if 1–2 million cells use 5 μL of RNA solution and 15 μL of RNase-free water; if 3–5 million cells use 3.3 μL of RNA solution and 16.7 μL of RNase-free water; if 6–9 million cells use 1 μL of RNA solution and 19 μL of RNase-free water.
3. Heat the RNA sample to 65°C for 10 min in a thermocycler, then chill on ice.

Reverse transcription

1. Pipette the bulk first-strand cDNA reaction mix to obtain a uniform suspension, and add 11 μL to a 0.2 mL PCR tube.

2. To this tube add 1 μL of DTT solution, 1 μL of pd$(N)_6$ primer and the heat-denatured RNA. Pipette up and down several times to mix.
3. Incubate at 37°C for 60 min in a thermocycler.

Polymerase chain reaction

The PCR process works by using multiple cycles of template denaturation, primer annealing, and primer elongation to amplify a DNA sequence.

Primer design

Appendix 10.1 is a list of primers used for commonly assayed genes in hESC.

Careful primer design is a key step in producing a successful RT-PCR. There are a number of guidelines that are important in producing an ideal primer pair that will anneal to unique sequences that flank the target. A convenient online tool that uses nearest-neighbor analysis and calculates other useful properties of the primers is located at http://www.basic.nwu.edu/biotools/oligocalc.html.

- Genomic contamination is of primary concern in RT-PCR. It is necessary to ensure that the primers do not amplify a fragment of genomic DNA. This is achieved by designing primers that are on different exons. Primers that do not flank an intron can amplify genomic DNA and produce a false positive result.
- Both primers are calculated in the product size, which should be less than 1 kb, ideally between 100 bp and 500 bp.
- Primer length should be between 18 and 24 nucleotides. Primers made of more than 24 nucleotides do not confer greater specificity.
- Each primer should be 40% to 60% in GC content.
- The 5′and central regions should contain the majority of GC residues. The 3′end should be composed of three A's or T's within the last five nucleotides.
- Primer dimers are prevented by avoiding complementary sequences at the 3′end of the primer pairs.
- Primers should have similar melting temperatures (T_m), which are most accurately determined by nearest-neighbor analysis.
- Each primer should also be checked for its potential to form internal secondary structures such as hairpin formations. The site http://www.basic.nwu.edu/biotools/oligocalc.html contains a check against self-complementarity, which also reports potential for primer dimer formation.
- Primers should amplify only sequences in the gene of interest. To ensure that the primers will not amplify an incorrect gene, input the primer sequences in the nucleotide–nucleotide database at http://www.ncbi.nlm.nih.gov/BLAST/.

Thermocycler set-up

Before making the master mix, program the thermocycler (Table 10.1).

NOTE: For hard to detect signals such as low abundant mRNA number of cycles can be increased to 35 or 40.

TABLE 10.1 Thermocycler program

	Temperature (°C)	Segment duration (min)	Number of cycles
Initial denaturation	94	4	1
Denaturation	94	1	
Annealing	Varies[a]	1	30
Extension	72	1	
Final extension	72	7	1
Hold time	4	Hold	–

[a] The best annealing temperature for a pair of primers is determined experimentally. Start at 5°C below the estimated T_m and in subsequent reactions vary the annealing temperature in 2°C increments. If the two primers have different T_m values, the annealing temperature should be set at 5°C below the lower T_m.

TABLE 10.2 PCR mixture

Component	Volume (50 μL reaction) (μL)	Final concentration
PCR-grade water	33.2	–
10× PCR buffer ($MgCl_2$-free)	5	1×
dNTP	4	1.25 mM
$MgCl_2$ (25 mM)	3	1.5 mM
Forward primer (20 μM)	0.75	0.3 μM
Reverse primer (20 μM)	0.75	0.3 μM

TABLE 10.3 Variation of PCR mixture

dNTP	0.2–1.5 mM
$MgCl_2$	0.5–2.5 mM
Primers	0.1–0.5 μM
Taq DNA polymerase	1–5 units

Reagent set-up and cycling

1. Prepare a master mix by multiplying the amount in the "Volume" column by the desired number of reactions, plus one additional reaction. Add reagents in the order as shown in the Table 10.2.
2. Mix gently and pipette 46.7 μL master mix into a 0.2 mL nuclease-free PCR tube on ice.
3. Add 3 μL of the cDNA template.
4. Add 0.25 μL of *Taq* DNA polymerase (1.25 units).
5. Immediately place into thermocycler and begin cycling.

NOTE: The above protocol can be varied so that the following reagents have final concentrations between the corresponding ranges (Table 10.3).

TABLE 10.4 $MgCl_2$ concentration

Volume of $MgCl_2$ (25 mM) in 50 μL (μL)	Final concentration (mM)
1	0.5
2	1
3	1.5
4	2
5	2.5

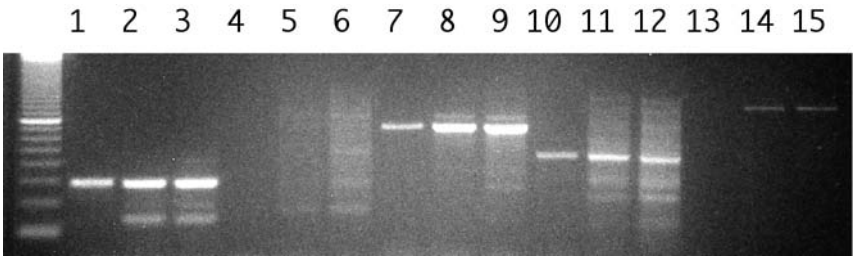

FIGURE 10.2 RT-PCR of five genes, each one done under three different magnesium concentrations (0.5 mM, 1 mM, and 1.5 mM) visualized on a 2% gel against a 100 bp ladder. Lanes 1 through 3 show mouse retina expression of *Mash1*, a retinal neurogenesis transcription factor (293 bp). Notice the appearance of unspecific product as magnesium concentration is increased from 0.5 mM (lane 1) to 1 mM (lane 2), and 1.5 mM (lane 3). One millimolar magnesium is not enough, however, for expression to be detected for the *Otx2* gene in lanes 4–6 and the *Prox1* gene in lanes 13–15. In both cases increased magnesium brings out the desired product (180 bp and 992 bp respectively), albeit with some unspecific binding for *Otx2*. The remaining lanes (7–9 and 10–12) (865 bp and 460 bp) provide more examples of how PCR is influenced by magnesium concentration.

Improving PCR specificity

It usually takes a number of trials before satisfactory PCR results are obtained. Specificity can be improved in the following ways.

Magnesium concentration

10× PCR buffers usually contain $MgCl_2$. The above protocol uses 10× PCR buffer without $MgCl_2$ to allow for a simple but powerful way of manipulating PCR specificity. Magnesium stabilizes the template and thus increases yield.

The best concentration for a set of primers is determined experimentally. The protocol described above brings the magnesium to a concentration of 1.5 mM. Table 10.4 helps in designing reactions with varying magnesium content.

The effect of magnesium concentrations on PCR specificity is shown in Figure 10.2.

Annealing temperature

Annealing temperature is a cruder method of influencing specificity, and as such it is the chosen approach when either there is no signal or there is excess of unspecific binding. Increased annealing temperature increases the specificity of the reaction and lowers unspecific binding. Decrease annealing temperature when signal is either weak or absent.

TABLE 10.5 Problems with RT-PCR

Problem	Solution	Methods
Unspecific binding	↑ Specificity	↑ Annealing temperature ↓ Magnesium
Low or absent signal	↓ Specificity	↓ Annealing temperature ↑ Magnesium

Table 10.5 summarizes what action to take when confronted with PCR specificity issues. Both temperature and magnesium can be varied at the same time.

Remember that in practice, especially when working with new primer sets, a few trials would be necessary before the best experimental conditions are determined.

Product visualization

A standard procedure of gel electrophoresis is used to visualize RT-PCR.

Agarose gel preparation

As noted previously, optimal length of a RT-PCR product is between 100 and 500 bp. Gels can be between 1.5 and 2.0% agarose.

1. Prepare 1 L of TAE (Tris/acetic acid/EDTA) working buffer by adding 20 mL of 50× buffer concentrate to 980 mL of deionized water.
2. To make a 2% gel, add 2 g of agarose powder to 100 mL of working TAE buffer, and heat in a microwave oven until completely melted.
3. Add 5 μL of ethidium bromide (final concentration should be 0.5 μg/mL). *Caution:* Ethidium bromide is a highly cancerous substance. Exercise extreme care to avoid contact.
4. Swirl to facilitate ethidium bromide distribution in the solution. When no traces of ethidium bromide are seen (red color) and the solution has cooled to about 60°C, pour into a casting tray and place a gel comb.
5. When gel has solidified, place it in the gel apparatus with the wells closer to the negative polarity. Pour enough TAE working buffer to just cover the top of the wells.

Sample preparation

1. If ready-to-use dye is unavailable dissolve 20 g of Ficoll into 100 mL of water and add enough Orange G to obtain a dark orange color.
2. On parafilm, combine 5 μL of dye to 2 μL of a marker (ladder). To facilitate transfer, add 20 μL of TAE working buffer, mix, and deposit into the first well.
3. The most efficient method of combining sample and dye is to add 5 μL of dye into the 50 μL of PCR product. Vortex to obtain a uniform color and transfer 20 μL of mixture to wells.

Gel running and visualization

1. Place apparatus lid and adjust voltage to 100 V.
2. Running time depends on gel length. Use the dye band as a guide for reproducibility.
3. Use an ultraviolet light source and photograph. *Caution:* Avoid looking at a UV source without protection.

Alternative Procedures

One-step RT-PCR

The above protocols involve setting-up (Table 10.6) and running the RT and PCR reactions separately. A faster and easier alternative method is offered in one-step protocols such as Invitrogen's "SuperScript III One-Step RT-PCR System" (Figure 10.3).

1. Add the reagents (Table 10.7) to a 0.2 mL, nuclease-free, PCR tube on ice. For multiple reactions, prepare a master mix to minimize reagent loss and enable accurate pipetting.

Table 10.6 Thermocycler settings

	Temperature (°C)	Segment duration	Number of cycles
cDNA synthesis	55	30 min	1
Initial denaturation	92	2 min	1
Denaturation	94	15 s	
Annealing	Varies[a]	30 s	40
Extension	68	40 s	
Final extension	68	5 min	1
Hold time	4	Hold	–

[a] Annealing temperature is specific to each primer used. Refer to the literature references for proper temperatures.

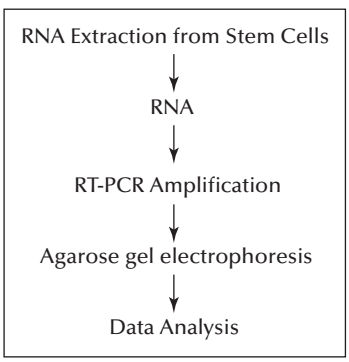

Figure 10.3 One-step PCR work flow protocol.

TABLE 10.7 PCR mixture

	Volume (50 μL reaction) (μL)	Final concentration
PCR-grade water	22.25	–
2× Reaction mix	25	1×
Forward primer (20 μM)	0.25	0.1 μM
Reverse primer (20 μM)	0.25	0.1 μM
RNA	0.25	1–3 ng[a]
SuperScript III RT/platinum *Taq* mix	2	–

[a] Template RNA can have concentrations from 0.01 pg to 1 μg.

2. Verify absence of genomic DNA in RNA samples by omitting the SuperScript III RT/Platinum *Taq* Mix and substituting 1.25 units of *Taq* DNA polymerase in the reaction.

PITFALLS AND ADVICE

- It is ideal to treat each RNA preparation with RNase-free DNase to avoid genomic DNA contamination. Even the best RNA extraction methods yield some genomic DNA. Of course, it is ideal to have primers not amplifying genomic DNA at all but sometimes this may not be possible.
- For optimal results, the reagents (before the preparation of the PCR mix) and the PCR mixture itself (before loading) should be vortexed and mixed well. Otherwise there may be shifting R_n values during the early (0–5) cycles of PCR. It is also important to add probe to the buffer component and allow it to equilibrate at room temperature prior to reagent mix formulation.

EQUIPMENT

- Micropipettors: 10, 20, 200 μL
- Aspirator
- Vortexer
- Microcentrifuge
- Thermal cycler such as from Perkin Elmer (Shelton, CT, USA)
- Agarose gel electrophoresis apparatus
- Centrifuge with 50 mL conical rotor.

Supplies and Reagents

- 0.2 mL PCR tubes
- 1% agarose in TBE
- 1.5 mL microcentrifuge tubes
- 10 mL Trizol (BRL catalog no. 15596-018)
- 100% isopropanol
- 10× PCR buffer ($MgCl_2$-free)
- 18 gauge needle
- 1× TBE buffer
- 2 mL chloroform (Sigma catalog no. C2432)
- 30 mL syringe
- 50 mL conical tube
- 70% ethanol diluted from 95%, not 100%
- dNTP
- Ethidium bromide
- Kits: PURESCRIPT RNA Purification Kit from Gentra Systems (Minneapolis, MN, USA); DNA-free Kit from Ambion (Austin, TX, USA), First-strand cDNA Synthesis Kit from Amersham (Piscataway, NJ, USA)
- $MgCl_2$ (25 mM)
- Micropipette tips, filtered. 2 and 200 μL
- PCR-grade water
- RNase-free water (i.e. DEPC treated)
- RNeasy Maxi Kit (QIAgen catalog no. 75162)
- Sterile, RNase-free 0.65 mL microfuge tubes
- Taq DNA polymerase.

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APPENDIX 10.1 PRIMERS FOR RT-PCR

Gene-specific forward and reverse primers can be designed using freely available web-based software (Primer3, Netprimer, Beacon Designer, mFold and Oligonucleotide Properties Calculator, e.g. http://www.basic.nwu.edu/biotools/oligocalc.html) and their specificity can be analyzed by alignment to the GenBank sequence (http://www. ncbi.nlm.nih.gov/BLAST).

Gene abbreviation	Gene name	Primers (forward/reverse)	Reference
ACTB	Actin, beta	CACCCTGAAGTACCCCATCGAGCA/ CAGGTCTTTGCGGATGTCCACGTCAC	Abeyta *et al.*, 2004
ACTB	Actin, beta	TGGCACCACACCTTCTACAATGAGC/ GCACAGCTTCTCCTTAATGTCACGC	Richards *et al.*, 2003
ACTB	Actin, beta	TGACGGGGTCACCCACACTGTGCCCATCTA/ CTAGAAGCATTGCGGTGGACGATGGAGGG	Vodyanik, *et al.*, 2005
ACTB	Actin, beta	TGGCACCACACCTTCTACAATGAGC/ GCACAGCTTCTCCTTAATGTCACGC	Xu *et al.*, 2005
ACVR2B	Activin A receptor, type IIB	ACACGGGAGTGCATCTACTACAACG/ TTCATGAGCTGGGCCTTCCAGACAC	Gonzalez *et al.*, 2006
AFP	Alpha-fetoprotein	ACTGCAATTGAGAAACCCACTGGAGATG/ CGATGCTGGAGTGGGCTTTTTGTGT	Abeyta *et al.*, 2004
AFP	Alpha-fetoprotein	AGAACCTGTCACAAGCTGTG/GACAGCAAGCTGAGGATGTC	Henderson, *et al.*, 2002
ALPL	Alkaline phosphatase, liver/bone/kidney	ATTGTGACCACCACGAGAGTGAAC/ ACGTCAATGTCCCTGATGTTATGC	Kren *et al.*, 1997
BIRC5	Baculoviral IAP repeat-containing 5(survivin)	GCATGGGTGCCCCGACGTTG/GCTCCGGCCAGAGGCCTCAA	Kamihira *et al.*, 2001
BMP4	Bone morphogenetic protein 4	GTGAGGAGCTTCCACCACGA/ACTGGTCCCTGGGATGTTCTC	D'Amour *et al.*, 2005

CD247	CD3zeta	CTCTGCCTCCCAGCCTCTTT/GCGTCGTAGGTGTCCTTGGT	Vodyanik *et al.*, 2005
CD3D	CD3delta	TTCCGGTACCTGTGAGTCAGC/GGTACAGTTGGTAATGGCTGC	Vodyanik *et al.*, 2005
CD3E	CD3epsilon	AGTTGGCGTTTGGGGGCAAGATGGTAATGAAGAAA/ CCCAGGAAACAGGGAGTCGCAGGGGGACTGGAGAG	Vodyanik *et al.*, 2005
CD3G	CD3gamma	GGGCTGCTCCACGCTTTTGC/TTTTCCCCAATAGGTGGCGC	Vodyanik *et al.*, 2005
CD79A	CD79a molecule, immunoglobulin-associated alpha Mb-1	TCCAAGCTCTGCCTGCCACCAT/GACTGCTGGTATGACTCGTTGC	Vodyanik *et al.*, 2005
CDX1	Caudal homeobox domain 1	TCAGAGCTGGCTGCCAATC/TGGAACCAGATCTTCACCTGC	D'Amour *et al.*, 2005
CDX2	Caudal homeobox domain 2	GGGCTCTCTGAGAGGCAGGT/CCTTTGCTCTGCGGTTCTG	D'Amour *et al.*, 2005
CDX2	Caudal homeobox domain 2	GAACCTGTGCGAGTGGATGCG/GGTCTATGGCTGTGGGTGGGAG	Gonzalez *et al.*, 2006
CDX2	Caudal homeobox domain 2	CCTCCGCTGGGCTTCATTCC/TGGGGGTTCTGCAGTCTTTGGTC	Matin *et al.*, 2004
CER	Cerberus 1	ACAGTGCCCTTCAGCCAGACT/ACAACTACTTTTTCACAGCCTTCGT	D'Amour *et al.*, 2005
CGB	Chorionic gonadotropin	CAGGGGACGCACCAAGGATG/GTGGGAGGATCGGGGTGTCC	Henderson *et al.*, 2002
CGB	Chorionic gonadotropin	ATGGGCGGGACATGGGCATCCA/GGCCCCGGGAGTCGGGATGG	Matin *et al.*, 2004
CGB	Chorionic gonadotropin, beta polypeptide	TGAGATCACTTCACCGTGGTCTCC/TTTATACCTCGGGGTTGTGGGG	Xu *et al.*, 2005
CGB5	Chorionic gonadotropin, beta polypeptide 5	AAGGATGGAGATGTTCCAGGG/CCATGTCCCGCCCATG	D'Amour *et al.*, 2005
CXCR4	Chemokine (C-X-C motif) receptor 4	CACCGCATCTGGAGAACCA/GCCCATTTCCTCGGTGTAGTT	D'Amour *et al.*, 2005
CYCG	Cyclophilin G	CTTGTCAATGGCCAACAGAGG/GCCCATCTAAATGAGGAGTTGGT	D'Amour *et al.*, 2005
DMNT3B	DNA methyltransferase	CGAAAGGATGTTTGGCTTTCC/ACCTTCCCAGCAGCTTCTG	Skottman *et al.*, 2006

(*Continued*)

Gene abbreviation	Gene name	Primers (forward/reverse)	Reference
DNMT3B	DNA methyltransferase	CTCTTACCTTACCATCGACC/CTCCAGAGCATGGTACATGG	Gonzalez *et al.*, 2006
DUSP6	Dual specificity phosphatase 6	GCTGTGGCACCGACACAGT/ACTCGCCGCCCGTATTCT	Skottman *et al.*, 2006
ECAD	E-Cadherin (epithelial)	AGGAATTCTTGCTTTGCTAATTCTG/CGAAGAAACAGCAAGAGCAGC	D'Amour *et al.*, 2005
EOMES	Eomesodermin homolog	ACCCCCTTCCATCAAATCTC/CCATGCCTTTTGAGGTGTCT	Skottman *et al.*, 2006
FABP2	Fatty acid binding protein	TGGTTGATTTTCCATCCCAT/TACTGGGCCAGGAATTTGAC	Jaiswal *et al.*, 2000
FGF4	Fibroblast growth factor 4	GACACCCGCGACAGCCT/TCACCACGCCCCGCT	D'Amour *et al.*, 2005
FGF4	Fibroblast growth factor 4	CTACAACGCCTACGAGTCCTACA/ GTTGCACCAGAAAAGTCAGAGTTG	Henderson *et al.*, 2002; Richards *et al.*, 2003
FGF8	Fibroblast growth factor 8	CGACCCCTTCGCAAAGCT/GGACTCGAACTCTGCTTCCAAA	D'Amour *et al.*, 2005
FLT3	Flk-2 fms-related tyrosine kinase 3 stem cell tyrosine kinase 1	ATGCACGGCATCTGGGAATC/GCTACTGTCCTGCAAGTTGCTGTC	Kaufman *et al.*, 2001
FOXA1	Forkhead box A1 HNF3alpha	CCAAGCCGCCTTACTCCTACA/CGCAGATGAAGACGCTGGAGA	Xu *et al.*, 2005
FOXA2	Forkhead box A2/ hepatocyte nuclear factor 3, beta	GGGAGCGGTGAAGATGGA/TCATGTTGCTCACGGAGGAGTA	D'Amour *et al.*, 2005
FOXF1	Forkhead box F1	GCCGAGCTGCAAGGCA/AACTCCTTTCGGTCACACATGC	D'Amour *et al.*, 2005
FST	Follistatin	GTAAGTCGGATGAGCCTGTCTGT/CAGCTTCCTTCATGGCACACT 5′-Probe-3′ for TaqMan: CCAGTGACAATGCCACTTATGCCAGC	Skottman *et al.*, 2006
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	ACCACAGTCCATGCCATCAC/TCCA CCACCCTGTTGCTGTA	Clark *et al.*, 2004

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	GTTCGACAGTCAGCCGCATC/GGAATTTGCCATGGGTGGA 5′-Probe-3′ for TaqMan: ACCAGGCGCCCAATACGACCAA	Skottman *et al.*, 2006
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	TCCAAAATCAAGTGGGGCGAT/TTCTAGACGGCAGGTCAGGTC	Vodyanik *et al.*, 2005
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	GAGTCCACTGGCGTCTTCAC/CTCAGTGTAGCCCAGGATGC	Xu *et al.*, 2005
GATA1	GATA binding protein 1	CTCCCTGTCCCCAATAGTGC/GTCCTTCGGCTGCTCCTGTG	Vodyanik *et al.*, 2005
GATA2	GATA binding protein 2	AGCCGGCACCTGTTGTGCAA/TGACTTCTCCTGCATGCACT	Kaufman *et al.*, 2001
GATA2	GATA binding protein 2	GCTTCCCTCTCTGAAATAGCCGA/ CAGAATCTAAGCTCGGGACACGTT	Vodyanik *et al.*, 2005
GATA4	GATA binding protein 4	CATCAAGACGGAGCCTGGCC/TGACTGTCGGCCAAGACCAG	Gonzalez *et al.*, 2006
GATA6	GATA binding protein 6	GAGCACCAATCCCGAGAACA/GCGAGACTGACGCCTATGTAGA 5′-Probe-3′ for TaqMan: CCCATCTTGACCCGAATACTTGAGCTCG	Skottman *et al.*, 2006
GATA6	GATA binding protein 6	CCATGACTCCAACTTCCACC/ACGGAGGACGTGACTTCGGC	Gonzalez *et al.*, 2006
GCM1	Glial cells missing homolog 1	GTGGACCCCATGAAGCTCTA/GCAGTGATCCAAACCCAAGT	Matin *et al.*, 2004
GDF3	Growth differentiation factor 3	AGACTTATGCTACGTAAAGGAGC/ CTTTGATGGCAGACAGGTTAAAGTA	Clark *et al.*, 2004
GSC	Goosecoid	GAGGAGAAAGTGGAGGTCTGGTT/CTCTGATGAGGACCGCTTCTG	D'Amour *et al.*, 2005
GUSB	Glucoronidase beta	ACGCAGAAAATATGTGGTTGGA/GCACTCTCGTCGGTGACTGTT	D'Amour *et al.*, 2005
HBZ	Hemoglobin-zeta	CTGACCAAGACTGAGAGGAC/ATGTCGTCGATGCTCTTCAC	Henderson *et al.*, 2002
HESX1	Homeobox, ES cell expressed 1	GGATTTCATTCCCTAGCGTGG/GTGATTCTCTATGGGACCTTTTC	Richards *et al.*, 2003
HNF4A	Hepatocyte nuclear factor 4, alpha	GCTTGGTTCTCGTTGAGTGG/CAGGAGCTTATAGGGGCTCAGAC-	Gonzalez *et al.*, 2006

(*Continued*)

Gene abbreviation	Gene name	Primers (forward/reverse)	Reference
IL6ST	Interleukin 6 signal transducer (gp130, oncostatin M receptor)	GCCTCAACTTGGAGCCAGATT/ GTTTAAGGTCTTGGACAGTGAATGAAG 5′ -Probe-3′ for TaqMan: CTCCTGAAGACACAGCATCCACCCGA	Skottman *et al.*, 2006
INHBA	Inhibin, beta A, activin A, activin AB alpha polypeptide	CTTGAAGAAGAGACCCGAT/CTTCTGCACGCTCCACTAC	Gonzalez *et al.*, 2006
KDR	Kinase insert domain receptor FLK1, VEGFR2	AAGGTGACAGGAAAAGACGAACT/TCCCCTCCATTGGCCCGCTTAAC	Abeyta *et al.*, 2004
KDR	Kinase insert domain receptor FLK1, VEGFR2	ACTTTGGAAGACAGAACCAAATTA/TCTCTGGGCACCATTCCACCA	D'Amour *et al.*, 2005
KDR	Kinase insert domain receptor FLK1, VEGFR2	ATGCACGGCATCTGGGAATC/GTCACTGTCCTGCAAGTTGCTGTC	Vodyanik *et al.*, 2005
KRT7	Keratin 7, SCL	ATGGTGCAGCTGAGTCCTCC/TCTCATTCTTGCTGAGCTTC	Vodyanik *et al.*, 2005
LHX1	LIM homeobox 1	TCCCCAATGGTCCCTTCTC/CGTAGTACTCGCTCTGGTAATCTCC	D'Amour *et al.*, 2005
LIFR	Leukemia inhibitory factor receptor alpha	ACTGTGGAAGATATAGCTGCAGAAGA/ CACTGTTGCTGTCTATGGATCTAGGA 5′ -Probe-3′ for TaqMan: ATAAAACTGCGGGTTACAGACCTCAGGCC	Skottman *et al.*, 2006
LIN28	Lin-28 homolog	AGTAAGCTGCACATGGAAGG/ATTGTGGCTCAATTCTGTGC	Gonzalez *et al.*, 2006
LPL	Lipoprotein lipase	ATGGAGAGCAAAGCCCTGCTC/TACAGGGCGGCCACAAGTTTT	Jaiswal *et al.*, 2000
MEOX1	Mesenchyme homeo box 1	AGGCGGAGAAAGGAGAGTTCAG/CTCCGGCTTCCCTCTGTTC	D'Amour *et al.*, 2005
MIXL1	Mix1 homeobox-like 1	CCGAGTCCAGGATCCAGGTA/CTCTGACGCCGAGACTTGG	D'Amour *et al.*, 2005
NANOG	Nanog homeobox	CAGCTGTGTGTACTCAATGATAGATTT/ CAACTGGCCGAAGAATAGCAATGGTGT	Clark *et al.*, 2004

NANOG	Nanog homeobox	GGCAAACAACCCACTTCTGC/TGTTCCAGGCCTGATTGTTC	Gonzalez *et al.*, 2006
NANOG	Nanog homeobox	TGATTTGTGGGCCTGAAGAAAA/GAGGCATCTCAGCAGAAGACA	Noaksson *et al.*, 2005
NANOG	Nanog homeobox	TGCAGTTCCAGCCAAATTCTC/ CCTAGTGGTCTGCTGTATTACATTAAGG 5′ -Probe-3′ for TaqMan: TCCAAAGCAGCCTCCAAGTCACTGG	Skottman *et al.*, 2006
NANOG	Nanog homeobox	AATACCTCAGCCTCCAGCAGATG/CAAAGCAGCCTCCAAGTCACTG	Xu *et al.*, 2005
NCAD	N-Cadherin (neuronal)	CCCACACCCTGGAGACATTG/GCCGCTTTAAGGCCCTCA	D'Amour *et al.*, 2005
NCAM1	Neural cell adhesion molecule 1	AGGAGACAGAAACGAAGCCA/GGTGTTGGAAATGCTCTGGT	Abeyta *et al.*, 2004
NCAM1	Neural cell adhesion molecule 1	ATGGAAACTCTATTAAAGTGAACCTGA/ TAGACCTCATACTCAGCATTCCAGT	Clark *et al.*, 2004
NEUROD1	Neurogenic differentiation 1	AAGCCATGAACGCAGAGGAGGACT/AGCTGTCCATGGTACCGTAA	Xu *et al.*, 2005; Henderson *et al.*, 2002
NKX2–5	NK2 transcription factor related, locus 5	TGCAGAAGGCAGTGGAGCTGGACAAGCC/ TGCACTTGTAGCGACGGTTCTGGAACCAG	Xu *et al.*, 2005
NODAL	Nodal homolog	CCGAGGGCAGACATCATCC/CCATCCACTGCCACATCTTCT	D'Amour *et al.*, 2005
PAX6	Paired box gene 6	CGTCCATCTTTGCTTGGGAAATC/GAGCCTCATCTGAATCTTCTCCG	Xu *et al.*, 2005
POU5F1	POU domain, class 5, transcription factor 1 OCT4	CTGCAGTGTGGGTTTCGGGCA/CTTGCTGCAGAAGTGGGTGGAGGA	Abeyta *et al.*, 2004
POU5F1	POU domain, class 5, transcription factor 1 OCT4	GAGAACAATGAGAACCTTCAGGA/TTCTGGCGCCGGTTACAGAACCA	Amit *et al.*, 2004
POU5F1	POU domain, class 5, transcription factor 1 OCT4	GAAACCCACACTGCAGCAGA/CACATCCTTCTCGAGCCCA	Carpenter *et al.*, 2004

(*Continued*)

Gene abbreviation	Gene name	Primers (forward/reverse)	Reference
POU5F1	POU domain, class 5, transcription factor 1 OCT4	ACATCAAAGCTCTGCAGAAAGAACT/ CTGAATACCTTCCCAAATAGAACCC	Clark *et al.*, 2004
POU5F1	POU domain, class 5, transcription factor 1 OCT4	TGGGCTCGAGAAGGATGTG/GCATAGTCGCTGCTTGATCG	D'Amour *et al.*, 2005
POU5F1	POU domain, class 5, transcription factor 1 OCT4	CGTGAAGCTGGAGAAGGAGAAGCTG/ CAAGGGCCGCAGCTTACACATGTTC	Gonzalez *et al.*, 2006
POU5F1	POU domain, class 5, transcription factor 1 OCT4	TCTGCAGAAAGAACTCGAGCAA/AGATGGTCGTTTGGCTGAACAC 5′-Probe-3′ for TaqMan: CCTCTTCTGCTTCAGGAGCTTGGCAA	Skottman *et al.*, 2006
POU5F1	POU domain, class 5, transcription factor 1 OCT4	GGGAAGGTATTCAGCCAAACG/GGTTCGCTTTCTCTTTCGGG	Xu *et al.*, 2005
PPARG	Peroxisome proliferative activated receptor, gamma	GCTGTTATGGGTGAAACTCTG/ATAAGGTGGAGATGCAGGTTC	Jaiswal *et al.*, 2000
PTCRA	Pre T-cell antigen receptor alpha	AGTACACAGCCCATGCATCTGTCA/ AATGCTCCAAGACTGGAGGAAGGA	Vodyanik *et al.*, 2005
PTPRC	Protein tyrosine phosphatase, receptor type, C, CD45	TTCAACTTATACCCTTCGTGTC/CCTGCTTTACTTTGTCCACTTC	Vodyanik *et al.*, 2005
RTN4	Reticulon 4, Nogo-A	TCAGAAATGGGATCATCGTTCA/GAGCTTCCACTGCAACTCTCT	Wang and Seed, 2003
SDF1	Stromal cell-derived factor 1	TGACCCGAAGCTAAAGTGGATTCCTCTCACATCTTGAACCTCTTGTT	D'Amour *et al.*, 2005
SERPINE1	Serpin pepidase inhibitor (nexin, plasminogen activator inhibitor 1)	TCCAGTTTTGTCCCAGATGA/ATCGAGGTGAACGAGAGTGG	Skottman *et al.*, 2006

SOX1	SRY (sex determining region Y)-box 1	ATGCACCGCTACGACATGG/CTCATGTAGCCCTGCGAGTTG	D'Amour *et al.*, 2005
SOX1	SRY (sex determining region Y)-box 1	CTCACTTTCCTCCGCGTTGCTTCC/ TGCCCTGGTCTTTGTCCTTCACC	Henderson *et al.*, 2002
SOX17	SRY (sex determining region Y)-box 17	GGCGCAGCAGAATCCAGA/CCACGACTTGCCCAGCAT	D'Amour *et al.*, 2005
SOX17	SRY (sex determining region Y)-box 17	CGCACGGAATTTGAACAGTA/GGATCAGGGACCTGTCACAC.	Gonzalez *et al.*, 2006
SOX17	SRY (sex determining region Y)-box 17	CGCACGGAATTTGAACAGTA/CACACGTCAGGATAGTTGCAG	Skottman *et al.*, 2006
SOX2	SRY (sex determining region Y)-box 2	CCGCATGTACAACATGATGG/CTTCTTCATGAGCGTCTTGG	Gonzalez *et al.*, 2006
SOX7	SRY (sex determining region Y)-box 7	ACGCCGAGCTCAGCAAGAT/TCCACGTACGGCCTCTTCTG	D'Amour *et al.*, 2005
SPP1	Secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)	CTAGGCATCACCTGTGCCATACC/ CAGTGACCAGTTCATCAGATTCATC	Jaiswal *et al.*, 2000
STELLAR	Germ and embryonic stem cell enriched protein STELLA	GTTACTGGGCGGAGTTCGTA/TGAAGTGGCTTGGTGTCTTG	Clark *et al.*, 2004
SULF1	Sulfatase 1	TCTTGGGGAGCTGAATAGGA/TGTAAGACCTCACCAAGTTCTGA	Skottman *et al.*, 2006
SYCP1	Synaptonemal complex protein 1	AAGATTTACAGATAGCAACAAACACA/ AATCTTTGCTGTTCTGTTCTCAATAA	Clark *et al.*, 2004
T	T, Brachyury	TGCTTCCCTGAGACCCAGTT/GATCACTTCTTTCCTTTGCATCAAG	D'Amour *et al.*, 2005
T	T, Brachyury	AACCCAACTGTGGAGATGATGCAG/ AGGGGCTTCACTAATAACTGGACG	Xu *et al.*, 2005
TAL1	T-cell acute lymphocytic leukemia 1	ATGGTGCAGCTGAGTCCTCC/TCTCATTCTTGCTGAGCTTC	Kaufman *et al.*, 2001

(*Continued*)

Gene abbreviation	Gene name	Primers (forward/reverse)	Reference
TBP	TATA binding protein	TGTGCACAGGAGCCAAGAGT/ATTTTCTTGCTGCCAGTCTGG	D'Amour *et al.*, 2005
TBX5	T-box 5	AGCACTTCTCCGCTCACTTC/CCGTGCACAGAGTGGTACTG	Skottman *et al.*, 2006
TDGF1	Teratocarcinoma-derived growth factor 1, Cripto	TGAGCACGATGTGCGC/TTCTTGGGCAGCCAGGTG	Carpenter *et al.*, 2004
TERT	Telomerase reverse transcriptase	CGGAAGAGTGTCTGGAGCAA/GGATGAAGCGGAGTCTGGA	Richards *et al.*, 2003
THY1	Thy-1 cell surface antigen, CD90	GACCCGTGAGACAAAGAAGC/GCCCTCACACTTGACCAGTT-3′	Lung *et al.*, 2005
UTF1	Undifferentiated embryonic cell transcription factor 1	GGCACCTGGGCGACATC/TCCACGTGCTGGTTCAAGGT 5′-Probe-3′ for TaqMan: AACATCCTGGGCCCGCTGCG	Skottman *et al.*, 2006
VPREB1	Pre-B lymphocyte gene 1	TTTGTCTACTGCACAGGTTGTGG / TGCAGTGGGTTCCATTTCTTCC	Vodyanik *et al.*, 2005
WNT1	Wingless-type MMTV integration site family, member 1	CCTCCTACCTGGGGACTCCT/CAGTGGAAGGAAATACTGAT	Walsh and Andrews, 2003
WNT2B	Wingless-type MMTV integration site family, member 2B	TGAGTGGTTCCTGTACTCTG/ACTCACACTGGGTAACACGG	Walsh and Andrews, 2003
ZFP42	Zinc finger protein 42 homolog Rex1	GCGTACGCAAATTAAAGTCCAGA/ CAGCATCCTAAACAGCTCGCAGAAT	Henderson *et al.*, 2002; Richards *et al.*, 2003
ZFP42	Zinc finger protein 42 homolog Rex1	CCTGGAGGAATACCTGGCATTG/TCTGAGGACAAGCGATTGCG	Xu *et al.*, 2005
ZFP42	Zinc finger protein 42 homolog Rex1	CCTGGAGGAATACCTGGCATTG/TCTGAGGACAAGCGATTGCG	Xu *et al.*, 2005
ZIC1	Zinc finger protein of the cerebellum 1	CTGGCTGTGGCAAGGTCTTC/CAGCCCTCAAACTCGCACTT	D'Amour *et al.*, 2005

CHAPTER

11

Gene Expression Profiling of Stem Cells by Microarray

Timothy McDaniel, Shawn Baker, Roy Williams, Franz-Josef Mueller, and David Barker

INTRODUCTION

The most fundamental questions in human embryonic stem cell (hESC) research concern how pluripotence and differentiation are controlled. One hope is that large-scale studies comparing the gene expression of hESCs to their differentiated progeny will lead to insights about these processes. Gene expression of undifferentiated hESCs has been investigated by a variety of techniques including MPSS (massively parallel signature sequencing), SAGE (serial analysis of gene expression), EST (expressed sequence tag) scans, and hybridization-based technologies such as focused cDNA arrays and genome-wide microarray platforms.

Currently, the most accessible, comprehensive, and reliable technology is the gene expression microarray. Microarray technology has a short history. Before the human genome was sequenced, the most reliable approach was to use the two-color competitive hybridization approach pioneered at Stanford University by Pat Brown and his colleagues in 1995. This approach relies on hybridization of fluorescently labeled double-stranded cDNA made from the mRNA of the samples of interest to cDNA collections that are spotted onto glass slides. Because of the requirement for a large

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ISBN: 978-0-1237-0465-8.

collection of correctly sequenced cDNAs to use as the probes, the competitive hybridization technique has not been successfully commercialized, and after the human genome was sequenced, it became more straightforward to use shorter oligonucleotides as probes. In the early 1990s, Affymetrix introduced the use of photolithography to synthesize short oligonucleotides that could be used in groups to represent transcript sequences. Agilent used inkjet technology to synthesize longer oligonucleotides (50–70 mers), and Illumina uses 50 mers for its bead-based platform. Hybridization of amplified transcripts continues to be the basis of these popular oligonucleotide-based methods.

Large-scale expression surveys are still vulnerable to criticism for being descriptive rather than hypothesis-driven. A counter-argument to this criticism is the growing evidence that a well-designed study that compares the activity of tens of thousands of markers (transcripts) across the samples under investigation can identify relevant genes or pathways that would have been missed by a search for markers based on more limited, mechanistic, hypotheses.

This chapter covers using microarrays to study gene expression in embryonic stem cells. Most laboratories use service providers or departmental core labs to carry out microarray experiments, so we focus on the design and analysis of array experiments that are necessary to guarantee meaningful biological results.

Overview

Microarray technology offers a unique combination of features that makes it well suited for most expression studies:

- It is comprehensive, allowing the monitoring of every annotated transcript in the human (or mouse) genome.
- It requires relatively little sample, approximately 100 ng (~10 000 cells) of total RNA for the most sensitive arrays.
- It is inexpensive compared with other comprehensive approaches, such as SAGE and MPSS, which cost thousands of dollars per sample. This economic advantage enables comparisons of large numbers of samples to account for biological variation among ESC lines and ensures that appropriate statistics can be applied to assess the significance of the findings.

Microarrays at the whole-genome scale are available from a variety of manufacturers. Although the details of formatting differ from platform to platform, all commercial arrays use immobilized oligonucleotides with sequences complementary to the sequence of a targeted RNA transcript. Most of these platforms also use the same labeling method, developed by James Eberwine in the 1990s (see Figure 11.2). The instruments and laboratory paraphernalia required to perform an experiment can cost tens to hundreds of thousands of dollars, so most laboratories that are not dedicated to large-scale gene expression projects generally have their RNA samples analyzed at commercial microarray service labs or academic core facilities, of which there are dozens throughout the world.

In this chapter we briefly describe the techniques used in these experiments, and offer more detail for the considerations that should be employed in designing and analyzing good microarray experiments. We take this approach because the precise details vary among the myriad platforms, and these details are generally covered in protocol manuals that come with the purchase of the arrays. Further, since the actual sample labeling and array handling tend to be carried out by core labs, the matter of concern for most researchers who make use of microarrays is how to appropriately design experiments and interpret their data.

PROCEDURES

Design of microarray experiments

As a first step, designing successful experiments to analyze stem cells by microarray requires consideration of their biological characteristics. While most stem cell researchers understand these issues, those who are not familiar with microarray analysis may be tempted to read more into the data that it actually reveals. Will a microarray study give you the information you seek? The concepts outlined below have been useful for us in the design of experiments. At the risk of sounding too preachy, we want to point out that these rather abstract considerations have profound practical consequences for the researcher designing microarray-based studies.

Have the stem cells been well characterized?

The cell preparation should be characterized as well as possible prior to any microarray experiment. Characterization of stem cells should include but not be limited to the experimental proof of self-renewal and differentiation potential, as well as the characterization of appropriate stem cell-associated transcripts and epitopes ("stem cell markers") by means of RT-PCR and antibody-based detection methods. Stem cells are defined operationally as a cell type that has the potential to self-renew and differentiate into more mature cell types. Although much effort has been invested in finding "stem cell markers," there is not yet a single or set of markers that defines stem cells exclusively. In practical terms, all stem cell markers that have been reported can serve as a means to enrich for cells with stem cell characteristics but not define unequivocally a stem cell a priori.

How heterogeneous is the population?

If only 0.1% of the cells in the population you are studying have the properties you are interested in, a microarray study will only confuse the issue by giving you information that will be impossible to interpret. It would be better to use the funds allocated for microarrays to better understand the biological characteristics of the population, using, for example, fluorescence activated cell sorting (FACS) or biological assays.

What question do you want to answer?

The design of a microarray experiment for biological questions can be a challenging task. We urge restraint in use of microarray data to make sweeping statements; a list of genes that are significantly up- or downregulated in a careful characterized experimental system

can serve as an excellent starting point for *in vivo*, *in vitro*, or *in silico* experiments to prove or disprove a biological hypothesis but will never replace the follow-up experiments. Most of the transcriptional profiling studies conducted by stem cell biologists so far revolve around two questions:

1. What are the differences/commonalities of certain stem cell preparations? In 2002 two large studies published in *Science* introduced a neologism into the scientific community that is still sometimes used: "stemness" (Ivanova *et al.*, 2002; Ramalho-Santos *et al.*, 2002). The studies asked: What transcribed genes do different stem cell preparations have in common and hence could be defined as the essence of a stem cell? Due to different sample selection and study design, there was no common set of "stemness" genes identified. Only one gene (integrin α6) was found to be shared in these studies and a similar follow-up study published a year later in *Science* (Fortunel *et al.*, 2003). Does this mean that α 6-integrin is the ultimate stemness gene? The data support this conclusion, but it is obviously simplistic. Unfortunately the reaction to the lack of consensus in these studies has been to blame microarray technology rather than to focus on the variations in interpretation and evaluation of the data.
2. How do stem cell populations change over time or as they differentiate? Since the first studies were published in 2001 (Loring *et al.*, 2001), several publications have compared gene expression in ESCs and their differentiated derivatives in an effort to identify genes that are associated with the pluripotent state and to try to understand what guides particular pathways of differentiation. In many cases, undifferentiated ESCs have been compared with embryoid bodies, which are formed as an aggregation step often used to induce differentiation. The challenge with such an analysis is to pick the right time points for the experiment. For example, while mouse ESCs are well differentiated after 10 days as embryoid bodies, at the same time point embryoid bodies derived from hESCs still strongly resemble undifferentiated hESCs. If microarray analysis were performed on each day of the 10-day period, it would yield interesting results for mouse cells, but would be a disappointment for human cells. Thus, because of the considerable investment in resources, it is essential to plan carefully for a time course experiment, using other markers or assays such as immunohistochemistry to choose the most appropriate time points for a large-scale microarray analysis. Because cultures are intrinsically variable, it is also essential that any time course have multiple, independent biological replicates. Always have replicates. *Always* have replicates.

Preparation of samples

The many steps involved in a microarray experiment are summarized in Figure 11.1. More detail on each step is shown below.

RNA isolation

Isolation of total RNA or polyadenylated RNA from the biological specimen is usually performed with commercial kits, produced, for example, by Ambion, Inc. and Qiagen.

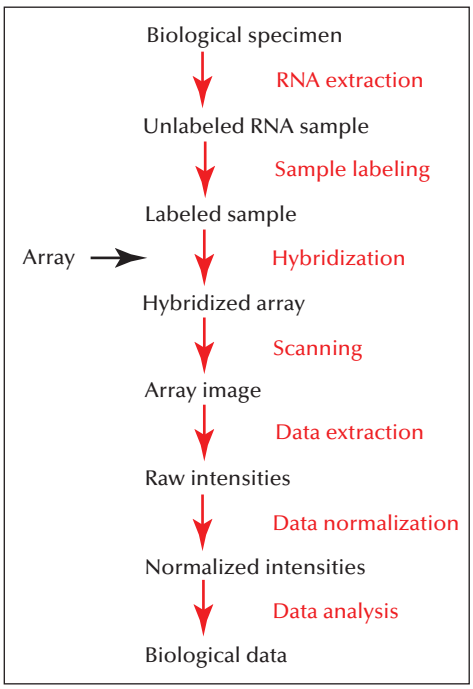


FIGURE 11.1 Steps of a microarray experiment. States of matter or information are shown in black, processes in red.

RNA labeling

This step is usually done by one of two general methods. In one method, RNA is converted to single-stranded cDNA using reverse transcriptase and a labeled nucleotide, which is incorporated into the cDNA during the reaction. This labeled cDNA is then used as the target hybridized to the array. The second technique involves converting RNA to cDNA in a reaction using no label. The primer used in the cDNA reaction is an oligo(dT) fused to a bacteriophage T7 promoter. T7 polymerase is then used to direct transcription from the incorporated promoter, driving the production of RNA from each cDNA. This reaction amplifies the product several hundred to >1000-fold. A labeled nucleotide is included during this reaction, providing the signal that is detected upon array hybridization. The first procedure offers the advantage of simplicity, while the second offers the advantage of amplification of product, which can be a significant benefit when starting materials are limited.

Hybridization to microarray/array washing

Arrays are generally hybridized overnight and washed the next day. The buffers and timing of the steps vary by manufacturers. All sell the buffers either as part of their array kits or as separate packages.

Scanning

A confocal scanning fluorescence microscope is used. These instruments are the most costly component of an array experiment, costing tens to hundreds of thousands of dollars. Scanning generally takes a few minutes per array.

Data extraction

The pixels corresponding to array elements are identified by software, which then extracts hybridization signals, generating a table of values for each gene. For most systems this is a fully automated process that takes no more than a few minutes.

Data processing and normalization

The extracted pixels are condensed to a single value for each transcript, often incorporating background subtraction or other data processing algorithms. Array signals are often normalized within an experiment to even out differences in overall intensity or other technical variation.

Data analysis

The data that is generated can be analyzed in many ways, using software as simple as spreadsheets to enterprise-wide array database and analysis systems. Currently certain standards in microarray analysis are emerging. This topic is too broad to survey adequately here, although we do cover some general guidelines for analysis below. For further reading we would recommend starting with the recent and excellent review by Allison and colleagues (2006).

Most biologists want to start with a "hit" list of up- or downregulated genes in their different experimental conditions. This approach is straightforward and freely available software packages for this type of question are available and have become accepted as standard for certain applications.

In this context, we suggest significance analysis for microarrays (SAM) as an excellent tool to obtain lists of genes that are up- or downregulated within a given dataset. The advantage of SAM is that it provides the experimenter not only with significance levels for results but also with sample size assessment – estimates of false discovery rate (FDR), false negative rate (FNR), type I error and power for different sample sizes and other features (see the SAM website: http://www-stat.stanford.edu/~tibs/SAM/).

Pitfalls and Advice

Do not rely on fold differences

A cautionary note to new users of microarray technology is that the popular method of using the ratios ("fold differences") to guess what genes might be significantly differentially regulated is no longer acceptable for publication of the data in most journals. The fold difference idea originated in the competitive hybridization studies of the late 1990s. Since this technique compared hybridization signals of two samples labeled with different fluorochromes, the output was a ratio of expression levels rather than a number based on arbitrary detection units. When single-color approaches later took hold, there was a tendency to use the same ratio approach to express differential expression. Instead of the ratio of expression, simple statistical analysis has now become the norm for discovering differences in gene expression.

Design in a sanity check

Differences detected in an microarray analysis can be caused by unnoticed variables, such as which technician produced the samples, and it is vital to include a "sanity check" in the design of the experiment. As an illustration, we present an experiment conducted recently in our laboratory as an example of how to go about the analysis of an array experiment. In our study 32 RNA samples were analyzed. These represented a variety of ES and ES-like teratocarcinoma lines, with each cultured in an undifferentiated state or differentiated to embryoid bodies, fibroblast-like feeders, neuronal lines, or other differentiated states. The samples were labeled by the Method 2 shown in Figure 11.2 and each was hybridized to a separate microarray containing >24 000 elements corresponding to human genes.

Figure 11.3 shows a dendrogram in which the 32 samples are classified based on the correlations of the hybridization intensities of the elements of the arrays. As shown in the diagram, the arrays correctly classify the samples based on their differentiation state, despite the fact that the cell lines had different laboratories of origin, culturing conditions, technician, etc. This would imply that the arrays are identifying expression differences that truly reflect the biological differences between undifferentiated stem cells and their differentiated derivatives, as opposed to technical variation induced by differences in laboratory technique.

Sanity checks such as these are very important, as there are many steps that intervene between the biological specimen and the microarray signal. Variation in any of these steps can introduce artifacts that may be confused for biological differences. In the design of an array experiment, it is important to bear these potential confounding variables in mind, and, wherever possible, design the sample processes such that the technical steps are scrambled with respect to the biological groups. For example, it would be a poor design to label the RNA from the undifferentiated cells on one day and the differentiated cells on another. It would be better to label the samples side by side on the same day, or, if one had more samples than one could handle in a single day, representatives from both sample types should be included in each batch of processing.

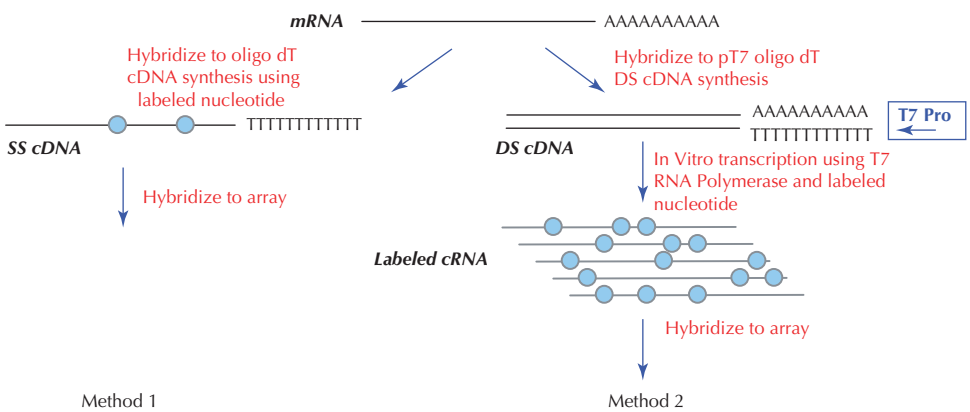


FIGURE 11.2 Labeling methods used in microarray experiments. Method 2 is the technique used most widely on all major commercial array platforms.

Checks such as the one shown in Figure 11.3 are an important first step in analysis, and, if samples cluster primarily according to some technical variable instead of biological classification it is best to redesign the study and start over. This implicitly raises an important reason why a relatively inexpensive technique is required in such studies: they allow the researcher to include an adequate number of samples to ensure that observed differences stem from differences in biology as opposed to some irrelevant technical difference. The section on statistics gives a further example of why adequate numbers are needed to draw valid conclusions.

Always include replicates

It cannot be overemphasized that if you plan to use only one replicate for a microarray experiment, it would better to spend your money on something else. Ideally, for statistical purposes, each sample should have five replicates to eliminate most sources of error. Practically, even when costs of arrays are modest, the costs of multiple replicates of experiments may be prohibitive. As a rule of thumb, always include two biological replicates for each microarray analysis. Biological replicates can be two wells of a culture dish, or two completely separate experiments. Technical replicates, which are multiple

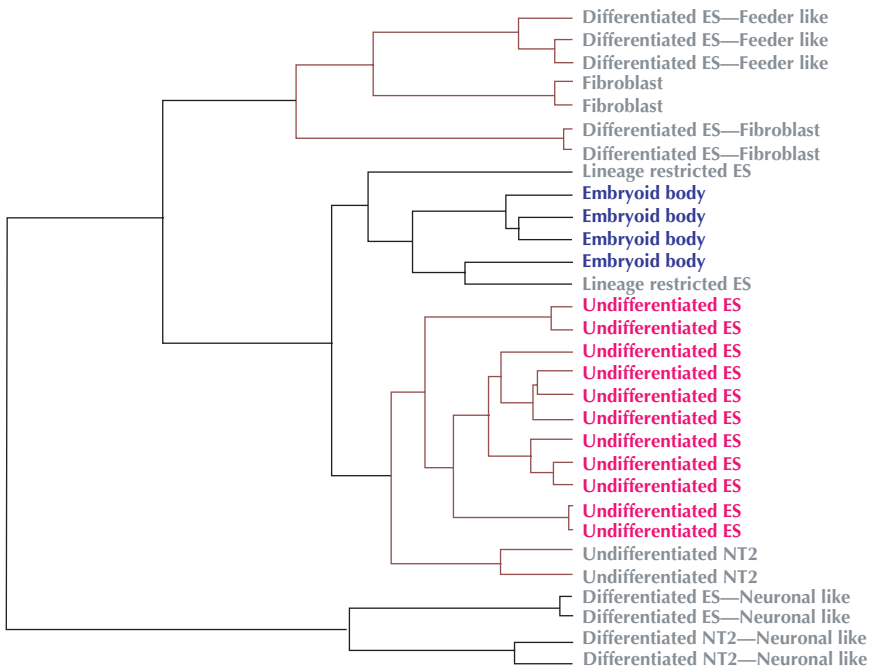


FIGURE 11.3 Relatedness of samples based on microarray data. Thirty-two RNA samples derived from embryonic stem cells, their differentiated progeny or other differentiated cell lines were analyzed on separate whole-genome gene expression arrays. The correlations of their gene expression intensity signals were used to generate this dendrogram, with more highly correlated samples clustering more closely on the tree. Note that all undifferentiated ESCs form a single cluster (red), as do the embryoid bodies (blue). Checks such as this, which establish that the arrays are differentiating samples based on biological differences, are a first step in analyzing array data.

analyses of the same biological sample, are generally unnecessary if the microarray facility has competent support.

Use statistics, not guesswork

Once initial analysis to validate the data is completed, the experimenter is ready to begin probing the data for the biological lessons. A popular analysis is the expression scatter plot (Figure 11.4A). In these plots each dot represents a transcript and the axis values are microarray hybridization signal intensities for that transcript, with each axis representing a different sample (or the averaged values for several samples from the same biological category). If all genes were expressed at the same levels in both sample types, then the points would lie on a perfect 45-degree angle. Thus, the

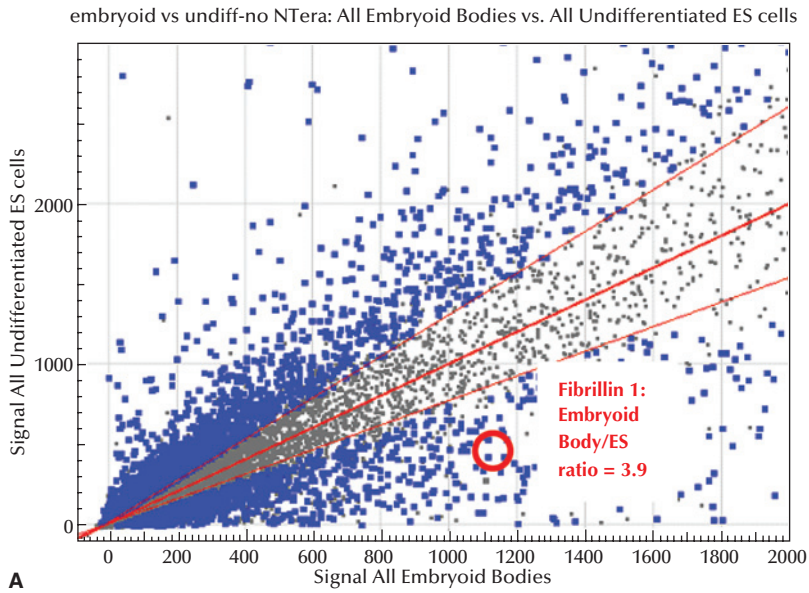
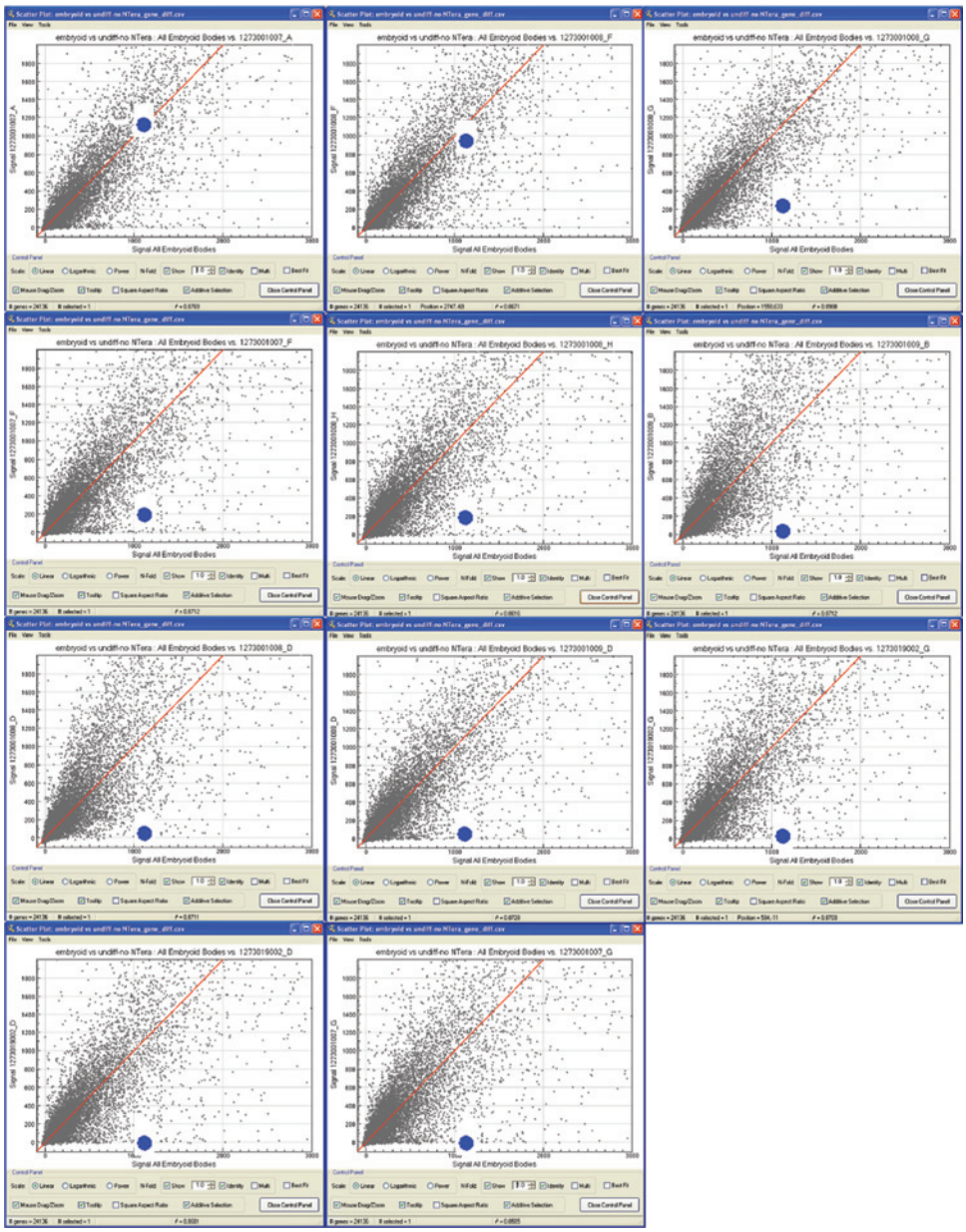


FIGURE 11.4 The difference between fold intensity change and statistical significance. (A) Scatter plot for the averaged intensities of all transcripts for the 11 undifferentiated ESC cultures (Y axis) versus the four embryoid body cultures (X axis) described in Figure 11.3. Transcripts showing statistically significant differential expression between the two categories ($P < 0.01$ based on a *T*-test) are represented by blue points, whereas those not showing statistical significance are gray. Note that many of the gray transcripts show, on average, a large difference in expression intensities between the two conditions, but are still not significantly significant. For example, the circled gene, fibrillin 1, shows a ratio of 3.8, but is still considered insignificant by the statistical test. The reason for this apparent discrepancy can be seen in B (overleaf), in which the 11 ESC hybridizations that are averaged together in A are broken out into the individual components. In six of the 11 ESC samples, there is little or no expression of fibrillin. However, three ESC samples show appreciable expression and two of the samples (upper left) show virtually no difference between the ESC samples and the averaged embryoid body data. Therefore, ESCs do not consistently show minimal expression of fibrillin compared with embryoid bodies, despite the large average difference. This inconsistency is why the difference did not meet statistical significance. The data illustrate why statistical analysis is needed for array data and why it is good to design experiments with several representatives of each biological condition.



B

FIGURE 11.4 (Continued)

plots can be used to easily visualize genes that are differentially expressed between the categories, as these will be outliers from the midline. In the example shown, the X and Y axes correspond to the averaged values of the 11 undifferentiated ESC samples and four differentiated into embryoid bodies.

A common but flawed way to extract the genes with the most significant changes is to sort them by the hybridization signal ratios from the two categories and select some cutoff (often two- or three-fold) above which changes are considered significant. The

problem with this approach is that, either due to technical or biological variation, there can be great differences in the expression of a given gene even within samples belonging to the same category. If these differences are large enough, they can lead to false conclusions unless one applies statistics.

The leading microarray manufacturers provide statistical tests as part of their analysis software, and you should always request statistical analysis from your core lab or service provider. In Figure 11.4 the points are color coded such that gene expression differences that pass statistical significance for being differentially expressed ($P < 0.001$ using a Mann–Whitney statistical test) are shown in blue, those failing in gray. Note that there are multiple examples of genes showing an average difference exceeding three-fold that fail the test, as well as many showing subtler fold changes that pass. The reason for this can be seen if one takes a closer look at one of these genes where statistics contradict the naïve conclusion: the fibrillin 1 transcript, which shows an average increase in expression of 3.8 in differentiated cells compared to ESCs, but fails the significance test. If one instead compares all 11 undifferentiated ESC samples to the average value from the embryoid bodies, the reason for the lack of statistical significance is clear (Figure 11.4B): in two of the 11 ESC samples the gene shows nearly equal expression between the two cell types, even though the remaining nine lines show a great difference.

At the risk of being repetitive, we note that at least three related lessons can be gleaned from this:

1. It is important to apply statistics when analyzing array data.
2. In order to satisfy point (1), one must analyze adequately large numbers of samples.
3. In order to satisfy point (2), one must have a technology that is inexpensive enough to make biological replicates affordable.

Equipment

Commercially available human whole-genome arrays are listed below. Each also requires hybridization cartridges, reagents, scanners and software, which are also provided by the suppliers. All of these manufacturers also make whole-genome arrays for mouse.

Item	Supplier	Catalog no.
Human-6 V2 BeadChip	Illumina	BD-25-101
Human Genome U133 Plus 2.0 Array	Affymetrix	900470
Human Genome Survey Microarray V2.0	Applied Biosystems	4359029
CodeLink Human Whole Genome	GE Healthcare	300026-6PK
Whole Human Genome Oligo Microarray	Agilent	G4112A

Supplies and Reagents

These vary from system to system. Unless one has a good reason for doing otherwise, one should use the labeling and hybridization methods recommended by the chip

manufacturer. This not only ensures that you will be using a method that has gone through extensive testing on your platform, but that you can receive customer support if the experiment encounters technical problems.

Quality Control Methods

Most array manufacturers make available unlabeled oligonucleotides for spiking into samples at know concentrations, labeled oligonucleotides and other kinds of controls to aid in troubleshooting failed hybridizations. However, the most important types of controls should be included by the experimenter in the design of the study. These controls include replicates at three levels of the experiment:

1. *Biological replicates:* For each category in your experiment (e.g. ESCs, embryoid bodies) include multiple samples. The samples should contain representatives that originate from different animals or donors, different labs, different cultures, etc. For example, if ESCs are being compared plus or minus treatment X, it is not enough to have one representative of each. If differences are seen between the individual samples, one does not know if X is responsible, or if it is merely the fact that they were different cultures. If, on the other hand, replicates of untreated and treated are included, the variation across cultures can be assessed independent of treatment and, therefore, any differences consistently seen in the treated but not in the untreated can be trusted. The number of replicates needed depends on the magnitude of change you wish to detect. If only a few replicates are included, only large changes in expression will prove statistically significant, while with more samples more subtle changes can be detected.
2. *Labeling replicates:* Using the same input RNA, do two separate labeling reactions and hybridize each to a different array. Establishing the correlation between the two samples will reveal how much variation originates from the labeling procedure.
3. *Hybridization replicates:* Using two aliquots of the same labeled RNA, perform two separate hybridizations. Comparing the hybridization replicates will indicate the reproducibility of the arrays themselves.

In a successful array experiment, the level of variation should be least in the hybridization replicates, followed by labeling replicates, followed by biological replicates. If not, one should be concerned that unintended technical variation is swamping biological differences, which will decrease the sensitivity to detect relevant changes and increase false positive rates.

Reading List

Microarray methods

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Microarray analysis method

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Stem cell microarray analysis

These papers are the first reports of analyzing stem cells using microarrays.

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CHAPTER

12

Generation of Human Embryonic Stem Cell-derived Teratomas

Robin L. Wesselschmidt

Introduction

When pluripotent embryonic stem cells (ESCs) are injected into immune-compromised mice they form teratomas, which are tumors made of cells and organized foci of complex tissues derived from all three germ layers. The generation of human ESC (hESC)-derived teratomas is an important technique used to assess the pluripotency of a hESC line and should be part of the routine evaluation of cell lines in culture as well as a critical step in the validation of newly derived lines or genetically altered hESC clones. For a detailed description of teratomas and photomicrographs of typical hESC-derived teratomas, see Chapter 13.

Overview

All procedures with animals should be performed under the appropriate institutional review and oversight. This chapter will describe several methods used to generate hESC-derived teratomas in immunodeficient mice. All of the strains of severe combined immunodeficient (SCID) mice listed below have been used successfully for the xenograft of hESCs.

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Each procedure described requires the injection of hESC into various sites in SCID mice, monitoring of mice for anywhere from 5 to 12 weeks, and harvest and analysis of the tumor. At least two animals are transplanted per culture or cell line to be tested. Injection under the testis and kidney capsule are major survival surgeries and require training and expertise, however these methods can be very effective in the production of teratomas and may offer an advantage in that fewer cells are required to seed the xenograft than for intramuscular injection.

Whichever method is chosen, the transplanted hESC should be representative of the culture to be tested.

We describe several methods for producing teratomas in mice:

1. Graft in the rear leg or thigh muscle
2. Graft under the testis capsule
3. Graft under the kidney capsule.

Strains of SCID mice

- C.B-17-Prkdc^{scid}
- NOD/SCID/γ_c^{null} (NOD/ShiJic-*scid* with γ_c^{null})
- SCID-beige mice (C.B-*Igh-1*^{b} GbmsTac-*Prkdc*^{scid}-*Lyst*^{bg}N7.

PROCEDURES

Xenograft into the rear leg or thigh muscle

A healthy hESC culture typical in morphology and growth characteristics of the line to be tested is harvested at the time of routine passage.

Cell preparation

Cells from a single well of a six-well dish are mechanically dissociated into clumps of 50–100 cells each. Collect the clumps in a 15 mL conical tube, wash twice with PBS and resuspend in about 100 μL of DMEM.

Mice

Two mice/cell line are injected with test cells:

1. Inject about 100 μL of cell suspension into the lower leg or thigh muscle using a 1 cc syringe and a 27G, ½ inch needle:
   - 1×10^6 cells, in lower leg muscle
   - 5×10^6 cells into the thigh muscle.
2. Observe the animal daily for changes in appearance and behavior. Monitor the injection site for tumor growth for 8–12 weeks.

3. When the tumor is palpable (about 5 mm in size), euthanize the mouse and remove the tumor.
4. Collect the tumor in PBS, wash twice and fix in 10% neutral buffered formalin.
5. Embed the tumor in paraffin, section to 5 μm, fix to slides, and stain with hematoxylin and eosin.
6. A pathologist should evaluate the sectioned tissue to identify the types of tissues present (see Chapter 13).

Xenograft into testis capsule

A healthy hESC culture of typical morphology and growth characteristics of the line or culture to be tested is harvested at the time of routine passage.

Cell preparation

Undifferentiated hESCs are collected by manually dissecting colonies. Cells are collected in clumps of about 200–400 cells: 10–15 clumps are injected per testis.

Surgery

Surgery requires review and approval of animal use protocols, specialized training of personnel, and planning prior to initiating the surgical procedure.

Mice

Two male SCID mice for each cell sample to be tested.

1. Prepare an appropriate surgical location where survival surgery can be performed aseptically. Assemble sterile surgery instruments and post-operative materials, such as clean cage and heating pad or lamp to aid in post-operative recovery.
2. Anesthetize the mouse: Weigh the mouse and inject intraperitoneally with 0.15–0.17 mL of 2.5% avertin/g body weight. Allow anesthesia to take effect, about 5 min.

NOTE: Check for reflex by gently squeezing the rear paw and monitoring response. When the mouse is under anesthesia, it will not withdraw its paw and breathing will be slow and shallow.

3. Place the mouse on its back on the prepared surface.
4. Shave the lower abdomen.
5. Swab shaven area with 70% ethanol or Betadine surgical scrub solution.
6. Make a small (1–2 cm) incision in the lower abdomen at the height of the knees through the skin and the abdomen wall.
7. Find the fat pad attached to the testis and, grasping the fat pad with blunt forceps, gently pull the testis out through the incision.
8. Clamp the fat pad with a serrefine clamp to hold the testis in place.

9. Under a dissecting microscope, carefully inject 50 μL of cell suspension under the testis capsule using a pulled glass micropipette (10–15 clumps of 200–400 cells/testis).
10. Place the testis back into the abdomen.
11. Suture the abdomen wall.
12. Close the skin with wound clips.
13. Place the animal in a pre-warmed, clean cage for post-operative observation.
14. Observe the animal daily for changes in appearance and behavior. Monitor the injection site for tumor growth for 8–12 weeks.
15. Remove wound clips after wound has healed, 7–10 days following surgery.
16. When the tumor is palpable, about 5 mm in size, euthanize the mouse and remove the tumor.
17. Collect the tumor in PBS, wash twice and fix in 10% neutral buffered formalin.
18. Embed the tumor in paraffin, section to 5 μm, fix to slides, and stain with hematoxylin and eosin.
19. A pathologist should evaluate the sectioned tissue to identify types of tissues present (see Chapter 13).

Xenograft under kidney capsule

NOTE: The following procedure is adapted from that described in Hogan *et al.* (1994).

A healthy hESC culture typical in morphology and growth characteristics of the cell line to be tested is harvested at the time of routine passage.

Cell preparation

Undifferentiated hESCs are collected by manually dissecting colonies. Cells are collected in clumps of about 200–400 cells: 10–15 clumps are injected per kidney capsule.

Surgery

This is major surgery that requires approval of animal use protocols, specialized training of personnel and planning prior to initiating the surgical procedure.

Mice

Two male SCID mice per cell sample to be tested.

1. Prepare an appropriate surgical location where survival surgery can be performed aseptically. Assemble sterile surgery instruments and post-operative materials, such as clean cage and heating pad or lamp to aid in post-operative recovery.

2. Anesthetize the mouse as described for testes capsule transfer with intraperitoneal injection of 0.6–0.7 mL of Avertin/30–35 gm mouse.

3. Shave a patch on the back of the lower right side of the mouse, right of the spine and caudal from the middle of the rib cage.

4. Place the animal in the lid of a 10 cm petri dish so it can be rotated as necessary without having to reposition the animal.

5. Swab the area with 70% ETOH or betadine solution.

6. Using high quality, sharp, sterile dissecting scissors make a small (1 cm) incision in the skin starting at the last rib in the direction of the hip and just to the right of the spine.

7. Put the animal on the stage of a stereo-dissecting microscope 4×–10× objectives and incident (fiber-optic) light source.

8. Looking through the small incision, find the kidney, which looks like a small solid dull red mass.

9. Make a 0.5–0.7 cm incision in through the muscle and peritoneal cavity exposing the kidney.

10. Using a pair of blunt forceps, gently grasp the fat pad on the rostral end of the kidney and pull the kidney out of the peritoneum.

11. If the incision is not too large, the kidney may not need further immobilization, however if the kidney is not stable resting against the outer body wall, it can be immobilized using a pair of Desmarres chalazion forceps.

12. Allow the exposed kidney to air dry for about 1 minute.

13. Using 2 pairs of watchmaker's forceps, gently pick up the membrane surrounding the kidney with one of the forceps, making a tent in the membrane where it is not laying against the kidney. With the other pair of forceps, poke a hole in the "tented" membrane by piercing the membrane with closed forceps and then allowing the tines to open slightly causing a small tear in the membrane.

14. Now pick up the pipette containing the hESCs and gently insert the transfer pipette into the opening, far enough away from the opening that the cells will remain in the capsule when the pipette is drawn. Slowly expel the cells and withdraw the pipette.

15. Release the membrane from the forceps.

16. Gently push the caudal end of the kidney back in to the body and the rest of the kidney should slip back in to the body.

17. Close the body wall with two stitches of absorbable suture.

18. Close the skin with staples.

19. Place the animal in a clean, warm cage for observation.

20. Remove the stitches in 7–10 days.

21. The tumor size should be checked regularly by palpation.

22. Euthanize the animal when the tumor is about 5 mm in size. Fix and embed tumor as described for testes capsule transplantation.

Tumor biopsy

Animals are observed daily when the tumor reaches about 5 mm in diameter, 6–12 weeks following injection, the animals are euthanized and tumors are harvested for analysis.

Typically, the mouse is euthanized and the tumor is dissected and fixed in 10% buffered formalin. Then it is embedded in paraffin, 5 μm sections are made and attached to slides that are stained with hematoxylin and eosin.

Tumor analysis

Tumors are usually sent out for processing and analysis by a pathologist, but it is worthwhile to try to identify the tissues yourself. Using the illustrations in Chapter 13, it should be possible to identify neural tube, gut, muscle, epithelial (retinal) pigment cells, and cartilage. A wide distribution of structures such as these means that the cells have differentiated into multiple derivatives and were therefore pluripotent before transplant. Although it is common in publications to state that all three embryonic germ layers are represented in a teratoma, this is a naïve assessment of the rich complexity of the tissues and structures that differentiate. Ectoderm, for example, gives rise to neural tissue and skin, but also the neural crest, which forms bone, cartilage, peripheral nervous system, and melanocytes.

Pitfalls and Advice

Cells

- The transferred cells should be representative of the culture, should be actively dividing and in log phase when harvested for transplant.
- A majority of the cells die upon transplant, especially when intramuscular xenografts are made. This may be less of a problem for transfer to testis and kidney capsule.

Animals

Monitoring tumor growth

Check with your Institutional Animal Care and Use Committee (IACUC) for institutional rules and guidelines governing tumor production.

In general, the animals should be observed daily for signs of distress and should be euthanized before the tumor causes debilitation of the animal.

Things to look for when observing the animals:

- Change in body weight: any change in food and water intake
- External physical appearance: scruffy, lack of grooming, hunched posture; ulcerated, enlarged tumor that interferes with movement
- Observable clinical signs: labored breathing, abnormal discharges, diarrhea
- Change in behavior: excessive sleeping, self-inflected trauma
- Change in behavior responses to external stimuli: aggression or unresponsiveness.

Avertin decomposition test

Avertin decomposes over time. There is a simple test to help assess whether or not it is time to make fresh anesthetic and avoid unnecessary discomfort for the animals.

1. Add one drop of Congo Red (0.1% w/v) to 5 mL avertin solution.
2. Observe color of solution. If a purple color develops, it indicates that the pH is below 5, indicating the decomposition of avertin to dibromoacetic aldehyde and hydrobromic acid. If this occurs, the solution should be discarded.

NOTE: Solutions should be tested once per month throughout storage.

EQUIPMENT

- Animal balance
- Animal clippers (for shaving fur prior to surgery)
- Stereo dissecting microscope
- Fiber-optic lamp
- Heating pad, slide warmer, or heating lamp to warm cages for post-op recovery
- Hot bead sterilizer (or other method to sterilize surgical instruments during surgery).

SUPPLIES AND REAGENTS

- Mice: SCID mice, Jackson Laboratory, male, 6–8 weeks old.
- Cells: Actively growing hESC cultures.

Surgical instruments and supplies

Item	Supplier	Catalog no.	Alternative
Micro dissecting scissors	Roboz	RS-5882	Many
Operating Scissor 5″	Roboz	RS-6806	Many
Serrefine clamp	Roboz	RS-5470	Many
Fine, blunt forceps "thumb dressing"	Roboz	RS-8102	Many
Watchmaker's forceps no. 5	Roboz	RS-4905	Many
Desmarres Chalazion forceps	Shileds/Dina	C62-2810	
Silk suture, size 5–0, with size 10 curved needle	Roboz	SUT-1074-21	Many
Wound applicator	Roboz	RS-9250	Many
Wound clips	Roboz	RS-9255	Many
Wound clip removing forceps	Roboz	RS-9263	Many
2,2,2-Tribromoethanol	Aldrich Chemical	T4,840-2	
2-Methyl-2-butanol (tertiary amyl alcohol)	Fisher Scientific	A-394-500	Many
100% Ethanol (absolute, 200 proof)	Fisher Scientific	AC61509-0020	Many
70% Ethanol	Henry Schein	1028715	Many
1 mL syringe with a 27G 13 mm needle	Fisher Scientific	14-826-87	BD 309623
Betadine surgical scrub	Henry Schein	6900581	
Glass capillary pipettes	Fisher Scientific	K 71900-50	Kimble Catalog no. 71900-50
Formalin, neutral phosphate buffered 10%		Fisher Scientific	SF100-4

Recipes

Avertin, 2.5% (100 mL)

Component	Amount	Final concentration
2,2,2-Tribromoethanol	2.5 g	2.5%
2-Methyl-2-butanol (tertiary amyl alcohol)	5 mL	
Distilled water	95 mL	

1. Add tribromoethanol to butanol and dissolve by gently heating and shaking tube.
2. Heat solution, briefly to ~50°C.
3. Add distilled water and continue to stir until butanol solution is totally dispersed.
4. Sterile filter through a 0.22 μm filter.
5. Aliquot into storage containers.
6. Store at 4°C.

Solution should be maintained in either brown glass or foil-covered bottles and refrigerated. Failure to protect from light or to store above at 4°C will result in the decomposition of tribromoethanol and the formation of irritants that can cause peritonitis and death.

Before use, warm to 37°C and shake well to make sure that there are no crystals remaining in the solution before injection.

Reading List

Reviews

Przyborski SA (2005). Differentiation of human embryonic stem cells after transplantation in immune-deficient mice. *Stem Cells* 23: 1242–1250.

Teratoma production in hind leg muscle

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM (1998). Embryonic stem cell lines derived from human blastocysts. *Science* 282: 1145–1147.

Xu C, Inokuma MS, Denham J, Golds K, Kundu P, Gold JD, Carpenter MK (2001). Feeder-free growth of undifferentiated human embryonic stem cells. *Nat Biotechnol* 19: 971–974.

Teratoma production in thigh muscle

Richards M, Fong CY, Tan S, Chan WK, Bongso A (2004). An efficient and safe xeno-free cryopreservation method for the storage of human embryonic stem cells. *Stem Cells* 22: 779–789.

Teratoma production in testis capsule

Rubenoff BE, Pera MF, Fong C-Y, Trouson A, Bongso A (2000). Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. *Nat Biotechnol* 18: 399–404.

Tissue transplant in the kidney capsule

Hogan B, Beddington R, Constantini F, Lacy E (1994). *Manipulating the Mouse Embryo*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Press.

Tam PL (1993). Histogenic potency of embryonic tissues. In: *Methods in Enzymology, Vol 225: Guide to Techniques in Mouse Development*. Edited by PM Wasserman and ML DePamphitis. CA: Academic Press.

Animal welfare web resources

Animal Welfare Information Center, US Department of Agriculture (www.nal.usda.gov/awic).

Institutional Animal Care and Use Committee (www.iacuc.org).

Pathology laboratories

IDEXX Laboratories, Preclinical Research Services, 2825 KOVR Drive, West Sacramento, CA 95605, USA (preclinicalresearch@idexx.com).

CHAPTER

13

Characterization of Human Embryonic Stem Cell-derived Teratomas

Ivan Damjanov and Lars Ahrlund-Richter

INTRODUCTION

Human embryonic stem cells (hESCs) are developmentally pluripotent as shown by their ability to differentiate into various somatic cell types *in vitro*. The differentiation abilities of hESC lines can be much further explored, however, by injecting them into immunodeficient mice, where they differentiate into highly organized tissues that resemble the structures found in fully developed organs.

Tumors produced from xenografted hESCs are called *teratomas*, in analogy with the human tumors spontaneously developing in the ovary, testis, and even in extragonadal sites. Most human teratomas are benign tumors of limited growth potential and are typically composed of mature somatic tissue derived from all three germ layers: the ectoderm, mesoderm, and endoderm. Typical recognizable structures in teratomas include hair follicles, respiratory epithelium, adipose tissue, and neurons.

In addition to these benign teratomas, which account for most of teratoid tumors in humans, some tumors contain immature fetal tissues and are called *immature teratomas*. Immature teratomas may grow indefinitely and spread to other tissues and are thus considered potentially malignant. Finally, it is important to realize that some teratomas behave like malignant tumors, especially those that are found in the testis and some extragonadal sites. These malignant teratomas, also known as *teratocarcinomas*,

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non-seminomatous germ cell tumors or *malignant mixed germ cell tumors*, contain undifferentiated malignant embryonic stem cells called *embryonal carcinoma cells*. Embryonal carcinoma cells are truly malignant cells in that they are capable of metastasizing and killing the host. These cells can be propagated indefinitely *in vitro*, retaining their undifferentiated phenotype as well their developmental pluripotency.

Human embryonal carcinoma cells (ECCs) are equivalent to the stem cells of murine teratocarcinomas, which are also called ECCs. Mouse embryonic stem cells (mESC) are a non-neoplastic equivalent of murine ECC. Likewise, it is thought that the hESCs derived from human blastocysts are the non-neoplastic equivalents of human ECCs that have been identified and isolated in form of permanent cell lines from human teratocarcinomas.

The generation of mESC- and hESC-derived teratomas has been used extensively as a tool to test the cells' developmental potential. Most of the tumors produced from ESCs have the appearance of benign teratomas. However, a considerable number of the tumors are histologically classified as immature teratomas, predominantly because of the prominence of immature fetal neural tissue within the tumors. The fact that hESCs form teratomas is the source of much concern about the safety of their application in clinical cell replacement therapies.

Teratomas produced from xenografted hESCs are an important quality assurance method for the developmental potential of hESCs. Currently there are no systematic studies on such xenografts and thus it is not possible to tell whether all existing hESC lines have the same developmental potential when injected *in vivo* to produce teratomas. Further studies under controlled and standardized conditions might answer that question as well as determine the significance of the persistent immature tissue and the foci of persistent undifferentiated hESCs in some of the grafts.

Overview

This chapter focuses on recognition and identification of the tissues arising from hESCs in experimental teratomas.

For experimental teratoma production, hESCs are typically transplanted to skeletal muscle, testes, or kidney capsule of immunodeficient mice (see Chapter 12). Teratomas are usually harvested 6–7 weeks after the injection of hESCs and are histologically composed of tissues derived from all three germ layers. The histology of teratomas is remarkable; the teratoma tissues often have the microscopic features of equivalent adult human somatic tissues, or fetal.

Most notably, many of the neural components of hESC-derived teratomas have the features of fetal neural tubes and immature brain tissue. An organoid arrangement of tissues may be seen occasionally but overall organogenesis is relatively uncommon in teratomas derived from hESCs. Most hESC teratomas do not contain undifferentiated hESCs, but occasionally foci of hESCs can be found in some xenografts. The significance of prolonged immaturity of neural components and the persistence of undifferentiated hESCs in some xenografts remains to be determined.

PROCEDURES

Detailed methods of producing teratomas from hESCs are described in Chapter 12. This chapter presents typical microscopic features of hESC-derived mature and immature teratomas through a series of histological sections of representative tumors. An overview of identifiable tissues and examples is outlined below. Details about the individual histological sections are provided in the figure legends.

Microscopic features of hESC-derived teratomas

Overall, the most common tissues found in hESC-derived teratomas in immunodeficient mice are mesenchymal tissues and neural tissue.

Mesenchymal tissues

Mesenchymal tissue may be present in the form of loose or dense connective tissue, cartilage, smooth and striated muscle cells, fat cells and occasionally bone. Cartilage is most readily recognized as it occurs in the form of discrete aggregates of chondroid cells surrounded by hyaline matrix (Figures 13.1 and 13.2). Such foci of cartilaginous differentiation are usually distributed at random and may be adjacent to other mesenchymal derivatives, ectodermal derivatives such as neural tissue or choroid plexus, or endodermal derivatives such as mucin-secreting glands.

Mesenchymal tissues may be composed of nondescript spindle cells, corresponding to fetal or adult connective tissue (Figure 13.3). Within such condensed mesenchymal tissue one may focally see areas of cartilage, bone, or striated muscle formation.

With the use of immunohistochemistry, such foci of abortive differentiation can be better visualized. For example, by using antibodies to desmin and myoglobin one may see foci of striated muscle differentiation (Figure 13.4).

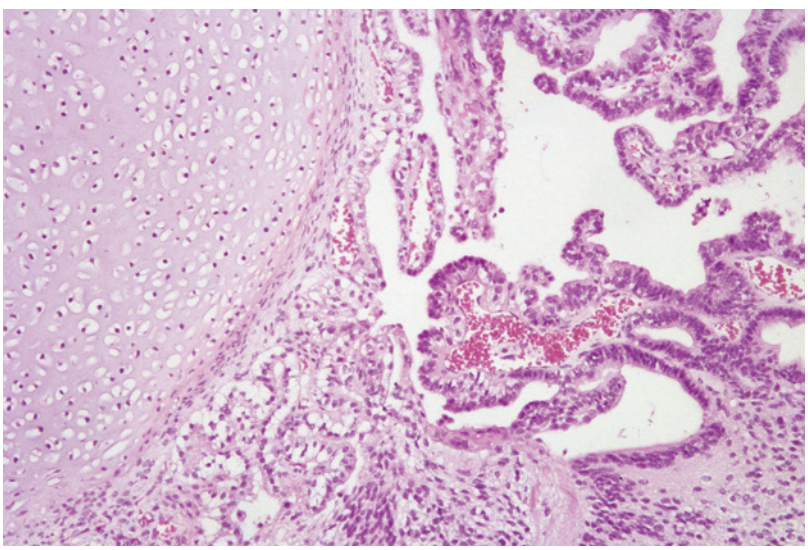

FIGURE 13.1 Teratoma composed of cartilage and choroid plexus-like tissue.

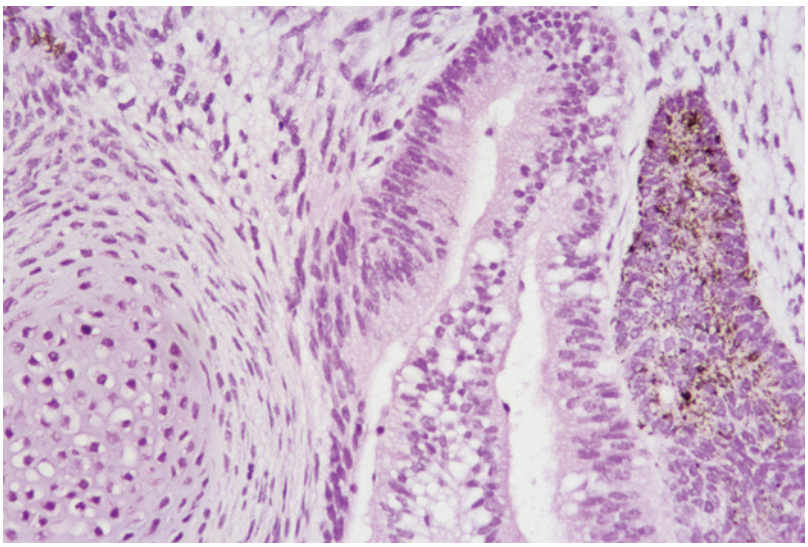

FIGURE 13.2 Teratoma composed of cartilage, primitive intestinal and neuroepithelial tissue, and a tube composed of pigmented retinal epithelium (right side).

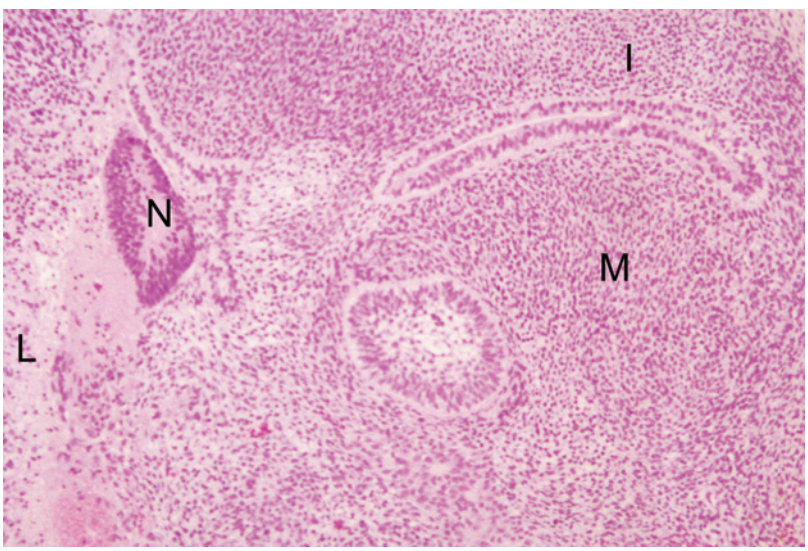

FIGURE 13.3 Teratoma composed of broad zones of mesenchymal stromal tissue (M), adjacent to fetal intestinal epithelium (I). Next to the neural tube (N) there is a small focus of lens-forming cells (L).

Neural tissues

Neural tissue is easiest to recognize when it is arranged into neural tubes or fetal neural rosettes (Figure 13.5).

Condensation of fetal neural cells may be associated with formation of glia-rich neuropil, indicative of maturation of fetal into adult-like cells (Figure 13.6). Specialized

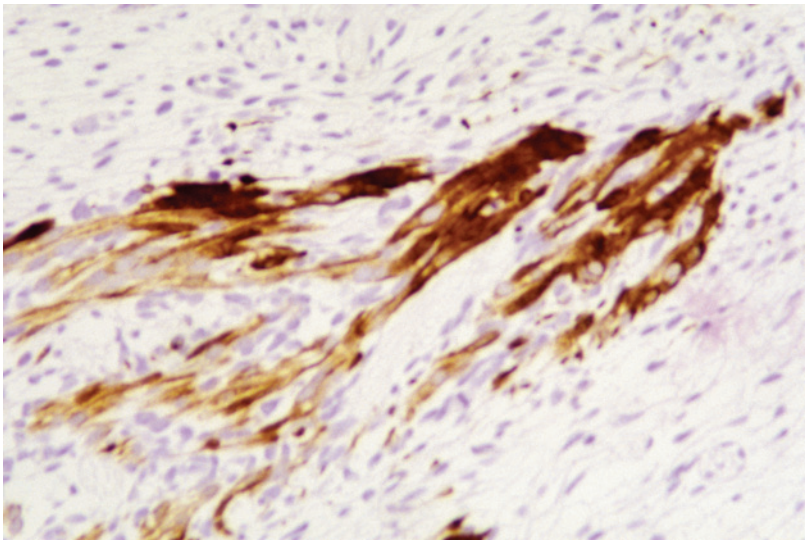

FIGURE 13.4 Teratoma containing striated muscle cells. These cells are immunohistochemically positive for desmin (brown).

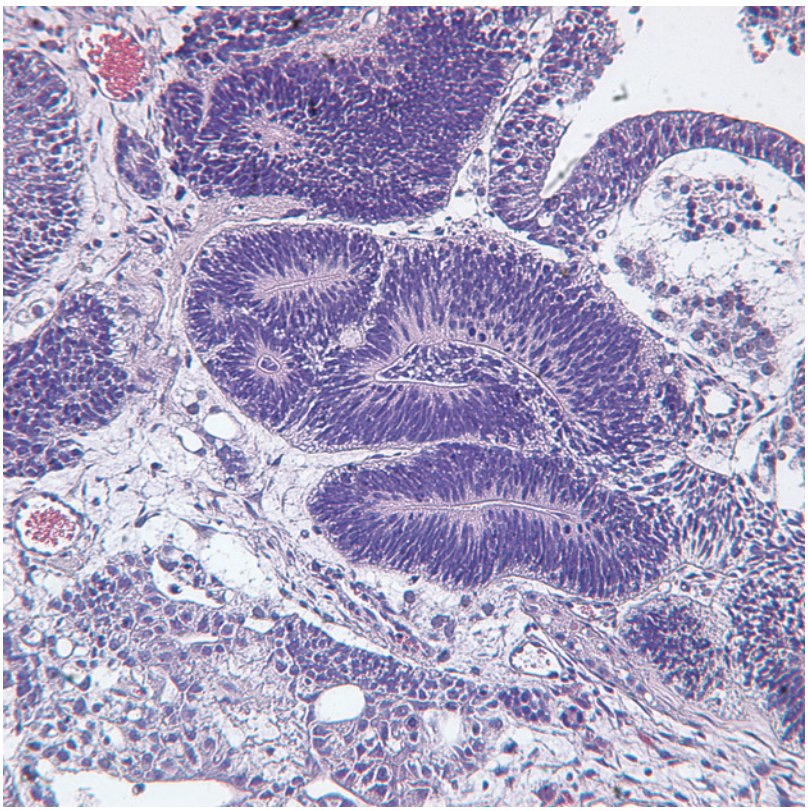

FIGURE 13.5 Immature teratoma composed of numerous fetal neural tube structures.

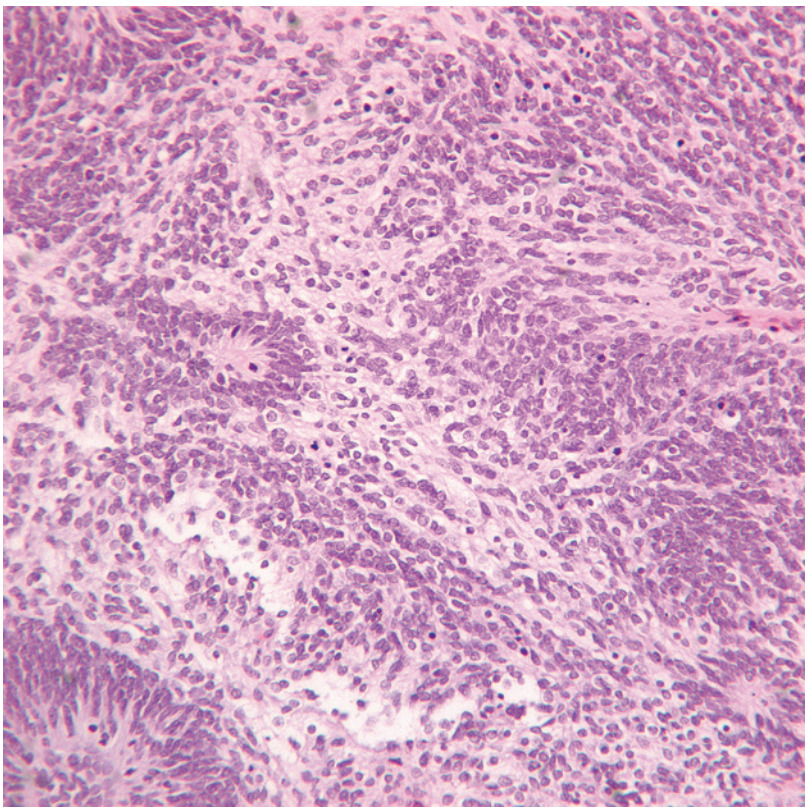

Figure 13.6 Immature teratoma composed of fetal neural tissue forming neural tubes or indistinct aggregates. The immature neural cells are surrounded by more differentiated neuropil that appears pink.

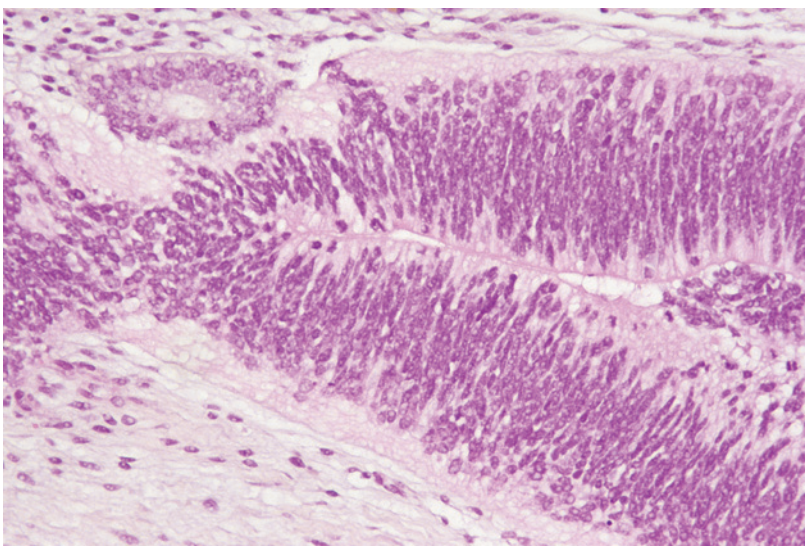

Figure 13.7 Immature teratoma composed of neural tissue. The neural tube appears multilayered.

neural axis-derived cells such as pigmented, melanin-containing retinal cells may be seen focally (Figure 13.2). Structures resembling the choroid plexus of the cerebral ventricles may be also seen (Figure 13.1). Foci composed of primitive lens-forming eye cells are also occasionally seen (Figure 13.3).

Some tumors are almost exclusively formed of neural tissue. Neural tubes in such tumors are often multilayered (Figure 13.7). Despite considerable proliferative activity these cells are often positive for neural markers (Figures 13.8 and 13.9).

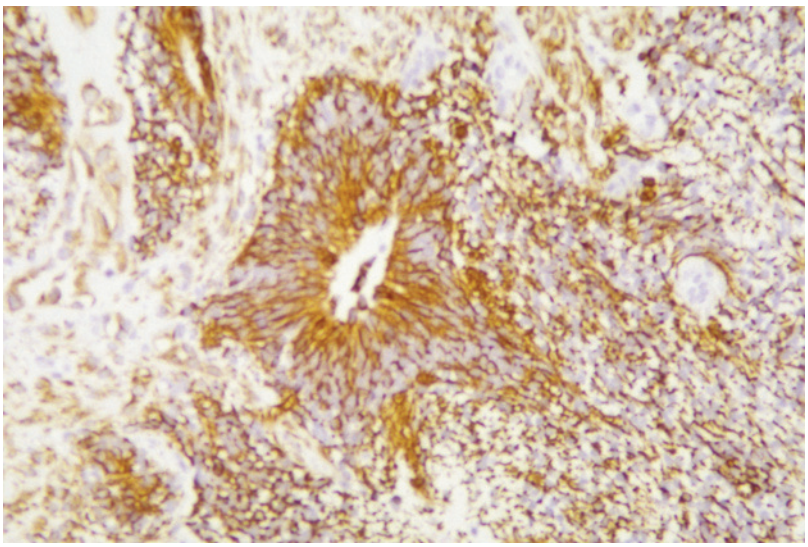

FIGURE 13.8 Immature teratoma immunohistochemically stained with antibodies to neurofilaments. Brown immunoreactants are signs of neural differentiation.

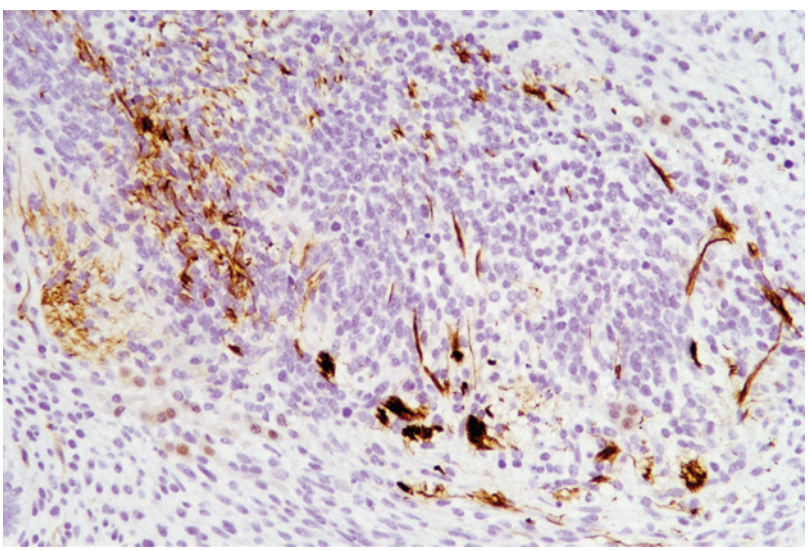

FIGURE 13.9 Immature teratoma immunohistochemically stained with antibodies to neurofilaments. Individual differentiated neural cells appear brown.

Other tissues

Among the other ectodermal derivatives the most common are cysts lined by squamous epithelium, and focally one may see structures resembling skin.

Endodermal structures include various glands and tubules lined by specialized epithelial cells. In some instances these cells resemble fetal or adult intestine (Figures 13.10 and 13.11). In other instances the glands cannot be positively identified (Figure 13.12).

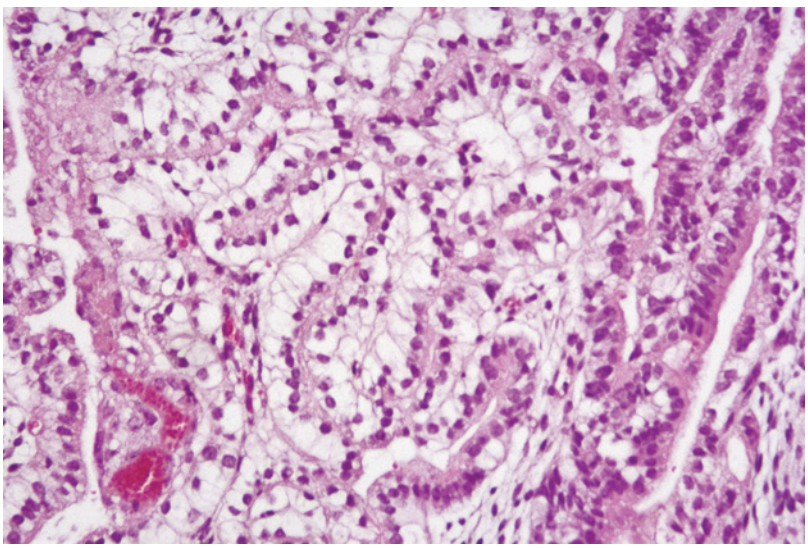

FIGURE 13.10 Teratoma containing clear cells resembling fetal intestine.

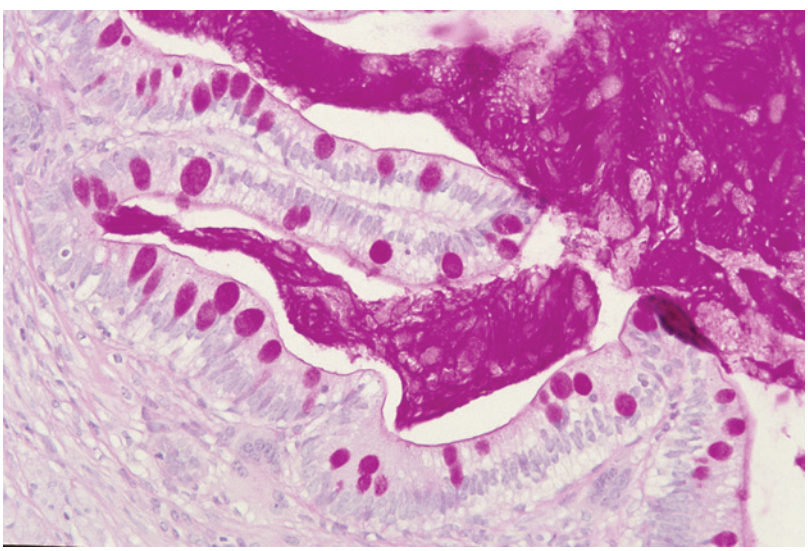

FIGURE 13.11 Teratoma containing intestinal epithelium. The slide was stained with the periodic acid-Schiff reaction outlining the mucus in the lumen (right upper quadrant) and individual mucus-secreting goblet cells in the intestinal epithelium.

The glandular structures can be surrounded by smooth muscle cells, suggesting intestinal development (Figure 13.13). Sometimes the glands are surrounded by nondescript mesenchymal cells and the juxtaposition of different cell types suggests complex organogenetic interaction of cells, similar to events that occur during fetal development (Figure 13.14).

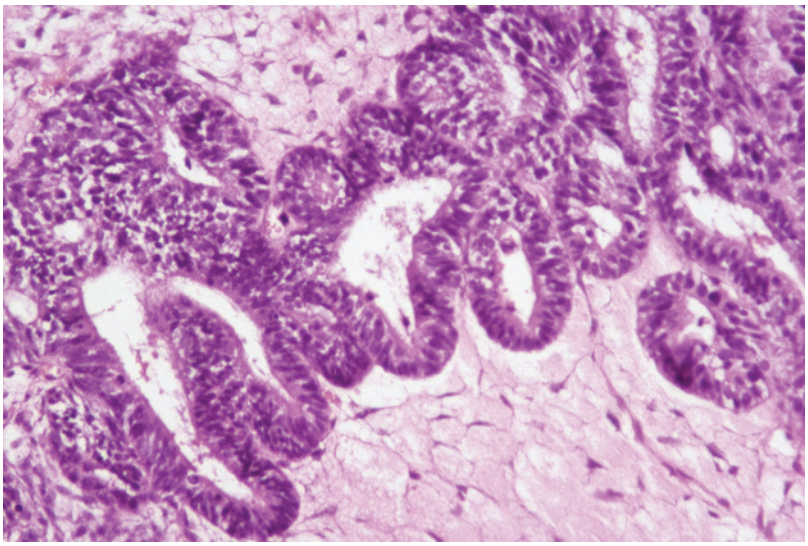

FIGURE 13.12 Teratoma-containing glands that cannot be further characterized.

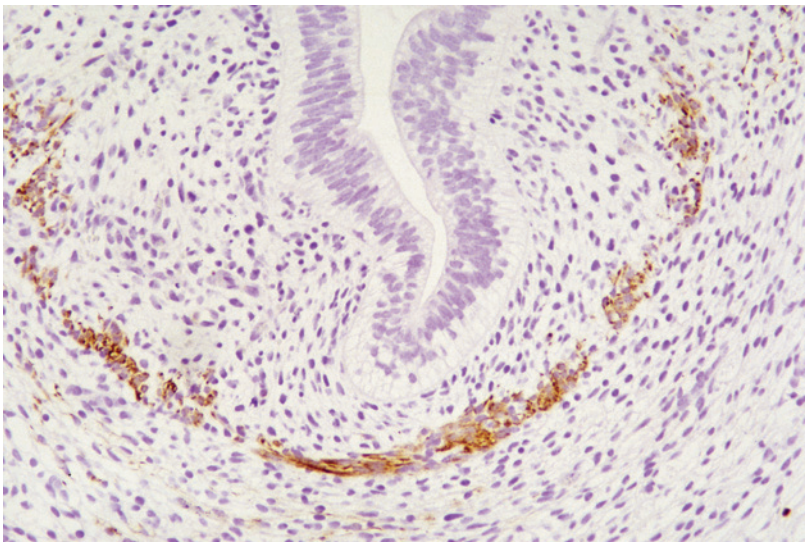

FIGURE 13.13 Teratomas containing an area of intestinal differentiation. The slide was immunohistochemically stained with antibodies to smooth mucle actin. With this approach one may see the layering of smooth muscle cells (brown) around the tubular intestinal epithelium, in a manner reminiscent of intestinal morphogenesis in the fetus.

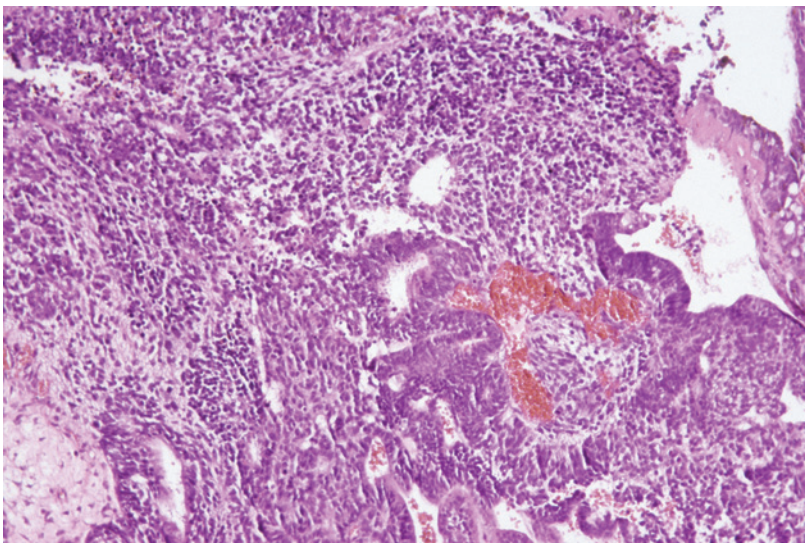

FIGURE 13.14 Teratoma composed of irregularly arranged nondescript glands and stromal tissue that cannot be further characterized on the basis of microscopy alone.

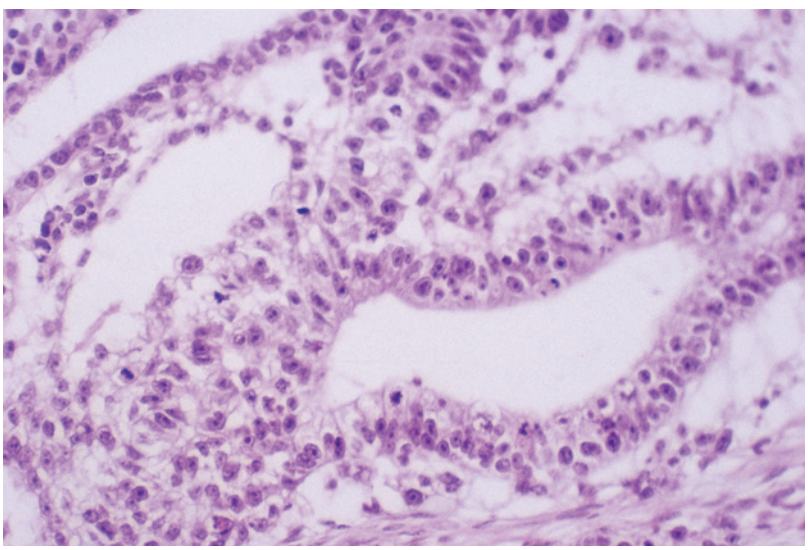

FIGURE 13.15 Immature teratoma containing undifferentiated hESCs. The cells are arranged into inter-anastomosing strands surrounding empty spaces. (Slide courtesy of Dr PW Andrews.)

Complex organogenesis may arise in some xenografts. Some tumors contain intestinal-like structures, juxtaposed neural tissue and choroid plexus, and some also contain signs of thyroid or early kidney formation. Bone or cartilage may be associated with skeletal muscles.

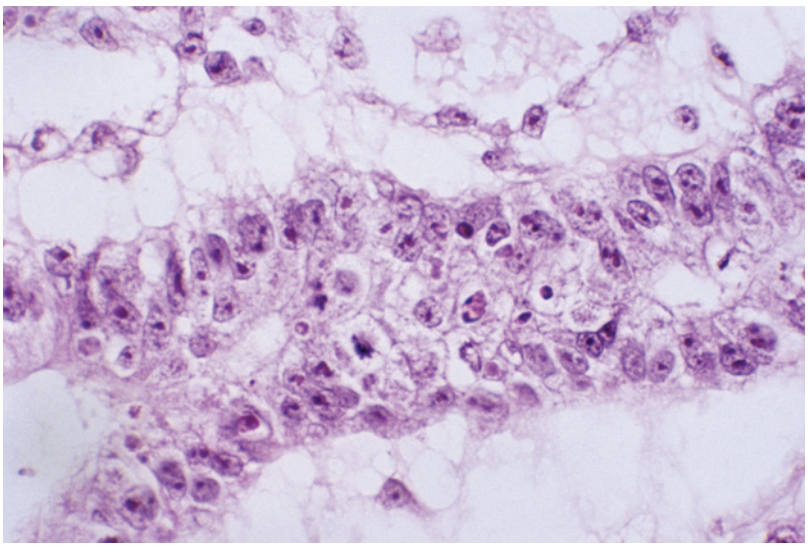

Figure 13.16 Immature teratoma containing undifferentiated hESCs. The hESCs are arranged into structures reminiscent of the early embryo and resemble embryoid bodies formed by hESCs *in vitro*. (Slide courtesy of Dr PW Andrews.)

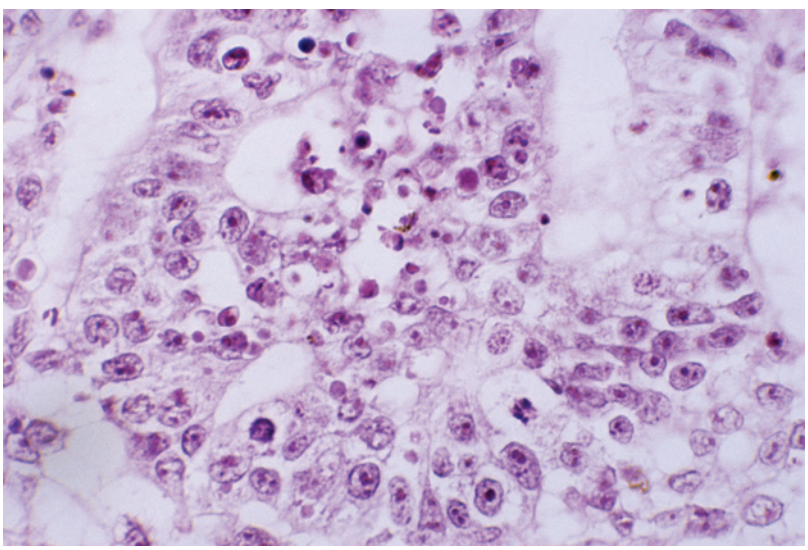

Figure 13.17 Immature teratoma containing undifferentiated hESCs. The hESC are loosely arranged and many of them have undergone apoptosis. (Slide courtesy of Dr PW Andrews.)

In a minority of xenografts one may occasionally see foci of undifferentiated hESCs (Figures 13.15–13.17). These cells have a high nucleus-to-cytoplasm ratio and their nuclei are slightly irregular, containing finely dispersed chromatin and prominent nucleoli. The cells are arranged into small groups, or in interconnected strands. Typically these foci contain numerous apoptotic bodies and also scattered mitoses.

Reading List

hESCs and hECCs

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PART

III

Differentiation of Human Embryonic Cells

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CHAPTER

14

Embryoid Bodies and Neuroepithelial Development

Matthew T. Pankratz and Su-Chun Zhang

Introduction

Unlocking the ability of human embryonic stem cells (hESCs) to differentiate to specific neural subtype cells will open a new and dynamic model system for studying the human nervous system. An efficient *in vitro* neural differentiation of hESCs will allow investigation of the otherwise inaccessible early phases of embryonic neural development. In addition, it will provide a source of cells for a wide spectrum of molecular and cellular neuroscience research questions ranging from ion channel properties to axon path-finding behaviors. Generation and/or enrichment of stage-specific neural cells, such as multipotential neuroepithelial cells, lineage committed neural progenitors, and post-mitotic neuronal and glial subtypes will serve as an invaluable tool for pharmaceutical screening and hopefully cell therapy.

Many of the approaches initially developed for the neural differentiation of mouse ESCs have been applied to hESCs with varying success. For example, mouse ESC, but not hESCs, can be efficiently differentiated to neural progenitors by treatment with retinoic acid at high concentrations. Mouse ESCs can also be differentiated to neuroepithelial cells by disassociating cultures to individual cells, a process that hESCs do not survive well. Instead of disassociation to single cells, neural differentiation of

Human Stem Cell Manual, edited by J. F. Loring, R. L. Wesselschmidt, and P. H. Schwartz.
ISBN: 978-0-1237-0465-8.

hESCs can be initiated through aggregation of small free floating clusters of ESCs, a process often referred to as "embryoid body" formation.

The ESC aggregates can then be differentiated to neuroepithelial cells either by co-culturing with stromal cells such as PA6 or MS5 cells or in a simple, serum-free culture medium. The stromal cell co-culture method is attractive in its simplicity and its tendency to generate mid/hindbrain cells more efficiently. However, one of the drawbacks to using such feeder-mediated induction protocols is the tendency to restrict the resultant neural progenitors to a mid/hindbrain fate, which may limit the ability to differentiate to neural cells with other regional identities. In addition, the neural inductive and patterning activities of the stromal cells are uncharacterized and there is potential for carryover of these tumor cells into subsequent neural cell cultures and transplants.

The serum-free differentiation protocol detailed below provides a simple methodology that avoids the complication of maintaining an additional stromal cell line. The major advantages of this approach includes its high efficiency and chemically defined media, which will be instrumental for dissecting molecular mechanisms underlying human neural specification. More importantly, this method allows control of developmental stages and generation of primitive neuroepithelial cells which can be further induced to neuronal and glial progenitors with forebrain, mid/hindbrain, and spinal cord identities. Thus, this neuroepithelial differentiation method can be used broadly to generate neural progenitors and mature neural subtypes, as well as being adapted to the needs of individual investigators who intend to differentiate hESCs to specific types of neurons and glial cells.

Overview

The following protocol was designed based on the timeline of human neural plate/tube formation at three weeks of human embryo gestation and our understanding of animal neuroepithelial induction in response to fibroblast growth factor (FGF) signaling. The protocol presented below is a simplified and optimized version of a previous report (Zhang *et al.*, 2001). It comprises three major steps: aggregation of ESCs ("embryoid body" formation), differentiation of primitive neuroepithelial cells, and generation of more definitive neuroepithelial cells. Each step is morphologically distinct and is readily identifiable under a regular phase contrast microscope and typical photos have been provided as a guideline.

The typical yield of neuroepithelial cells, defined by immunostaining for the neuroepithelial transcription factors PAX6, SOX1, and SOX2, is about 90% of the total differentiated progeny. In the case of neural differentiation of hESCs, it is essential that the ESC culture is free of partially differentiated cells, which is unfortunately not common in the community at present. Finally, the neuroepithelial cells can be further enriched using an enzymatic method developed in our lab and the hESC-derived neuroepithelial cells can then be further differentiated to neurons and glial cells. The timeline in Figure 14.1 outlines the protocol and will serve as a reference for planning experiments.

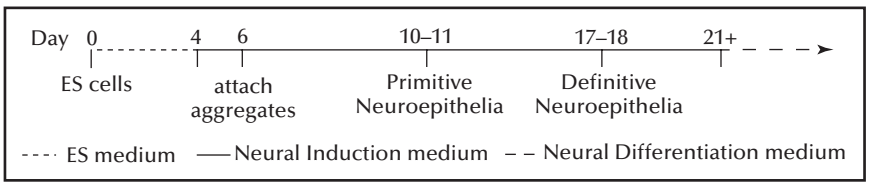

FIGURE 14.1 Timeline.

PROCEDURES

Making ESC aggregates/"embryoid bodies" (days 1–4)

1. Prepare dispase at 1 mg/mL in D-MEM/F12 (collagenase can also be used at the same concentration). Warm in a 37°C water bath to dissolve (7–15 min) and filter sterilize with a Steri-Flip.
2. Aspirate ESC medium off ESCs and add dispase (0.5 mL/well of a six-well plate). ESCs should be grown to the same density as those prior to passaging/splitting.
3. Incubate and wait 2–5 min until the edges of cell colonies begin to curl off of the plate. Tap or swirl the plate to dislodge colonies.
4. Add 3 mL of ESC medium and gently pipette all of the ESC colonies from one entire six-well plate and transfer them to a 15 mL tube. Gently triturate 3–5 times to break cell colonies into smaller clusters. Clusters should be roughly twice the size as clusters for passaging ESCs (Figure 14.2A).
5. Allow the ESC clusters to settle to the bottom of the tube (2–3 min). Aspirate off the medium with caution so as to not aspirate the entire pellet.
6. Wash the cells once by adding 5–6 mL of fresh ESC medium and then centrifuge for 2 min at 200× ***g***.
7. Aspirate off supernatant and resuspend cells in ESC medium (for six wells use 60 mL ESC medium) and transfer to flasks.

NOTE: Cell aggregates will initially look unhealthy from shock of separation from feeders. To speed cell recovery, feed for the first time within ~12 h and replace most of the medium to remove debris. Switching cells to a new flask is also useful to remove mouse embryonic fibroblasts (MEFs) that may have attached during the first 12 h.

8. Continue feeding with ESC medium every day for 4 days.

NOTE: When feeding, use a 5 mL pipette to gently pull aggregates up and then blow them back into the medium 2–3 times. This will help clean dead cells off the aggregate surface. Let the clusters settle to the bottom in a standing flask and aspirate off the medium.

See the Alternative procedures section for a discussion of ESC aggregates vs. embryoid bodies.

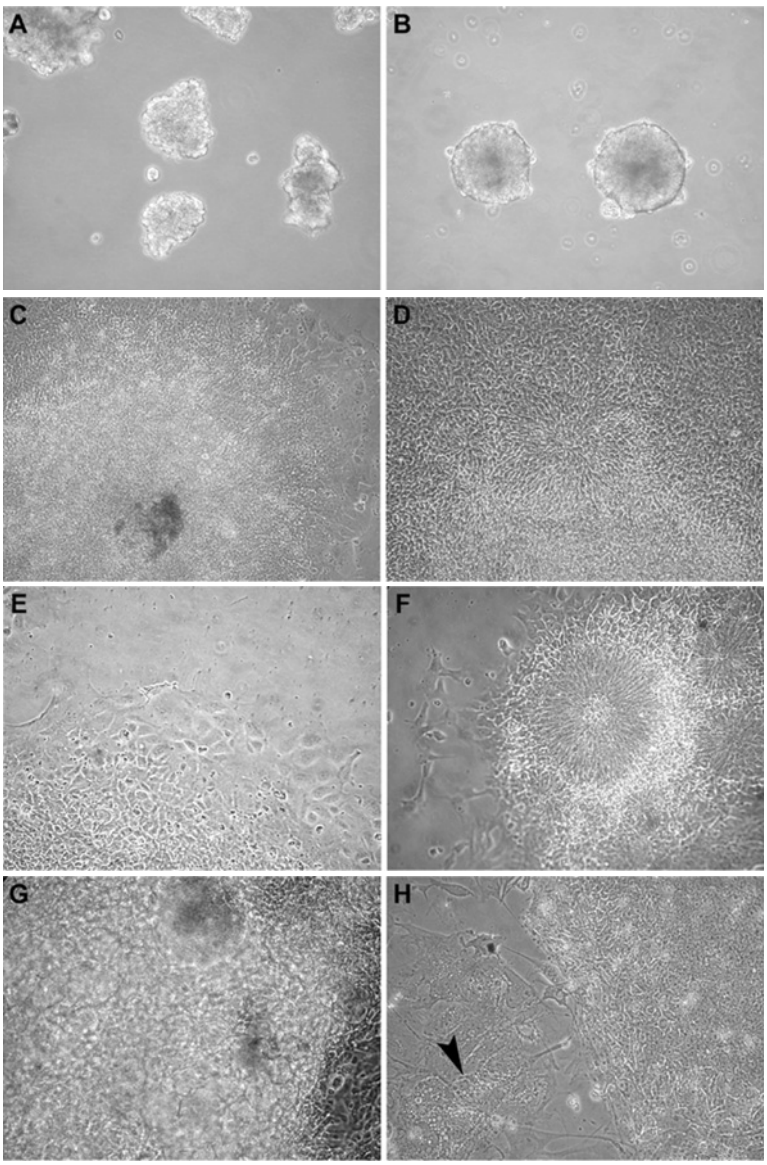

FIGURE 14.2 (A) hESC aggregates 1 h after separation from MEFs, 10× objective. (B) Six day ESC aggregates. Clusters need to be agitated gently to remove loose cells at the edges, which will promote attachment. (C) Eight days of differentiation (2 days following attachment), the aggregate has attached to a laminin substrate and collapsed to a monolayer colony. (D) Eleven day primitive neuroepithelial cells found in the center of colonies. Cells have a characteristic elongated morphology. (E) Flat non-neuroepithelial cells at the edge of a differentiating colony, the objective is to isolate the central primitive neuroepithelial cells from these cells (see figure 1F in Zhang *et al.*, 2001). (F) Definitive neuroepithelial cells in a rosette structure resembling the neural tube at 16 days of differentiation. (G) One of the typical types of non-neuroepithelial cells that can form during differentiation. Colonies containing these types of cells should make up less than 5–10% of the overall colonies and can be removed with manual selection. (H) hESC colony with normal morphology on the right, arrowhead pointing to the type of differentiation commonly seen when cultures are infected with mycoplasma.

Differentiating to primitive neuroepithelial cells (days 5–10)

After free floating culture in ESC medium for 4 days, the aggregates are ready for further differentiation.

1. Collect the ESC clusters, centrifuge for 1 min at 200×*g* and wash once with 5 mL of neural induction medium.
2. Resuspend cells in 50–60 mL of neural induction medium supplemented with 20 ng/mL bFGF and transfer to a new flask (see Alternative procedures section for a discussion of FGFs in culture). Cells in neural induction medium can be fed every other day.
3. After 2 days, aggregates should be bright and clear and are ready for attachment (Figure 14.2B). Induce attachment by plating cells on a laminin-coated substrate. Use 20 μg/mL mouse or human laminin in D-MEM/F12 or neural induction medium on either plastic or plain glass or glass coated with polyornithine (see Alternative procedures section for more information).

NOTE: Laminin-coated surfaces should be incubated at 37°C overnight for best results.

4. When plating aggregates, provide enough space for colonies to grow out without contacting one another. Aggregates should be transferred to a 15 mL tube and agitated gently with a 5 mL pipette to remove loose cells. About 20–30 aggregates should be deposited in fresh neural induction medium + bFGF in each well of a six-well plate or 2–4 aggregates/coverslip. If plating on a six-well plate, shake the plate on the incubator shelf up and down twice and then left and right twice gently to evenly distribute the clusters, the same method as for splitting ESCs. Cells should attach overnight (minimize jarring plates, including frequent incubator closing and opening to improve attachment).
5. Attached aggregates will collapse to form a monolayer colony after 1–2 days (Figure 14.2C). Continue feeding with neural induction medium + 20 ng/mL bFGF every other day.
6. After 10–11 total days of differentiation (4–5 days following attachment), over 95% of the colonies should take on a morphology in which the center cells exhibit an elongated, columnar morphology (Figure 14.2D). We have called these columnar epithelial cells primitive neuroepithelial cells because they express a range of early neuroectodermal markers. Immunostaining with PAX6 (dilute 1:5000), SOX2 (1:1000), and N-cadherin (1:1000) can be used to confirm a primitive neuroepithelial phenotype at this stage (see Pitfalls and advice section for comments on nestin).

NOTE: After 10–11 days of differentiation, primitive neuroepithelium is receptive to neural patterning signals. Attempts to add patterning signals (notably retinoic acid) prior to this time point can lead to differentiation to non-neural fates. At this point cells can continue to be cultured in neural induction medium supplemented with bFGF or alternatively switched to conditions designed to regionally specify the neuroepithelial cells to more specific cell fates (see Chapter 15).

Generating definitive neuroepithelial cells (days 11–17)

1. Scratch off any colonies that do not contain any primitive neuroepithelial cells (this should be less than 10% of colonies, see the Pitfalls and advice section for additional help if this is not the case). The "bad" colonies can be marked by an objective marker lens under a phase contrast microscope and then scraped away with a pipette tip in a sterile hood.
2. Feed neuroepithelial cells with the same medium every other day and culture for 7 days. During this period starting at day 14–15 the columnar neuroepithelial cells will further compact and proliferate, often forming ridges or rings of cells outlining a distinct lumen (Figure 14.2F). The overall morphology is reminiscent of the neural tube and cells at this stage are often referred to as neural tube-like rosettes.
3. After 17–18 days of differentiation under these conditions the neuroepithelium that makes up the rosettes will stain positive for the definitive neural tube stage marker SOX1 (dilute at 1:500).

Isolating definitive neuroepithelial cells (days 17–18)

To increase the purity of neuroepithelial cells generated, we have added a subculture step after the formation of neural tube-like rosettes (see notes on neuroepithelium isolation timing in the Alternative procedures section).

1. Treat the culture with 0.5 mg/mL dispase in D-MEM/F12 or neural induction medium. Incubate at 37°C ~2–3 min, monitoring closely for when inner neuroepithelial cells start to peel away from the flat peripheral cells at the edges of colonies (Figure 14.2E; Zhang *et al.*, 2001).
2. Once the rosettes start to peel off, tap the plate to speed the process while trying to keep flat peripheral cells attached.
3. Once the neuroepithelial cells are detached, collect the neuroepithelial cell clusters in a 15 mL centrifuge tube.

NOTE: Use gentle pipetting during rosette isolation as the cells separate easily and it is best to not break up clusters of neuroepithelial cells initially.

4. Spin at 100×*g* for 2 min and wash once with fresh neural induction medium.
5. Aspirate the medium and resuspend the clusters of definitive neuroepithelial cells in 5 mL of neural induction medium + 20 ng/mL bFGF, supplement with B27 to improve cell survival.
6. Over the next 24 h the rosette aggregates will roll up to form round spheres while any flat non-rosette peripheral cells will usually attach to the culture vessel. After this period rosette aggregates should be switched to a new flask with neural induction medium + bFGF.
7. After several days in neural induction medium and bFGF, neuroepithelial aggregates are ready for further differentiation to neural cells. To induce further differentiation, plate spheres on laminin/polyornithine-coated coverslips in the

presence of neural differentiation medium supplemented with cAMP (1 μM), ascorbic acid (200 ng/mL), laminin (1 μg/mL), and BDNF, GDNF, and IGF-I (10 ng/mL) (see Chapter 15 for further details).

Alternative Procedures

ESC aggregates vs. embryoid bodies

The term embryoid body or EB was originally used to describe how mouse ESCs or embryonal carcinoma cells (ECCs) could form clusters of ectoderm, mesoderm, and endoderm cells surrounding a fluid-filled cavity when cultured long term as suspended aggregates. The initial stage in this protocol details how to generate such aggregates, yet refrains from referring to them as EBs. It is true that if these aggregates were cultured long term in ES medium they would become true EBs, however the media and timing of this protocol are designed instead to direct the cells to an ectoderm fate so few to no endoderm or mesoderm cells are generated. During the first 4 days of culture the clusters of cells truly are best described as ES aggregates since re-plating on MEF results in largely ES colonies with some differentiated cells at the edges.

FGFs in culture

FGFs are important patterning and proliferative signals during the development of the nervous system. However, in an attempt to study certain aspects of human neural development *in vitro* it may be desirable to avoid the use of exogenous FGFs in culture. The protocol outlined above can be used to generate neuroepithelial cells without exogenous FGFs. However, in our experience the omission of FGFs results in decreased neuroepithelial proliferation and an increase in the percentage of ESC aggregates that fail to form neuroepithelial cells.

Polyornithine-coated coverslips

Coverslip sterilization

1. Empty coverslips (Bellco Catlog no. 1943-10012) into a pre-assigned beaker for nitric acid use.
2. Add approximately 50 mL nitric acid, or enough to cover the coverslips. (Wear gloves and operate in a fume hood.)
3. Shake for 1 h on a shaker.
4. Pour out as much nitric acid from the beaker as possible without pouring out any coverslips. Rinse a few times with distilled water.
5. Leave beaker under running distilled water for at least 15 min.
6. Under a sterile hood, store coverslips in 95% ethanol. Two 50 mL centrifuge tubes comfortably hold one case of sterilized coverslips. Shake for 30 min before using, or store at room temperature.

Polyornithine coating

1. In a sterile hood, pour out contents of 50 mL tube containing sterilized coverslips into the lid of a six-well plate. (This lid works well as a basin to pick up coverslips.)
2. Using sterilized forceps, pick up one coverslip at a time and set upright in a well of a 24-well plate. Repeat for the whole plate. Be careful that there are not two coverslips stuck together and try not to scratch the coverslips.
3. Allow coverslips to dry completely.
4. Tap plates until coverslips have fallen flat in each well.
5. Add 100 µL polyornithine (0.1 mg/mL in sterile dH_2O) to each coverslip. Keep the drop on the coverslip alone.
6. Incubate plates at 37°C overnight.
7. Remove plates from the incubator and allow to cool to room temperature before proceeding.
8. Aspirate polyornithine off of each coverslip. Do not scratch/touch the center of the coverslip. Touch only the edge of the coverslip when aspirating.
9. Allow coverslips to dry for approximately 30 min before washing.
10. Add 1 mL sterile dH_2O to each well.
11. Let sit for 10 min.
12. Aspirate water from each well.
13. Repeat steps 10 and 11 two more times, for a total of three washings.
14. Allow plates to completely dry by removing the lids and leaving them in the hood as long as necessary.
15. Cover plates, wrap in foil, label with date and store at −20°C.

Alternatives to laminin-enhanced adhesion

Attachment of ESC aggregates or neuroepithelial clusters can be accomplished with a variety of different adhesion molecules including fibronectin and Matrigel™. Another quick and cost-effective method to facilitate adhesion is to supplement neural induction medium with 10% fetal bovine serum (FBS) for 12–24 h. The serum should then be washed away after the aggregates have attached. Although serum should be avoided for neuroepithelial differentiation, this short exposure to enhance adhesion does not significantly reduce the overall efficiency of the culture system for generating neuroepithelial cells. It should be noted that the use of serum may affect some gene expression patterns.

Timing for neuroepithelium isolation

Neuroepithelial cells can technically be isolated at any point after the primitive neuroepithelial cell stage at 10 days of differentiation and grown in suspension. The benefit

of isolating neuroepithelial cells at the primitive stage is that it allows for early selection of neuroepithelial cells and limits cell death and differentiation that result from high-density culture. However, culture of neuroepithelial cells as free floating clusters as opposed to a monolayer affects the exposure of cells to patterning signals and mitogens. If after 17–18 days of culture, neural tube-like rosettes are difficult to observe and there are too many non-neuroepithelial cells in culture, try enzymatically isolating neuroepithelial cells at the primitive neuroepithelial stage (day 10). Grow cells as aggregates for 1–2 days in neural induction medium with bFGF (20 ng/mL) and then re-plate the cells at a lower density. Take care not to break up neuroepithelial clusters too much. If neuroepithelial clusters attach and form monolayer colonies of larger flat cells, like the ones seen at the edges of colonies at 10 days, the clusters are too small. Breaking neuroepithelial clusters less initially or keeping clusters in culture longer will allow more cell proliferation and should solve this problem.

Mechanical neuroepithelial cell isolation

If you are having trouble enzymatically isolating neuroepithelial cells at the primitive (10 day) or definitive (17–18 day) stage from the flat surrounding cells, try isolating them mechanically. Very gentle pipetting with a 1000 μL tip can usually dislodge the neuroepithelium which is denser in the center of colonies, as opposed to the flat, tightly bound cells at the periphery.

Pitfalls and Advice

hESC colonies will not come off the plate

Variations in ESC lines, plastic culture dishes and differences in MEF and gelatin can all lead to variability in how tightly ESC colonies are adhered. Using a higher concentration of enzyme may be necessary; also make sure the enzyme is fresh and fully dissolved in solution. Using excess ESC medium pipetted onto cells with a gentle clockwise swirling motion can help lift colonies as well. MEFs are inevitably lifted in this step in the procedure as well, however they usually die or attach to the flask overnight and do not contribute significantly to subsequent stages of differentiation.

hESC aggregates are black and dying or clump together too much

Separation of ESC colonies from feeders is a shock to cells and does induce some cell death. Pre-warming media and minimizing the time cells remain out of the incubator can improve cell health. Using more media and larger flasks and changing media more often can also bolster cell survival. Making aggregates too large or small can also be a problem. A 5 mL pipette tip pushed against the bottom of a 15 mL tube will effectively shear ESC colonies into smaller clusters of cells with 3–5 up and down pipettings. Excess or overzealous pipetting can lead to large, stringy clumps of cells stuck together with DNA from lysed cells. A few such clumps of cells are normal and can be easily removed. It is better to remove a few clumps of cells than try and break everything up and risk overpipetting.

Avoid using too much enzyme or treating cells enzymatically for too long to prevent MEF and ESC colonies from coming off the plate in a single sheet, which makes breaking colonies into individual aggregates far more challenging.

Aggregates will not attach

As long as aggregates are relatively bright and clear after 6 days in culture they should be ready for attachment. If aggregates do not look healthy they can be kept in neural induction medium for an additional 1–2 days. Loose cells on the aggregate surface can inhibit attachment. Washing cells several times with neural induction medium will help remove all the loose cells. An alternate attachment method such as a 12–24 h supplement of 10% FBS in neural induction medium can be used (see Alternative procedures section). It often works best to set aggregates up for attachment at the end of the day to minimize traffic in and out of the incubator.

Attached aggregates fail to form neuroepithelial cells

Aggregates that have differentiated to other non-neural cell types are readily identifiable in culture (Figure 14.2G). A small percentage (5–10%) of aggregates that fail to form neuroepithelial cells can be removed through simple scrapping of colonies with a sterilized Pasteur pipette tip. A failure of the majority of aggregates to form neuroepithelial cells is usually indicative of a problem in the initial ESC culture. The presence of differentiated cells, often at the periphery of ESC colonies, can influence neural induction and block neuroepithelial cell formation. ESC cultures should be largely free of cells differentiated to non-neural lineages such as endoderm (AFP+) or mesoderm (T(brachyury+)). Switching to a new preparation of MEFs or using a lower passage of ESCs can improve the overall undifferentiated status of the culture.

If ESC colonies appear properly undifferentiated, try screening for mycoplasma contamination (VenorGeM, Sigma). Mycoplasma contamination, even at low levels that do not visibly affect ESC morphology or growth, will drastically impair the ability of cells to form neuroepithelium. A typical type of differentiation often observed in ESC cultures contaminated with mycoplasma is pictured in Figure 14.2H.

Does nestin label human neuroepithelial cells?

Nestin is an intermediate filament protein that is expressed in early neuroepithelial/neural precursor cells. It is rapidly and specifically turned on in mouse ESCs as they differentiate to neural fates. It is less reliable as a neural maker in hESC work, because hESCs also express nestin. Therefore nestin alone should not be used as the sole determinant of neural identity, additional early neural markers such as PAX6, CDH2, SOX1, and SOX2 (which is also expressed in ESCs) should also be used.

Can neuroepithelial cell clusters be cultured long term?

Clusters of neuroepithelial cells can be propagated for several months in neural induction medium + 20 ng/mL bFGF. Every 5–7 days as aggregates become large, aggregates should be broken up. To break clusters, take a sterilized glass Pasteur pipette with a cotton filter, flame polish the end and narrow the opening slightly; also

flame treat the narrow part of the shaft to introduce a 20–30° bend. Suck up neuroepithelial clusters into the pipette and push them out (the narrow opening and bend in the pipette should help shear the clusters into smaller pieces of roughly uniform size). Following breaking of the clusters, supplement medium for 1 day with B27 and switch the cells to a new flask the following day to eliminate attached cells. Cells cultured in this fashion will continue to give rise to neurons and glia with plating and growth in a neural differentiation medium with trophic factors. However, the cells' response to patterning signals and the percentage and types of neural cells generated will inevitably change with long-term culture.

Supplies and Reagents

Supplies

Item	Supplier	Catalog no.	Alternative
T25 flasks (polystyrene flasks with polyethylene filter cap)	Fisher Scientific	12-565-57	Nunc catalog no. 136196
T75 flasks	Fisher Scientific	12-565-31	Nunc catalog no. 178891
Six-well polystyrene plates	Fisher Scientific	12-565-73	Nunc catalog no. 140675
24-well polystyrene plates	Fisher Scientific	12-565-75	Nunc catalog no. 143982
Polystyrene conical tube, 15 mL	Fisher Scientific	05-527-90	BD Biosciences catalog no. 352095
Polystyrene conical tube, 50 mL	Fisher Scientific	14-432-23	BD Biosciences catalog no. 352073
Serological pipettes 5, 10, and 25 mL	Fisher Scientific	13-678-11	
9″ Pasteur pipettes, cotton plugged	Fisher Scientific	13-678-8B	
9″ Pasteur pipettes	Fisher Scientific	13-678-20D	
50 mL Steri-Flip	Fisher Scientific	SCGP00525	
500 mL filter unit (0.22 μm sterilizing low protein binding membrane)	Corning Inc.	430513	
60 × 15 mm Petri dish	Fisher Scientific	08-757-13A	
50 mL Steri-Flip filters	Millipore	SCGP 005 25	

Reagents

Item	Supplier	Catalog no.
L-Glutamine solution (200 mM)	Sigma	G-7513
MEM non-essential amino acids solution	Invitrogen	11140-050
KnockOut™ serum replacement (KSR)	Invitrogen	10828-028
Dulbecco's modified Eagle's medium: nutrient mixture F12 1:1 (D-MEM/F12)	Invitrogen	11330-032
Dulbecco's modified Eagle's medium (D-MEM)	Invitrogen	11965-092

(*Continued*)

Item	Supplier	Catalog no.
Neurobasal medium	Invitrogen	21103-049
2-Mercaptoethanol (14.3 M)	Sigma	M-7522
N2 supplement	Invitrogen	17502-048
Laminin from human placenta	Sigma	L6274
Bovine serum albumin (BSA)	Sigma	A-7906
Cyclic AMP	Sigma	D-0260
Ascorbic acid	Sigma	A-4403
Retinoic acid	Sigma	R-2625
Sonic hedgehog	R&D Systems	1845-SH
Recombinant human FGF8	PeproTech	100-25
TGFβ3	R&D Systems	243-B3
Dispase	Invitrogen	17105-041
Acutase	Innovative Cell Technologies	AT104
Trypsin-EDTA (1×)	Invitrogen	25300-054
Trypsin inhibitor (1 mg/mL dissolved in D-MEM/F12 and sterile filtered)	Invitrogen	17075-029
Heparin	Sigma	H3149
Recombinant human bFGF	R&D Systems	233-FB
Fibronectin from human plasma	Invitrogen	16000-044
Recombinant human BDNF	PeproTech	450-02
Recombinant human GDNF	PeproTech	450-10
Recombinant human IGF-I	PeproTech	100-11
Poly-2-hydroxyethylmethacrylate (poly-HEME)	Sigma	P-3932
Polyornithine	Sigma	P-3655
Fetal bovine serum (FBS)	Sigma	F-2006
VenorGeM mycoplasma screening kit (PCR based)	Sigma	MP0025

Antibodies

Item	Supplier	Catalog no.
PAX6 monoclonal	Developmental Studies Hybridoma Bank (DSHB)	
SOX2 monoclonal	R&D Systems	MAB2018
N-Cadherin monoclonal, D-4	Santa Cruz Biotechnology	sc-8424
SOX1 rabbit polyclonal	Chemicon International	AB-5768
Goat anticholine acetyltransferase (ChAT) affinity-purified polyclonal antibody	Chemicon International	AB144P
Rabbit anti-Olig2	Santa Cruz	sc-19969
Monoclonal antibody against MNR2 (HB9)	Development Studies Hybridoma Bank	81.5C10
Rabbit anti-βIII-tubulin	Covance	PRB-435P
TH monoclonal	Sigma	T-2928
Lmx1b rabbit polyclonal	Gift from Yuqiang Ding	
Alexa fluor secondary antibodies	Molecular Probes/ Invitrogen	Match to desired primary and fluorescence your microscope can detect

Recipes

Stock solutions

Component	Amount	Stock concentration
Recombinant human FGF basic	Dissolved in sterilized PBS with 0.1% BSA and 2 μg/mL heparin	10 μg/mL, aliquot and store at −80°C
Heparin	Dissolve 20 mg heparin in 10 mL D-MEM medium	2 mg/mL, aliquot and store at −80°C
Retinoic acid	Dissolve 3.004 mg in 10 mL ethanol	1 mM, store at −20°C
Sonic hedgehog	Dissolve 1 mg in 10 mL sterilized PBS with 0.1% BSA	100 μg/mL, aliquot and store at −80°C
Recombinant human BDNF, GDNF, IFG-I	Dissolve 200 μg growth factors in 2 mL sterilized distilled water	100 μg/mL, aliquot and store at −80°C
Ascorbic acid	Dissolve 2 mg in 10 mL PBS	200 μg/mL, aliquot and store at −80°C
TGFβ3	Dissolve 10 μg in 10 mL PBS	10 μg/mL, aliquot and store at −80°C
Recombinant FGF8	Dissolved in sterilized PBS with 0.1% BSA and 2 μg/mL heparin	100 μg/mL, aliquot and store at −80°C
Cyclic AMP	Dissolved in sterilized water	1 mM, aliquot and store at −80°C

ESC medium (500 mL)

Component	Amount	Final concentration
D-MEM/F12	392.5 mL	
KnockOut serum replacement	100 mL	20%
MEM non-essential amino acids solution	5 mL	0.1 mM
2-Mercaptoethanol (14.3 M)	3.5 μL	0.1 mM
L-Glutamine (200 mM)	2.5 mL	1 mM

Sterile filter with a 0.22 μm filter, add 4 ng/mL bFGF just prior to feeding cells. Medium is stored at 4°C for up to two weeks.

Neural induction medium (500 mL)

Component	Amount	Final concentration
D-MEM/F12	490 mL	
N2	5 mL	1×
MEM non-essential amino acids solution	5 mL	0.1 mM
Heparin (2 mg/mL)	500 μL	2 μg/mL

Sterile filter with a 0.22 μM filter, add cytokines and signaling molecules (such as FGFs) just prior to feeding cells.

Neural differentiation medium (500 mL)

Component	Amount	Final concentration
Neurobasal medium	490 mL	
N2	5 mL	1×
MEM non-essential amino acids solution	5 mL	0.1 mM

Sterile filter with a 0.22 μm filter, add cytokines and signaling molecules just prior to feeding cells.

Dispase solution (10 mL)

Component	Amount	Final concentration
Dispase	10 mg	1 mg/mL
D-MEM/F12	10 mL	

Leave in a 37°C water bath for 15 min and filter sterilize the dispase solution with a 50 mL Steri-Flip before use.

Reading List

Du ZW, Zhang SC (2004). Neural differentiation from embryonic stem cells: Which way? *Stem Cell Dev* 13: 372–381.
A review of neural differentiation protocols focusing on mouse ESCs.

Li XJ, Du ZW, Zarnowska ED, Pankratz M, Hansen LO, Pearce RA, Zhang SC (2005). Specification of motoneurons from human embryonic stem cells. *Nat Biotechnol* 23: 215–221.
Use of the culture system outlined above to generate posteriorly patterned neuroepithelial cells; contains pictures of staining for early neuroepithelial cell markers.

Perrier AL, Tabar V, Barberi T, Rubio ME, Bruses J, Topf N, Harrison NL, Studer L (2004). Derivation of midbrain dopamine neurons from human embryonic stem cells. *Proc Natl Acad Sci USA* 101: 12543–12548.
A stromal cell-based neural differentiation protocol used to generate midbrain patterned neuroepithelial cells.

Yan Y, Yang D, Zarnowska ED, Du Z, Werbel B, Valliere C, Pearce RA, Thomson JA, Zhang SC (2005). Directed differentiation of dopaminergic neuronal subtypes from human embryonic stem cells. *Stem Cells* 23: 781–790.
Use of the chemically defined culture system to generate midbrain patterned neuroepithelial cells.

Zhang SC, Wernig M, Duncan ID, Brustle O, Thomson JA (2001). In vitro differentiation of transplantable neural precursors from human embryonic stem cells. *Nat Biotechnol* 19: 1129–1133.
Basis for the culture system outlined above, the paper contains differentiation of cells to mature neuron and glia fates and transplantation of neuroepithelial cells.

CHAPTER

15

Motor Neuron and Dopamine Neuron Differentiation

Xue-Jun Li, Dali Yang, and Su-Chun Zhang

INTRODUCTION

Motor neurons and dopamine neurons have been the prime targets for differentiation from embryonic stem cells (ESCs) given their potential application in studying and treating amyotrophic lateral sclerosis (ALS) and Parkinson's disease. Motor neurons are located throughout the CNS in the cerebral cortex, brainstem, and spinal cord and each of these neurons projects to distinct targets controlling movement in different organs and tissues. Similarly, dopamine neurons are present in the olfactory bulb, cerebral cortex, hypothalamus, midbrain, and retina, and are involved in diverse functions from sense and locomotion to emotion. Neurons expressing the same neurotransmitter but in different brain regions are specified through very different molecular pathways during embryonic development. Thus, protocols aimed at generating a specific type of neuron need to be differentially tailored.

Specification of neuronal subtypes requires patterning of naïve neuroectodermal cells to regional progenitors, each of which resides in a unique position along the anterior–posterior (A-P) and dorsal–ventral (D-V) axes of the developing nervous system. This is achieved via response of naïve neuroectodermal cells to a set of organizing molecules such as bone morphogenetic protein (BMP), WNTs, fibroblast growth factors (FGFs), retinoic acid (RA), and sonic hedgehog (SHH) in a temporal and

Human Stem Cell Manual, edited by J. F. Loring, R. L. Wesselschmidt, and P. H. Schwartz.
ISBN: 978-0-1237-0465-8.

spatial restricted manner. Using this principle, human ESCs (hESCs) have been differentiated to spinal motor neurons in response to RA and SHH, and midbrain dopamine neurons in response to FGF8 and SHH. Most protocols for dopamine neuron differentiation employ a co-culture system with stromal cells such as PA6 or MS5 cells since stromal cell-derived signals appear to preferentially promote differentiation of midbrain/hindbrain progenitors. The drawbacks of such a co-culture system are that the neural inductive and patterning activity of the stromal cells is uncharacterized, and that there is potential for carryover of these tumor cells into subsequent neural cell cultures.

In Chapter 14, we described an efficient, chemically defined system for differentiating hESCs to neuroepithelial cells and have identified a primitive neuroepithelial cell stage that is responsive to morphogens for neural subtype differentiation. Thus, we feel that this system will be well suited as a template protocol that can be readily modified for differentiating hESCs to a versatile range of neuronal subtypes. Here we use differentiation of spinal motor neurons and midbrain dopamine neurons to illustrate the use of this template protocol.

Overview

This generalized protocol was designed based on the developmental principles that neuroectoderm is patterned to ventral spinal cord progenitors in response to RA and SHH and ventral midbrain progenitors in response to FGF8 and SHH. Another key to the creation of this protocol was the identification of a primitive neuroepithelial cell stage that is responsive to morphogens for further differentiation to region-specific neural progenitors. No matter what type of neurons you are trying to generate the same three fundamental steps must be addressed: (1) differentiation of primitive neuroepithelial cells (see Chapter 14); (2) specification of region-specific neural progenitors (ventral spinal progenitors for motor neurons and ventral midbrain progenitors for dopamine neurons); and (3) generation of postmitotic functional neurons (Figure 15.1A). The only difference, which is also critical for specification of other neuronal subtypes, is the application of appropriate morphogens to the primitive neuroepithelial cells. When the primitive neuroepithelial cells are cultured in the presence of RA and SHH, Olig2-expressing spinal motor neuron progenitors appear at around four weeks of hESC differentiation. Postmitotic HB9-expressing motor neurons appear at around five weeks. This time course corresponds to the program of motor neuron generation in the human embryo and most of the motor neurons exhibit characteristics of cervical/brachial motor neurons.

The present protocol, modified from our published report, has significantly increased the yield of HB9-expressing motor neurons from 20% to 50% of the total differentiated progenies. In the presence of FGF8 and SHH, the primitive neuroepithelial cells generate neurons that express tyrosine hydroxylase (TH) at about five weeks. The typical yield of TH-expressing dopamine neurons is about 20–35% of the total differentiated progenies. At present, not all the TH-expressing neurons contain all the transcription factors that a midbrain dopamine neuron possesses. There is thus a need for optimization of the protocol to improve the generation of authentic midbrain, especially substantia nigra, dopamine neurons.

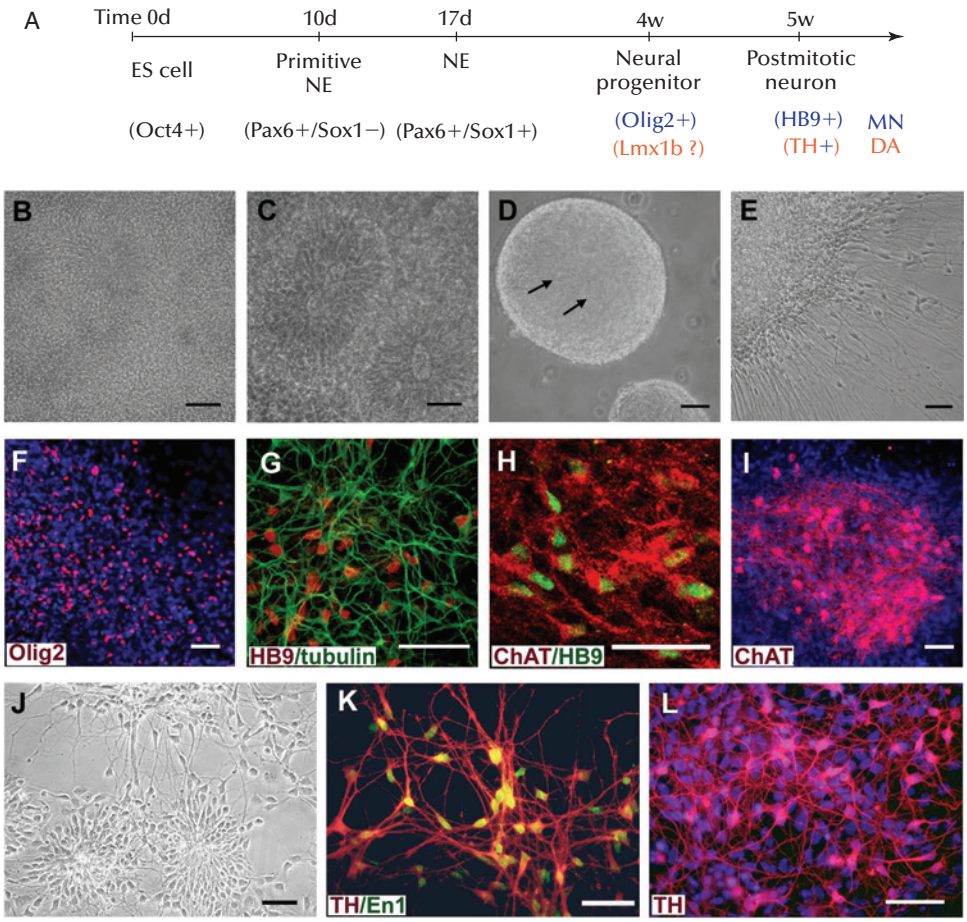

FIGURE 15.1 Generation of spinal motoneurons and midbrain dopaminergic neurons from hESCs. (A) Schematic procedure for neuronal differentiation. Primitive neuroepithelial cells were generated at 10 days of differentiation (B). After further culturing for 7 days, columnar epithelial cells developed multiple rosettes in the colony center (C). Rosette cells were separated from peripheral flat cells and cultured in neural induction medium. They formed spheres, which have rosettes inside indicated with arrows (D). For motor neuron differentiation, primitive neuroepithelial cells were treated with RA (0.1 μM) for one week before the rosettes were isolated from their surrounding cells. After 3–5 day suspension (days 20–22), neuroepithelial clusters were plated on the polyornithine and laminin substrate for differentiation in the presence of RA and SHH. Within 2 days after plating, numerous neurites extended out of the neuroepithelial cluster (E). After four weeks of differentiation many cells expressed Olig2 (F), a motoneuron progenitor marker and further differentiated to HB9+ post-mitotic motoneurons in another week (five weeks, G). After seven weeks of differentiation from ESCs, some cells became ChAT+ with the support of neurotrophic factors, these same cells double stained with HB9 (H, I). Neurons can differentiate immediately after plating neuroepithelial cells and gradually mature along time (J). Many TH-positive neurons express En-1 (K) and exhibit a complex neuronal morphology. TH-positive neurons induced by this protocol are about 30% of total cells. Blue indicates Hoechst-stained nuclei (L). (Reproduced in part from Li *et al.*, 2005 and Yan *et al.*, 2005, with permission.)

PROCEDURES

Differentiation of spinal motor neurons from hESCs

1. ESCs are differentiated to neuroepithelial cells in a six-well plate according to the procedure described in Chapter 14 for 10 days.
2. On day 10 of differentiation, primitive neuroepithelial cells characterized by their elongated, columnar morphology make up the majority of the inner cells in differentiating colonies (Figure 15.1A,B). At this stage, aspirate off the medium and add 3 mL of fresh neural induction medium containing retinoic acid (RA, 0.1 μ M) to each well of the six-well plate.

NOTE: RA is sometimes difficult to dissolve in solution and a yellow powder will persist in the ethanol solution. To facilitate the process add a small volume of DMSO to dissolve RA first, then add ethanol (do not exceed a DMSO:ethanol ratio of 1:3).

3. Feed the cultures every other day for one week by exchanging all of the medium with fresh neural induction medium + RA. Over the course of culture, the area of columnar epithelial cells increases and these cells pile up to form multiple cell layers in the center of the colony. Multiple neural-tube like rosettes are now obvious in each colony center (Figure 15.1C).

NOTE: Addition of RA will not affect the morphology of rosettes. RA should be added after the appearance of primitive neuroepithelial cells at 10 days. If it is added too early, RA will decrease the neuroepithelial induction efficiency.

4. On day 17–18, the differentiating neuroepithelial cells need to be subcultured. Add dispase at 0.5 mg/mL to the cells to isolate neuroepithelial clusters as described in Chapter 14. Gently triturate the neuroepithelial clusters with a 5 or 10 mL serological pipette up and down twice, but avoid breaking up the clusters. Centrifuge at 50×***g*** for 2 min at room temperature before aspirating off the supernatant.
5. Rinse the neuroepithelial clusters by adding 5 mL of neural induction medium (without growth factors), resuspend the cell pellet, and centrifuge at 50×***g*** for 2 min at room temperature.
6. Aspirate off the supernatant, resuspend the neuroepithelial cluster in 5 mL of neural induction medium containing B27, SHH (100 ng/mL), and RA (0.1 μM), and transfer the culture to a T25 flask (cells from three wells may be added to one T25 flask or 35 mm Petri dish).
7. The neuroepithelial clusters will form spheres in suspension culture (Figure 15.1D). They will be maintained in suspension culture for 5 days in the same medium as step 6. During this culture period the spheres may be broken into smaller clusters (100–200 μm in diameter) using a flame-polished Pasteur pipette. Following breaking, one T25 flask of cells should be split into two T25 flasks.
8. After 5 days, plate the neuroepithelial clusters onto laminin-coated culture dishes. For immunostaining, the cells may be plated onto glass coverslips treated

with polyornithine and laminin (see Chapter 14) (2–4 clusters/coverslip). After attachment cells should be fed with a neural differentiation medium which should initially be supplemented with RA (0.1 μM), SHH (100 ng/mL), cAMP (1 mM), ascorbic acid (200 ng/mL), and laminin (1 mg/mL).

NOTE: In our hands, when neuroepithelial cells were dissociated to single cells and plated, there are very few HB9+ motor neurons after differentiation. We usually plate small clusters (100–200 μm) for differentiation, which will attach and flatten out to an almost monolayer culture after a couple of days.

9. On day 24 (or 2 days after plating the cells on an adhesive substrate), numerous neurites extend out of the cluster. Some neuronal cells may also be seen surrounding the cluster (Figure 15.1E). Immunostaining of this culture on day 26–28 will reveal numerous cells expressing Olig2 (goat IgG, 1:400), a transcription factor expressed by spinal motor neuron progenitors (Figure 15.1F).
10. Continue the differentiation by feeding the culture with the same neural differentiation medium and additives, also now supplemented with neurotrophic factors (BDNF, GDNF, and IGF-I, 10 ng/mL). This medium should be changed every other day (half medium change). In about one week (or about five weeks of total differentiation), large axons extend out from the cluster and some neurons migrate to the periphery. Immunostaining at this stage will show at least 20% of the total neurons express HB9 (mouse IgG, 1:50) (Figure 15.1G), the motor neuron-specific transcription factor (Li *et al.*, 2005). Cells can also be stained for the general neuronal marker β III-tubulin (rabbit IgG, 1:5000).
11. After five weeks the adherent culture can be maintained to generate mature motor neurons. The culture medium will be the same base neural differentiation medium but the amount of RA and SHH is reduced to 0.01 μM and 10 ng/mL, respectively. We observed choline acetyltransferase (ChAT)-expressing (goat IgG, 1:100) mature motor neurons appearing at about six weeks after hESC differentiation (days 40–42) (Figure 15.1H,I).

Differentiation of dopaminergic neurons from hESCs

1. To start dopaminergic neural differentiation, ESCs are differentiated to neuroepithelial cells under FGF2 treatment (20 ng/mL) in a six-well plate according to the procedure described in Chapter 14 for 10 days.
2. On day 10 primitive neuroepithelial cells characterized by their elongated, columnar morphology make up the majority of the inner cells in differentiating colonies (Figure 15.1A,B). At this stage, aspirate off the medium and add 3 mL of fresh neural induction medium containing FGF8 (20 ng/mL) and SHH (100 ng/mL) to each well of the six-well plate.

NOTE: Heparin should always be added along with FGFs to stabilize their activity. FGFs are readily degraded in culture media. Addition of heparin at 2 μg/mL in neural induction medium will stabilize the activity of FGFs.

3. Feed the culture every other day for one week with fresh neural induction medium with FGF8/SHH replacing half of the medium. Over the course of culture the area of columnar epithelial cells increases and these cells pile up to form multiple cell layers in the center of the colony. Multiple neural-tube like rosettes are now obvious in each colony center (Figure 15.1C).

4. On day 17–18, the differentiating neureopithelial cells need to be subcultured. Add dispase at 0.5 mg/mL to the cells to isolate neuroepithelial clusters as described in Chapter 14. Gently triturate the neuroepithelial clusters with a 5 or 10 mL serological pipette up and down twice, but avoid breaking up the clusters. Centrifuge at 50×***g*** for 2 min at room temperature.

5. Aspirate off the supernatant and resuspend the neuroepithelial clusters in 5 mL of neural induction medium containing FGF8 (50 ng/mL), SHH (100 ng/mL), B27, ascorbic acid (200 μM) and suspend the cultures in a flask. (In general two wells of clusters from a six-well plate should be suspended in one T25 flask.)

6. The neuroepithelial clusters will form spheres (Figure 15.1D) and should be grown in suspension culture for 6 days in the same media as step 5, replacing half of the medium when feeding. During this culture period, the spheres may be broken into smaller clusters (100–200 μm) using a flame-polished Pasteur pipette and split to additional T25 flasks. (See the Pitfalls and advice section in Chapter 14 for further instructions on long-term culture of neuroepithelial cells.)

7. To differentiate mid/hindbrain patterned neuroepithelial cells, the clusters can be dissociated (unlike motor neuron differentiation). Collect the neuroepithelial clusters into a 15 mL tube and spin at 50×***g*** for 3 min. Aspirate medium and wash the spheres once with neural induction medium (without growth factors). Add accutase and trypsin (1:1, 1 mL for each T25 flask) and incubate at 37°C with occasional agitation. When clumps lose their outline (usually within 2–3 min), stop digestion by adding an equal volume of trypsin inhibitor. Spin at 100×***g*** for 3 min. Aspirate supernatant. Resuspend the cells in neuronal differentiation medium at the density of 200 000 cells/mL.

8. Evenly plate the cell suspension onto laminin-coated culture dishes or glass coverslips pre-coated with polyornithine/laminin (see Chapter 14) (about 10 000 cells in 50 μL for each coverslip, 60 000 cells in 300 μL for each well of a six-well tissue culture plate). Wait (1–2 h) for cells to attach, then add 400 μL for each coverslip and 1.2 mL for each well of six-well plates of neuronal differentiation medium containing FGF8 (50 ng/mL), SHH (100 ng/mL), B27, ascorbic acid (200 μM), cAMP (1 μM), laminin (1 μg/mL), TGFβ 3 (1 ng/mL) and trophic factors (BDNF 20 ng/mL, GDNF 50 ng/mL).

9. Continue the differentiation culture by feeding the culture with the same neuronal differentiation medium as detailed in step 8 every other day (half medium change). Six days later, withdraw FGF8. SHH is decreased to 10 ng/mL after two weeks (or about 38 days of total differentiation). At this point, individual neuroepithelial cells often re-aggregate to form small monolayer rosettes with differentiating neurons surrounding the clusters (Figure 15.1J). In a further two weeks, more mature dopamine neurons will be present. Immunostaining of the cultures will reveal numerous TH+ neurons expressing En-1, a transcription factor expressed by midbrain cells (Figure 15.1K). Many of the TH+ neurons exhibit a complex neuronal morphology (Figure 15.1L).

Alternative Procedures

Motor neuron differentiation in suspension

Neuroepithelial clusters can also be differentiated in suspension in the presence of RA and SHH. Olig2 and HB9 positive cells can be observed at similar time points as outlined above.

Pitfalls and Advice

Retinoic acid

RA is sensitive to light and should be protected in tinted 1.5 mL tubes or foil-wrapped 15 mL tubes. Experiments containing RA should be exposed to light for a minimal amount of time. We recommend making a fresh RA stock on a two-week basis.

Removal of dead cells in the neuroepithelial cluster suspension culture

The best way to remove debris from the culture medium is to let the flask stand and let the cell clusters settle to the bottom. Aspirate the medium containing debris and feed the culture with fresh medium. In addition, a low concentration of bFGF (1–10 ng/mL) in the culture medium will help the survival and proliferation of neuroepithelial cells without affecting the final outcome of motor neuron populations.

Mitogens and growth factors in the culture system

Neurotrophic factors (BDNF, GDNF, IGF1) were added to the culture at around four weeks after differentiation, which corresponds to the appearance of Olig2+ cells. At the same time, SHH is decreased to 10 ng/mL in long-term cultures since SHH is no longer needed for patterning the neural progenitors but is not completely omitted because of its potential role as a survival factor for motor neuron progenitors.

Enrichment of neuroepithelial cells

The presence of non-neural cells may interfere with neuroepithelial differentiation and subsequent positional patterning. Scratch off any colonies that do not contain any neuroepithelial cells (this should be less than 10% of colonies). The "bad" colonies can be marked by an objective marker lens under a phase contrast microscope and then scraped away with a pipette tip in a sterile hood.

Quality Control Methods

Motor neuron marker confirmation

Because all the antibodies for motoneuron-related markers are developed for avian and rodent species, we screened the expression of these markers on embryonic monkey (E34–36) spinal cord sections to confirm the specificity of these antibodies.

Supplies and Reagents

Supplies

Item	Supplier	Catalog no	Alternative
T25 flasks (polystyrene flasks with polyethylene filter cap)	Fisher Scientific	12-565-57	Nunc catalog no. 136196
T75 flasks	Fisher Scientific	12-565-31	Nunc catalog no. 178891
Six-well polystyrene plates	Fisher Scientific	12-565-73	Nunc catalog no. 140675
24-well polystyrene plates	Fisher Scientific	12-565-75	Nunc catalog no. 143982
Polystyrene conical tube, 15 mL	Fisher Scientific	05-527-90	BD Biosciences catalog no. 352095
Polystyrene conical tube, 50 mL	Fisher Scientific	14-432-23	BD Biosciences catalog No. 352073
Serological pipettes, 5, 10, and 25 mL	Fisher Scientific	13-678-11	
9″ Pasteur pipettes, cotton plugged	Fisher Scientific	13-678-8B	
9″ Pasteur pipettes	Fisher Scientific	13-678-20D	
50 mL Steri-Flip	Fisher Scientific	SCGP00525	
500 mL filter unit (0.22 μm sterilizing low protein binding membrane)	Corning Inc.	430513	
60 × 15 mm Petri dish	Fisher Scientific	08-757-13A	
50 mL Steri-Flip filters	Millipore	SCGP 005 25	

Reagents

Item	Supplier	Catalog no.
L-Glutamine solution (200 mM)	Sigma	G-7513
MEM non-essential amino acids solution	Invitrogen	11140-050
KnockOut™ serum replacement	Invitrogen	10828-028
Dulbecco's modified Eagle's medium: Nutrient mixture F12 1:1 (D-MEM/F12)	Invitrogen	11330-032
Dulbecco's modified Eagle's medium (D-MEM)	Invitrogen	11965-092
Neurobasal medium	Invitrogen	21103-049
2-Mercaptoethanol (14.3 M)	Sigma	M-7522
N2 supplement	Invitrogen	17502-048
Laminin from human placenta	Sigma	L6274
Bovine serum albumin (BSA)	Sigma	A-7906
Cyclic AMP	Sigma	D-0260
Ascorbic acid	Sigma	A-4403
Retinoic acid (RA)	Sigma	R-2625
Sonic hedgehog (SHH)	R&D Systems	1845-SH
Recombinant human FGF8	PeproTech	100-25
TGFβ 3	R&D Systems	243-B3

(*Continued*)

Item	Supplier	Catalog no.
Dispase	Invitrogen	17105-041
Acutase	Millipore	SCR005
Trypsin-EDTA (1×)	Invitrogen	25300-054
Trypsin inhibitor (1 mg/mL dissolved in D-MEM/F12 and sterile filtered)	Invitrogen	17075-029
Heparin	Sigma	H3149
Recombinant human FGF basic	R&D Systems	233-FB
Fibronectin from human plasma	Invitrogen	16000-044
Recombinant human BDNF	PeproTech	450-02
Recombinant human GDNF	PeproTech	450-10
Recombinant human IGF-I	PeproTech	100-11
Poly-2-Hydroxyethylmethacrylate (Poly-HEME)	Sigma	P-3932
Polyornithine	Sigma	P-3655
Fetal bovine serum	Sigma	F-2006
VenorGeM mycoplasma screening kit (PCR based)	Sigma	MP0025

Antibodies

Item	Supplier	Catalog no.
PAX6 monoclonal	Developmental Studies Hybridoma Bank	
SOX2 monoclonal	R&D Systems	MAB2018
N-Cadherin monoclonal, D-4	Santa Cruz Biotechnology	sc-8424
SOX1 rabbit polyclonal	Chemicon International	AB-5768
Goat anti-ChAT affinity purified polyclonal antibody	Chemicon International	AB144P
Rabbit anti-Olig2	Santa Cruz	sc-19969
Monoclonal antibody against MNR2 (HB9)	Development Studies Hybridoma Bank	81.5C10
Rabbit anti-β III-tubulin	Covance	PRB-435P
TH monoclonal	Sigma	T-2928
Lmx1b rabbit polyclonal	Gift from Yuqiang Ding	
Alexa fluor secondary antibodies	Molecular Probes/ Invitrogen	Match to desired primary and fluorescence your microscope can detect

Stock solutions

Component	Amount	Stock concentration
Recombinant human FGF2	Dissolved in sterilized PBS with 0.1% BSA and 2 μg/mL heparin	10 μg/mL, aliquot and store at –80°C
Heparin	Dissolve 20 mg heparin in 10 mL D-MEM medium	2 mg/mL, aliquot and store at –80°C

(*Continued*)

Component	Amount	Stock concentration
RA	Dissolved 3.004 mg in 10 mL ethanol	1 mM, store at –20°C
SHH	Dissolved 1 mg SHH in 10 mL sterilized PBS with 0.1% BSA	100 μg/mL, aliquot and store at –80°C
Recombinant human BDNF, GDNF, IFG-I	Dissolve 200 μg growth factors in 2 mL sterilize distilled water	100 μg/mL, aliquot and store at –80°C
Ascorbic acid	Dissolve 2 mg ascorbic acid in 10 mL PBS	200 μg/mL, aliquot and store at –80°C
TGFβ 3	Dissolve 10 μg TGFβ3 in 10 mL PBS	10 μ g/mL, aliquot and store at –80°C
Recombinant FGF8	Dissolved in sterilized PBS with 0.1% BSA and 2 μg/mL heparin	100 μg/mL, aliquot and store at –80°C
Cyclic AMP	Dissolved in sterilized water	1 mM, aliquot and store at –80°C

ESC medium (500 mL)

Component	Amount	Final concentration
D-MEM/F12	392.5 mL	
KnockOut serum replacement (KSR)	100 mL	20%
MEM non-essential amino acids solution	5 mL	0.1 mM
2-Mercaptoethanol (14.3 M)	3.5 μL	0.1 mM
L-Glutamine (200 mM)	2.5 mL	1 mM

Sterile filter with a 0.22 μm filter, add 4 ng/mL bFGF just prior to feeding cells, Medium is stored at 4°C for up to two weeks.

Neural induction medium (500 mL)

Component	Amount	Final concentration
D-MEM/F12	490 mL	
N2	5 mL	1×
MEM non-essential amino acids solution	5 mL	0.1 mM
Heparin (2 mg/mL)	500 μL	2 μg/mL

Sterile filter with a 0.22 μm filter, add cytokines and signaling molecules (such as FGFs) just prior to feeding cells.

Neural differentiation medium (500 mL)

Component	Amount	Final concentration
Neurobasal medium	490 mL	
N2	5 mL	1×
MEM non-essential amino acids solution	5 mL	0.1 mM

Sterile filter with a 0.22 μm filter, add cytokines and signaling molecules just prior to feeding cells.

Dispase solution (10 mL)

Component	Amount	Final concentration
Dispase	10 mg	1 mg/mL
D-MEM/F12	10 mL	

Leave in a 37°C water bath for 15 min and filter sterilize the dispase solution with a 50 mL-Steri-Flip before use.

Reading List

Jessell TM (2000). Neuronal specification in the spinal cord: inductive signals and transcriptional codes. *Nat Rev Genet* 1: 20–29.
This review summarizes the mechanisms underlying the formation of a diverse set of neural types in the spinal cord, including the gradient of patterning signals, transcriptional factors and extrinsic factors from mesoderm that control this patterning.

Li XJ, Du ZW, Zarnowska ED, Pankratz M, Hansen LO, Pearce RA, Zhang SC (2005). Specification of motoneurons from human embryonic stem cells. *Nat Biotechnol* 23: 215–221.
This study first reported successful generation of spinal motor neurons from hESCs by applying a set of morphogens in a specific time window.

Perrier AL, Tabar V, Barberi T, Rubio ME, Bruses J, Topf N, Harrison NL, Studer L (2004). Derivation of midbrain dopamine neurons from human embryonic stem cells. *Proc Natl Acad Sci USA* 101: 12543–12548.
This is the first study reporting generation of dopaminergic neurons exhibiting some midbrain properties from hESCs. A co-culture with stromal cells was employed in this study which potentially introduces foreign cells when these cultures are applied as donor cells for transplantation.

Yan Y, Yang D, Zarnowska ED, Du Z, Werbel B, Valliere C, Pearce RA, Thomson JA, Zhang SC (2005). Directed differentiation of dopaminergic neuronal subtypes from human embryonic stem cells. *Stem Cells* 23: 781–790.
This study reports efficient generation of midbrain and non-midbrain dopaminergic neurons from hESCs with a chemically defined medium and avoids the use of stromal feeders.

CHAPTER

16

Oligodendrocyte Differentiation from Human Embryonic Stem Cells

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INTRODUCTION

Oligodendrocytes are glial cells that play a critical role in supporting the central nervous system. Specifically, they insulate axons and nerve cells within the CNS by wrapping them with myelin sheaths. The myelin sheath enables fast, saltatory conduction of impulses along the axons of neurons, controlling functions such as walking, perception of visual stimuli, and cognitive processes. When axons become demyelinated (i.e. lose their myelin sheath), as occurs in multiple sclerosis (MS) and spinal cord injury (SCI), axons cannot properly function. This may be due to loss and/or damage of oligodendrocytes. Therefore, replacement of oligodendrocytes or oligodendrocyte progenitor cells (OPCs) by cellular replacement therapies may in part restore axonal conduction and normal neuronal function. One approach to producing oligodendrocytes is through differentiation from embryonic stem cells (ESCs).

Human Stem Cell Manual, edited by J. F. Loring, R. L. Wesselschmidt, and P. H. Schwartz.
ISBN: 978-0-1237-0465-8.

One of the greatest challenges facing human ESC (hESC) cellular therapy is the derivation of high-purity lineages. The protocol provided in this chapter addresses this challenge by deriving high-purity oligodendrocyte progenitor lineages from pluripotent hESCs. The ability to control differentiation of hESCs into highly pure populations of specific derivatives not only enables generation of specific cells for transplantation therapies but also provides powerful tools to study normal cellular development. Although other methods for generating OPCs are available, this protocol is reliable and results in OPCs of greater than 90% purity.

Overview

This chapter describes an efficient way to produce OPCs from hESCs; specific examples are given for the WA01 and WA07 lines. Differentiation into oligodendroglial progenitors is attained by using specialized media supplemented with specific growth and differentiation factors at key time points. The resulting oligodendroglial progenitors are then amplified and positively selected using mechanical enrichment.

We recommend that the same person follow these procedures from start to finish for the sake of consistency. It is also extremely important that there are no deviations from the original procedures. This process is tightly regulated, highly sensitive, and even a single skipped feeding can be detrimental to the differentiation protocol and cell yield. Once high-purity OPCs are produced, the cells can be used in a variety of *in vitro* or *in vivo* assays.

Procedures

hESC cultures for oligodendrocyte production

Human embryonic stem cells are grown and expanded on Matrigel™-coated flasks until the appropriate numbers for the differentiation protocol are obtained. (See Chapters 1 and 2).

One day prior to cell culture: Preparation of Matrigel-coated flasks

1. Coat T75 flasks by adding growth factor-reduced Matrigel diluted 1:30 in knockout D-MEM to flask. Swirl flask to ensure even spread of solution and set in a 4°C refrigerator overnight.
2. Before use, aspirate the Matrigel solution, replace with 20 mL of MEF-conditioned medium (MEF-CM; can be prepared in advance and frozen for at least one month) and place the flask for at least 1 h in a tissue culture incubator for thermal and pH balance.

Day 1: Thawing hESCs and initial growth

1. Warm 35 mL of MEF-CM in a 37°C water bath.
2. Quickly thaw a 1 mL vial of hESCs containing approximately 1.5×10^6 cells and add to 9 mL of MEF-CM. Spin cells down at $200 \times g$ for 4 min and

resuspend in 5 mL of MEF-CM medium. Carefully break up the pellet by trituration. Do not break up the cell clusters.

3. Add the resuspended cells to the flask, and add 8 ng/mL of bFGF (Add 0.8 μL of 10 μg/mL (10 ng/μL) stock solution for each milliliter of medium). Place flask in incubator at 37°C with 5% CO_2.

Days 7–14: Expansion of hESC cultures

1. Feed cells with 20–30 mL of new MEF-CM containing 8 ng/mL bFGF *every* day.
2. Passage cells once a week 1:4 or 1:6.
3. Before passaging, coat plates with Matrigel matrix as described above 24 h in advance.
4. On the day of passage, prepare MEF-CM + 8 ng/mL bFGF.
5. Dissociate cells by adding 10 mL of 1 mg/mL collagenase IV to the flask and leave it in the incubator for 2–5 min.
6. Aspirate collagenase and wash cells with 10 mL D-PBS. Aspirate D-PBS and add 10 mL of new MEF-CM to the flask. Scrape the hESCs in the medium with a cell scraper.
7. Collect the cells and distribute according to the desired splitting ratio into Matrigel-coated flasks and add sufficient medium plus 8 ng/mL of bFGF to each.
8. Return flasks to incubator.
9. Feed the cells daily with fresh medium plus 8 ng/mL of bFGF.

Cellular aggregates (start of differentiation)

Differentiation is started by removing the cells from the adherent substratum and treating with a new medium ("transition medium") for the first 2 days, which induces neural differentiation, and then continuing with glial restrictive medium (GRM) for the rest of the protocol to induce oligodendroglial differentiation.

Day 1: Transfer of hESCs to non-adhesive substratum in transition medium with FGF

1. Prepare 30 mL of transition medium + 4 ng/mL bFGF for each T75 flask (use 0.4 μL of 10 μg/mL bFGF stock for each milliliter of medium). Pre-warm and pH balance medium in the CO_2 incubator.
2. Treat hESCs with 10 mL per flask of 1 mg/mL collagenase IV for 2–5 min in incubator. Aspirate collagenase and wash cells with 10 mL of D-PBS.
3. Aspirate D-PBS, add 30 mL of pre-warmed transition medium, and scrape cells with a scraper to dislodge. Collect cells into a 50 mL centrifuge tube and pipette cell aggregates (about 3–5 times) to slightly break up large clumps.
4. Distribute cells to Costar low attachment six-well plates (5 mL cell suspension in each well). Incubate at 37°C, 5% CO_2.

Day 2: Feed cells with transition medium with FGF, EGF, and RA

1. Prepare 30 mL of transition medium + 4 ng/mL bFGF + 20 ng/mL EGF and 10 μM RA for each six-well plate (use 1000× EGF stock and 2000× RA stock).
2. Collect cells from each well of the six-well plate and combine in a 50 mL centrifuge tube. Spin cells at 200×*g* for 4 min.
3. Aspirate old medium and add 30 mL of new transition medium with supplements. Resuspend gently, without breaking up the cell clusters.
4. Distribute 5 mL to each well in the plate. Return cells to incubator.

Yellow sphere formation: neural progenitors

Days 3–10: Formation of aggregates – growth with RA

At the beginning of this period small clusters of 20–50 cells can be seen floating in the medium. At the end of this time period yellow spheres containing neuralized cells will be clearly observed as the only growing elements in the culture.

NOTE: The following procedure must be done every day.

1. Prepare 30–35 mL of GRM + 20 ng/mL EGF + 10 μM RA for each six-well plate of cells.

NOTE: Use minimal light during feeding since RA is light sensitive. For RA, use 1 μL for each milliliter of medium from the 10 μM/mL stock solution. Discard the vial after use.

2. Remove debris from the culture by low force centrifugation: collect cells in a 50 mL conical tube and centrifuge at 200×*g* for 2 min.
3. Aspirate supernatant and add 30 mL of new GRM + EGF + RA medium. Do not dissociate clumps.
4. Redistribute 5 mL in each well of a six-well dish.

Days 11–15: Medium aggregates – growth without RA

Yellow spheres are the only regularly shaped cellular aggregates growing in the cultures and are very likely to be visible macroscopically. Darker clusters in the culture have an irregular shape and a loose composition. Individual floating cells are discarded at every feeding.

1. Feed the cultures every other day (M-W-F).
2. Prepare 30 mL of GRM + 20 ng/mL EGF for each six-well plate of cells.
3. Collect cells and perform the same procedures as described for days 3–10 with a low centrifugation force (200×*g* for 1–2 min).

Days 16–28: Large clusters

Yellow sphere growth continues as unhealthy cells are discarded.

1. Change medium three times a week (usually M-W-F) and always return cells to the incubator as soon as possible.

2. Prepare 30 mL of GRM + 20 ng/mL EGF for each six-well plate of cells.
3. Collect the cells in 50 mL tubes and leave to settle without centrifugation for 5–10 min.
4. Aspirate supernatant and add new medium on top.
5. Redistribute the clusters: agitate the tube and immediately collect 15 mL using a 25 mL pipette and distribute suspension quickly to the first three wells.
6. Agitate the tube again, collect the remaining 15 mL, and distribute it in the other three wells.

Oligodendrocyte progenitors

A purification method is used by plating the yellow spheres on Matrigel-coated flasks. Plating will eliminate dead or non-adherent cells and promote outward migration from yellow spheres.

Day 28: Plating spheres on Matrigel

1. Prepare Matrigel matrix-coated T75 flask with 1:30 Matrigel in knockout D-MEM 24 h in advance.
2. Prepare 30 mL of GRM + 20 ng/mL EGF, place the medium in the coated flask and establish proper temperature and pH for at least 1 h in the CO_2 incubator.
3. Collect cells from the six-well plate into a 50 mL centrifuge tube. Let the spheres settle for 5 min.
4. Aspirate old medium and add a small amount (5 mL) of pre-warmed GRM + EGF.
5. Place cells into the coated T75 flask with the balanced medium.
6. Return to the incubator at 37°C, 5% CO_2.
7. The next day, gently shake the plated flask to dislodge non-adherent debris.

Day 29–34: Oligodendrocyte progenitors migrate out of yellow spheres

1. Change medium every M-W-F.
2. Prepare 30 mL of GRM + 20 ng/mL EGF for each Matrigel-coated flask.
3. Aspirate old medium.
4. Add 30 mL of new GRM + EGF to flask. Return to incubator.

Day 35–42: Purification, replating and imaging

The cultures will go through a panning process in which adherent cells (astrocytes, fibroblasts) attach to tissue culture plastic and the less-adherent OPCs are collected and recultured for *in vivo* use. At the same time, the cell population is sampled for immunocytochemistry by plating them on laminin or Matrigel-coated imaging

slides (Permanox Lab-Tek Chamber Slides, Nunc catalog no. 177437) or glass coverslips.

1. Prepare the required Matrigel-coated T75 flasks with 1:30 Matrigel in knockout D-MEM 24 h in advance. Prepare enough flasks for splitting the cells at a 1:2 ratio.
2. Add 25 mL of GRM + 20 ng/mL EGF to each flask; pre-warm and balance the pH of the medium in the CO_2 incubator for 1 h before use.
3. Prepare Matrigel 1:30 or laminin (10 μg/mL/cm^2)-coated imaging slides or coverslips in wells a day in advance. Replace the coating solution with GRM (without EGF) and place the slides or dishes in the CO_2 incubator for temperature and pH balance 1 h before use.
4. Aspirate medium from each cell-containing flask. Wash with 10 mL D-PBS. Aspirate again.
5. Add 7 mL of warm trypsin/EDTA to flask. Incubate 5–10 min at 37°C.
6. Add 7 mL of anti-trypsin solution to the flask. Collect the dispersed cells into a 15 mL centrifuge tube.
7. Take a small sample of dissociated cells to count cells using a hemocytometer. Take a 50 μL sample of the cell suspension and mix with 50 μL of Trypan Blue. Count live (unstained) and dead (blue-stained) cells.
8. Spin the cells at 250×***g*** for 5 min.
9. Panning for adherent cells: Aspirate medium and resuspend in 50 mL of GRM (without EGF) medium. Transfer cells to two T75 or one T150 uncoated tissue culture plastic flasks and incubate for 1 h at 37°C. This step allows astrocytes and other adherent cells to attach to the plastic bottom, while the less adherent oligodendrocytes will float in the medium.
10. Collect medium with a gentle shake of the flasks and transfer to centrifuge tubes.
11. Take a sample from the purified cell population for immunocytochemistry. Plate cells at 50 000 cells/cm^2 on imaging slides or coverslips. After 2 days cells are ready to be fixed and stained.
12. Split the rest of the purified cells into two Matrigel-coated T75 flasks prepared earlier containing GRM + 20 ng/mL EGF. After 7 days of growth, they are ready to be used for transplantation.

Monitoring cultures by morphology and immunocytochemistry

At each stage of the procedure, cultured cells exhibit distinct morphological characteristics. Observing these changes is a simple way to determine whether the protocol is working. However, morphological observations should always be confirmed by immunocytochemistry.

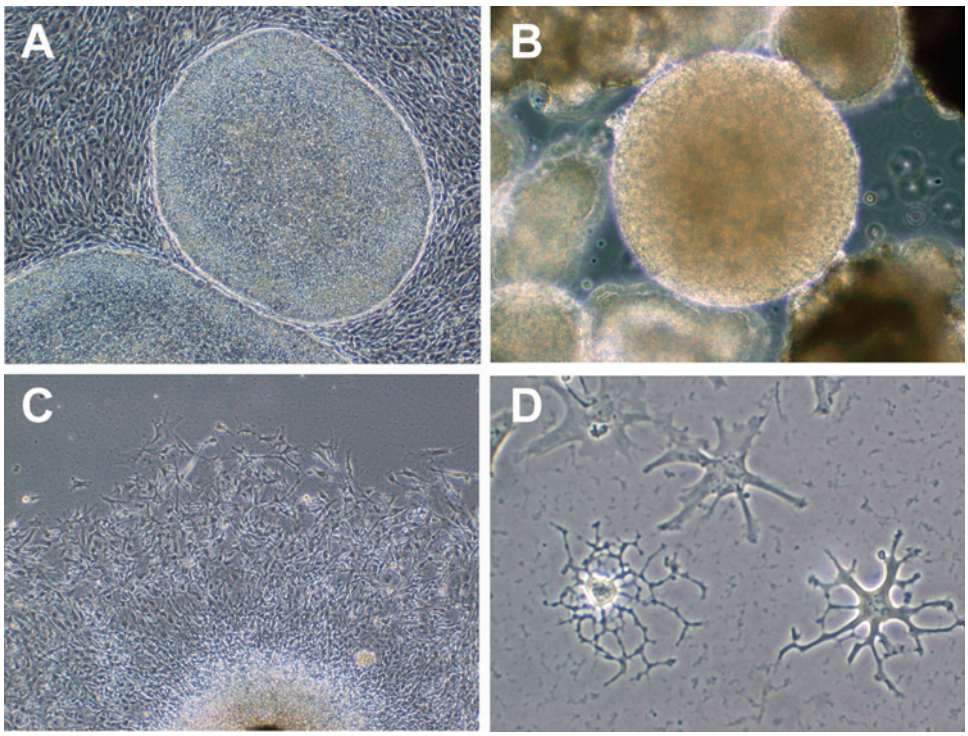

FIGURE 16.1 Morphological characterization. (A) Undifferentiated hESCs. (B) Yellow spheres/neural progenitors. (C) Oligodendrocyte progenitors. (D) Differentiated oligodendrocytes.

Morphological characterization

Undifferentiated hESCs (Figure 16.1A)

Undifferentiated hESCs grow in colonies with a smooth-looking surface. Some colonies will show some multilayered areas or areas of spontaneous differentiation. In the absence of an exogenous feeder layer, hESCs generate a subpopulation of migratory fibroblastic cells (extraembryonic endoderm derivatives).

Cellular aggregates

Aggregate clusters appear after plating partially dissociated hESC colonies in non-adhesive culture dishes. The aggregates will initially be inconsistent in size and not perfectly spherical. Isolated floating cells are usually not viable and are discarded at feeding with low force centrifugation. These aggregates are not "embryoid bodies," as defined for mouse ESCs, although some published reports use this term for hESC aggregates. Embryoid bodies are highly structured and have discernable layers such as Reichert's membrane. Consequently, we refer to the hESC aggregates as "cellular aggregates."

Yellow spheres/neural progenitors (Figure 16.1B)

Spheres form from the aggregate clusters, acquire a near-perfect spherical morphology, and are bright yellow. There will be small and large yellow spheres surrounded by cellular debris that includes dying cells. The spheres should be homogeneous without visible dark, necrotic centers. During this stage more and more yellow spheres are produced and fewer contaminants are seen. Establishment of healthy yellow spheres is critical for the next steps.

Oligodendrocyte progenitors (Figure 16.1C)

Plating of yellow spheres/neural progenitors allows selection of viable cells, dissociation of the spheres and further differentiation of neural progenitors into OPCs. During this stage migrating cells can exhibit either an epithelial or a bipolar morphology with short thick branches. Most importantly, they are positive for oligodendrocyte markers Olig1 and NG2. Some plated yellow spheres will extend long processes first and then OPC will migrate along such radial branches over the next few days.

Differentiation (Figure 16.1D)

After plating at low density in growth factor-free medium, some cells will acquire the shape of fully mature oligodendrocytes with branches and sheets and stain positive for oligodendroglial markers.

Immunocytochemical markers

This list offers suggestions for antibodies that are useful for characterizing the cells at each stage of the procedure. All of these antibodies require slightly different protocols and specific dilutions; refer to Chapter 9 on immunocytochemistry and the manufacturer's suggestions for specific protocols. A general immunocytochemistry protocol is provided.

Undifferentiated hESCs

- SSEA-4: a glycolipid epitope that is used as a marker of many pluripotent cells (Chemicon catalog no. MAB4304)
- POU5F1/OCT4: a transcription factor characteristic of pluripotent cells (Santa Cruz Biotechnology catalog no. sc-9081).

Cellular aggregates

This is a transition stage. Plated on an adherent substrate many cells will stain positive for embryonic markers (SSEA-4, POU5F1/OCT4) but some will express markers such as NESTIN or A2B5 after approximately 3 days. Occasionally neurogenic cores can be observed surrounded by non-labeled cells.

Yellow spheres/neural progenitors

Early yellow spheres (up to day 21) plated on adherent substrate will stain positive for:

- PAX6: a transcription factor indicative of neural commitment (Chemicon catalog no. AB5409)

- A2B5: marker of early neural progenitors (Chemicon catalog no. MAB312R)
- NESTIN: intermediate filament often used as a marker of neural commitment (Chemicon catalog no. MAB353)
- OLIG1/2: transcription factors expressed during oligodendroglial and motor neuron development (Chemicon catalog no. MAB345).

Oligodendrocyte progenitors

- OLIG1/2 (Chemicon catalog no. MAB345)
- NG2: chondroitin sulfate proteoglycan expressed in OPCs (Chemicon catalog no. AB5320)
- PDGFαR: growth factor receptor on immature oligodendrocytes (Chemicon catalog no. MAB3091)
- SOX10: a transcription factor (both nuclear and cytoplasmic) expressed in neural crest and both immature and mature oligodendrocytes.

Markers of contaminating cells

- GFAP: a marker of some neuronal precursors and of astrocytes (usually less than 5%)
- Neuron-specific cytoskeletal proteins (βtubulin, MAP2, TuJ1) (usually less than 5%)
- SMA (smooth muscle actin): occasionally seen in single cells (less than 0.1%).

Staining procedure

1. Fix cultures for 10 min at room temperature in freshly prepared 4% paraformaldehyde.
2. Wash three times in PBS for 5 min each.
3. Wash in 1% BSA + 0.1% Triton-X100 in PBS for 30 min at room temperature.
4. Wash three times in PBS for 5 min each.
5. Dilute primary antibodies in 1% BSA in PBS. Incubate overnight in 4°C.
6. Wash three times in PBS for 5 min each.
7. Block with 10% goat serum in PBS for 30 min at room temperature.
8. Wash three times in PBS for 5 min each.
9. Add secondary antibody corresponding to the primary host species and isotype, diluted 1:200 in PBS for 1 h at room temperature.
10. Wash three times in PBS for 5 min each.
11. Counterstain with Hoechst (1:1000) for 5 min at room temperature.
12. Wash three times in PBS for 5 min each and once with dH_2O.
13. Coverslip with an aqueous mounting medium that preserves fluorescence.

Note: We recommend AlexaFluor-conjugated secondary antibodies, because we have obtained persistent fluorescence after 1 year when slides were kept at room temperature.

Alternative Procedures

We recommend strict adherence to the protocol because timing and sequence of growth factor treatment is critical. Cultures can tolerate some differences in supplement concentration, but those changes may be reflected in the oligodendrocyte vs. contaminant cell yield.

Pitfalls and Advice

Contamination

Due to the length of the protocol (42 days), most problems that arise in stem cell differentiation result from improper sterile technique and subsequent contamination. It is best to always keep everything as sterile as possible and watch for unusual growth.

If contamination is caught early, cells may be salvaged. Repeated washing with sterile saline (HBSS) at feeding and addition of antibiotic for a week can sometimes restore the cultures. If contamination persists, cultures must be destroyed and the incubator decontaminated. We prefer to decontaminate the entire cell culture room.

The following preventative measures should minimize contamination.

- Work in a certified biosafety hood and calibrate incubators at least once a year.
- Spray anything that goes into the hood with generous amounts of 70% ethanol or alternative disinfectant (bleach or commercially available products).
- Spray down the hood with 70% ethanol before and after each use.
- Ethanol treat gloves often.
- Use barrier tips for all pipeting.
- Do not leave wrappers from tips, used tubes, or extra debris in the hood. Always remove items from hood immediately after use.
- Never leave the incubator door open.
- Rinse aspiration tubing after you're finished by aspirating 30% bleach.
- Keep all flask caps and six-well plate lids clear of medium or liquid. Replace wet caps and lids with new, clean ones.
- Keep all areas separate from other tissue/cellular use. If possible use the room only for stem cells.
- Aliquot reagents or supplements in one-dose vials.
- Do not work with the cells when you are sick (with flu, cold) and always wear a mask during the epidemic season.

- Water baths are a major source of contamination, and we have completely eliminated them from critical cell culture areas. Instead, medium is equilibrated in the incubator at 37°C for 1 h for pH and temperature balance.

NOTE: Check the water levels in the incubator to prevent evaporation of medium resulting in hyperosmolarity and sudden cell death.

Extensive cellular death at the culture dissociation step

The cause is usually overgrowth of the stem cell culture. Consider adding more medium or make two six-well plates from one flask of cells.

Yellow spheres are not forming

The quality of the stem cell culture is crucial for good differentiation. A good starting hESC culture should have large colonies clearly delineated with a smooth surface surrounded by abundant migrating ("stomal" or extraembryonic endoderm) cells. In our experience cultures that do not contain spontaneously differentiating cells generate very low yields of OPCs. In these cultures, hollow cystic cell aggregates form and fail to further differentiate. Cultures with too much spontaneous differentiation and multilayered colonies generate more contaminating neurons.

Retinoic acid quality can also be incriminated in failure of differentiation. The stock should be replaced after three months. Good, active RA is bright yellow but yellow intensity fades very quickly upon exposure to fluorescent light.

Failed terminal differentiation

The plating density for terminal differentiation in the absence of growth factors is critical. If the density is high, cells in culture will continue to proliferate. If density is too low, the culture will not survive. Mature oligodendrocytes do not survive for longer than 2–3 days. During characterization of plated imaging slides, we often see a mixture of young and semi-mature oligodendrocytes with few cells displaying a mature morphology.

SUPPLIES AND REAGENTS

- 15 and 50 mL conical tubes
- 1.5 mL centrifuge tubes
- T75, T150, and T225 cell culture flasks (Corning, Falcon, BD)
- Six-well low attachment plates (Costar catalog no. 3471)
- Barrier tips: 200 μL and 1000 μL
- 1 L Vacuum filters 0.22 μM polystyrene (Corning catalog no. 431205)
- Permanox Lab-Tek Chamber Slides (Nunc catalog no. 177437).

RECIPES

Stock solutions

Culture medium supplement stocks

Component	Supplier (Catalog no.)	Stock concentration	Final concentration
Progesterone	Sigma (P-6149) 1.0 mg	63 μg/mL	63 ng/mL
Putrescine	Sigma (P-6024-1MG)	100 μg/mL	0.1 μg/mL
Sodium selenite	Sigma (S9133-1MG)	50 μg/mL	50 ng/mL
Transferrin (human)	Sigma (8158-100MG)	50 mg/mL	50 μg/mL
T3 (triiodotyronine; triiodo-1-thyrosine)	Sigma (T67407-100MG)	40 μg/mL	40 ng/mL
Insulin, bovine	Sigma (I1882-100MG)	10 mg/mL	10 μg/mL
Retinoic acid – all *trans*	Sigma (R2625-50MG)	20 mM in DMSO	10 μM
Human bFGF	Invitrogen (13256-029) 10 μ g	10 μg/mL	4 ng/mL
Human EGF	Sigma (E9644-0.2MG)	20 μg/mL	20 ng/mL
B27 Supplement	Invitrogen (17504-044)	50×	

Other recommended reagents

Component	Supplier (Catalog no.)
Matrigel, growth factor reduced	BD Biosciences (356231)
D-MEM/F12	Invitrogen (10565-018)
Knockout D-MEM	Invitrogen (10829-018)
D-MEM medium	Invitrogen (12430-047)
Water for embryo transfer (WET)	Sigma (W1503)
D-PBS	Invitrogen (14190-144)
Trypsin/EDTA 0.05%/0.53 mM	Invitrogen (25300-054)

Culture medium supplement stock: Progesterone 1000× (16 mL)

Component	Supplier (catalog no.)	Stock concentration	Final concentration
Progesterone	Sigma (P-6149) 1.0 mg	63 μg/mL	63 ng/mL

Progesterone (1 mg): To solubilize add 1 mL absolute ethanol, gently swirl, and then add 15 mL D-MEM. Freeze as 1 mL aliquots at −20°C.

Culture medium supplement stock: Putrescine 1000×

Component	Supplier (Catalog no.)	Stock concentration	Final concentration
Putrescine	Sigma (P-7505)	10 mg/mL	10 μg/mL

Putrescine (100 mg): Dissolve in 10 mL sterile D-MEM. Freeze as 1 mL aliquots at −20°C.

Culture medium supplement stock: Sodium selenite 1000× (20 mL)

Component	Supplier (Catalog no.)	Stock concentration	Final concentration
Sodium selenite	Sigma (S9133-1MG)	50 μg/mL	50 ng/mL

Sodium selenite (1 mg): To prepare a 0.1 mg/mL stock, add 10 mL water (water for embryo transfer or "WET"). From this make a 1000× stock (50 μg/mL) by diluting 1 mL into 20 mL water. Freeze aliquots.

Culture medium supplement stock: Transferrin (human) 1000× (2 mL)

Component	Supplier (Catalog no.)	Stock concentration	Final concentration
Transferrin (human)	Sigma (8158-100MG)	50 mg/mL	50 μg/mL

Transferrin (10 mg): To prepare a 50 mg/mL stock solution add 2 mL sterile tissue culture medium. Gently swirl to dissolve.

Culture medium supplement stock: T3 (triiodotyronine) 1000× (100 mL)

Component	Supplier (Catalog no.)	Stock concentration	Final concentration
T3 (triiodothyronine; triiodo-1-thyronine)	Sigma (T67407-100MG)	40 μg/mL	40 ng/mL

T3 (triiodothyronine; triiodo-1-thyronine) (100 mg): To prepare a 40 μg/mL stock add 1 mL 1 N NaOH to dissolve (to 100 mg/mL) and then dilute 40 μL into 100 mL D-MEM (to 40 μg/mL).

Culture medium supplement stock: Insulin 1000× (10 mL)

Component	Supplier (Catalog no.)	Stock concentration	Final concentration
Insulin, bovine	Sigma (I1882-100MG)	10 mg/mL	10 μg/mL

Insulin, bovine (100 mg): To prepare a 10 mg/mL stock, add 100 μL glacial acetic acid to powder and then bring to 10 mL with water ("WET").

Culture medium supplement stock: Retinoic acid 2000× (8.3 mL)

Component	Supplier (Catalog no.)	Stock concentration	Final concentration
Retinoic acid – all *trans*	Sigma (R2625-50MG)	20 mM in DMSO	10 μM

Retinoic acid (50 mg): RA is supplied in sealed vials. This recipe is for a vial containing 50 mg of powder. Add 1 mL of DMSO to the vial and swirl to dissolve. Transfer the solution to a 15 mL tube and wash the ampoule with 200 μL of DMSO. Add DMSO to a final volume of 8.3 mL. Make 100–300 μL aliquots of this mixture in light protected vials and store at −80°C.

Culture medium supplement stock: Human basic FGF (bFGF) 10 μg/mL (1 mL)

Component	Supplier (Catalog no.)	Stock concentration	Final concentration
Human bFGF	Invitrogen (13256-029)	10 μg/mL PBS with 0.5% BSA (10 ng/μL)	4–8 ng/mL

Human bFGF (10 μg): Dissolve 10 μg in 1 mL PBS containing 0.2% BSA. Aliquot in 50–100 μL samples and store frozen aliquots at −20°C. Store thawed aliquots at 4°C for up to two weeks.

Culture medium supplement stock: Human EGF 20 μg/mL 1000× (10 mL)

Component	Supplier (Catalog no.)	Stock concentration	Final concentration
Human EGF	Sigma (E9644-0.2 mg)	20 μg/mL in HOAC/BSA	20 ng/mL

Human EGF (0.2 mg): Reconstitute the contents of the vial using 10 mL of 0.2 μm-filtered 10 mM acetic acid containing 0.1% BSA.

Culture medium supplement: B27

Item	Supplier (Catalog no.)	Concentration of stock
B27 Supplement	Invitrogen (17504-044)	50×

B27 is modification of serum-free Neurobasal medium. Its exact composition is proprietary, but a list of ingredients is given below. The reference to its composition is Brewer *et al.* (1993).

Components of Neurobasal	B27 added: Antioxidants	B27 added: Other
Linoleic acid	Catalase	Corticosterone
Linolenic acid	Superoxide dismutase	Biotin
Progesterone	DL-Tocopherol acetate	L-Carnitine
Albumin, bovine	DL-Tocopherol	D(+)-galactose
Putrescine	Glutathione (reduced)	Retinyl acetate
Selenium		Ethanolamine
Insulin		T3 (triiodo-1-thyronine)
Transferrin		

Modified from Podratz *et al.* (2004).

Collagenase IV (200 units/mL) (100 mL)

Component	Amount	Stock concentration
Collagenase IV (Invitrogen Catalog no. 17104-019)	20 000 units (typically 1 mg/mL)	200 U/mL in D-MEM

Dissolve 20 000 units of collagenase IV in 100 mL of D-MEM (usually 1 mg/mL). Filter using a 250 mL filter unit. Aliquot in 5–10 mL tubes and store at −20°C until use.

- Collagenase is isolated from *Clostridium histolyticum*. Type IV is selected because of low tryptic activity and is recommended for isolation of pancreatic islets.
- This is a crude product, so expect lot-to-lot variation.
- EDTA inhibits this enzyme's activity.
- A unit is defined as the amount of enzyme required to liberate 1 μM of L-leucine equivalents from collagen in 5 h at 37°C at pH 7.5.

Working solutions

MEF-conditioned medium

MEF-conditioned medium is made by incubating ES medium (without bFGF) for 24 h over a layer of MEFs.

The MEFs are plated at 5×10^5 per well of a six-well plate. This is 2.5 mL of a 2×10^5 cells/mL suspension. (See Chapter 3 on producing MEFs.)

bFGF is added fresh before using the medium for hESCs.

Glial restrictive medium (1000 mL)

Component	Amount (mL)	Final concentration
D-MEM/F12 (containing glutamine or Glutamax)	1000	
B27 supplement	20	1×
Insulin stock (1000×)	1	25 μg/mL)
Progesterone stock (1000×)	1	63 ng/mL
Putrescine stock (1000×)	1	10 μg/mL
Sodium selenite stock (1000×)	1	100 ng/mL
Transferrin stock (1000×)	1	50 μg/mL
T3 stock (1000×)	1	40 ng/mL

Mix the components in a filter cup/bottle and vacuum filter. Mix by gently swirling the bottle. Warm up only the required volume.

Transition medium

Mix glial restrictive medium and MEF-conditioned ES medium 1:1.

Matrigel coating

NOTE: Matrigel must be kept cold until ready for use because it gels instantly when it warms to room temperature. Manipulations after thawing have to be done quickly on ice. If it gels prematurely, Matrigel may be re-liquefied on ice at 2–8°C for 24–48 h.

1. Place the bottle in an ice-cold water bath and before completely thawing, open the cap and add 10 mL knockout D-MEM.
2. Dissolve the remaining frozen block by pipetting up and down (avoid foaming) and quickly aliquot 2 mL of 1:2 Matrigel into 15 mL centrifuge tubes. These aliquots must be immediately frozen and kept at –20°C until needed.
3. To prepare the working solution, 8 mL of knockout D-MEM are added to each 2 mL frozen aliquot for a total volume of 10 mL of 1:10 Matrigel.
4. For the final dilution, add 20 mL more knockout D-MEM (1:30) and add the pre-diluted Matrigel directly into the flask (5 mL in each). Swirl the coating

solution to cover the bottom and place the flasks in a 4°C refrigerator. The flasks can be used after 3 h or the next day.

5. One hour before the flasks are to be used, the coating Matrigel solution is discarded from the flask and replaced with working medium. The flask is placed in a CO_2 incubator for pH and temperature balance.

Additional information

Matrigel is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma. Its major component is laminin, followed by collagen IV, heparan sulfate proteoglycans, entactin and nidogen. It also has ECM-degrading enzymes such as tissue plasminogen activator. Reduced growth factor Matrigel contains reduced levels of TGFβ and FGF.

Information from Becton, Dickinson:

Growth factor	Typical growth factor concentration in GFR BD Matrigel matrix
EGF	<0.5 ng/mL
bFGF	n.d.
NGF	<0.2 ng/mL
PDGF	<5 pg/mL
IGF-I	5 ng/mL
TGF-β	1.7 ng/mL

n.d., not determined.

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CHAPTER

17

Cardiac Development of Human Embryonic Stem Cells

Maria Barcova, Victor M. Campa, and Mark Mercola

INTRODUCTION

Human embryonic stem cells (hESCs) offer an unprecedented opportunity to study human cardiomyogenesis and to produce a wide array of human cardiomyogenic cells for use in research applications and, potentially, for treatment of heart disease. hESCs can produce atrial and ventricular myocytes, as well as cells of the central and peripheral conducting systems in the heart. Despite recent advances, the possibility for successful clinical translation requires a concerted research effort because current production of cardiomyocytes from hESCs is far too inefficient and the methods must still be developed for integrating ESC-derived cardiomyocytes into damaged tissue so that they are compatible with host function and able to restore heart function.

In embryoid body (EB) cultures, hESCs spontaneously differentiate into multiple cardiac cell types, including atrial and ventricular myocytes, and endothelial and pacing cells. This complex differentiation most likely reflects a recapitulation of the inductive interactions between tissues that specify the heart development during early post-implantation development. Thus, the study of cardiogenesis in EBs presents an opportunity to understand these interactions at a molecular and genetic level. In vertebrate animal embryos such as mouse, frog, and chick, the heart becomes specified to develop

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in a region of the primitive streak mesoderm in response to signals from adjacent tissues, in particular from the anterior endoderm. Active research in a number of laboratories is directed toward characterizing the mechanisms that direct cardiogenesis and identifying natural and synthetic molecules that might be used to enhance the yield of cardiomyocytes.

Overview

This chapter presents procedures to produce and isolate cardiomyocytes from hESC cultures. Success at inducing cardiomyocytes depends critically on the first step, producing EBs. The EBs must be as free as possible from differentiating hESCs and residual feeder cells. Since the fraction of cardiomyocytes within an EB is relatively low, generally only a few tenths of a per cent, we focus on procedures for enrichment of cardiomyocytes, based on expression of the cardiomyocyte marker α-myosin heavy chain (MYH6/αMHC), using flow cytometry or antibiotic drug selection.

Fortunately, a hallmark of the immature, fetal cardiomyocytes that arise in EBs is spontaneous rhythmical contraction of about 1 Hz that can be readily detected visually in culture by light microscopy, making successful cardiogenesis readily apparent. Cardiomyocytes are also characterized by gene and protein marker expression that can be detected through immunocytochemistry or by reverse transcription polymerase chain reaction (RT-PCR). Over time, the immature cardiomyocytes mature into characteristic atrial, ventricular, and pacing cells that can be distinguished by gene and protein expression profiles and by characteristic action potentials. Physiologic recording from cardiomyocytes using either a current clamp or on a microelectrode array device allows definitive characterization of the cardiomyocyte type.

Procedures

Culture of hESCs

WA01 and WA09 or other lines are expanded on irradiated early passage (p3) mouse embryonic fibroblasts (MEFs) feeder layer (50 000 cells/cm^2) on Matrigel^{TM}-coated tissue culture dishes in hESC maintenance medium.

Note: We have observed that hESC maintained in MEF-conditioned medium, rather than in co-culture with MEFs, gradually lose their ability to form compact EBs, with resulting failure of cardiogenic differentiation. However, it is important to note that complete removal of MEFs from the undifferentiated hESC cultures immediately before EB formation is critical for successful spontaneous differentiation.

Genetically modified hESCs (MYH6/αMHC-GFP + MYH6/αMHC-Puro)

A simple approach for generation of pure cultures of cardiomyocytes from differentiating hESCs is to genetically engineer the cells to allow selection for the cells that

express cardiac-specific markers. We created a derivative of the WA09 hESC line that expresses two selectable markers under control of the MYH6/αMHC promoter: enhanced green fluorescent protein (eGFP) and a puromycin resistance gene.

We generated this cell line by a two-step process.

NOTE: See Chapter 19 for methods for infection of hESC with lentivirus.

In the first step, we introduced a reporter gene (eGFP) that would be expressed only when cells differentiate into cardiomyocytes. To allow selection of undifferentiated hESCs that contain this reporter, we included a drug-resistance cassette (NeoR) that is expressed under control of the promoter for a gene expressed in undifferentiated hESCs (ZFP42, aka REX1).

The cells were infected with a double-cassette lentiviral vector (Figure 17.1A) carrying the well-characterized −5.45 kb MYH6/αMHC promoter fragment directing eGFP expression and a ZFP42 (REX1)-neomycin resistance gene in a HIV-derived vector backbone.

The neo-resistance cassette expressed from the −700 bp ZFP42 (REX1) promoter confers resistance to the antibiotic G418 on pluripotent undifferentiated hESCs, while MYH6/αMHC-eGFP allows the visual identification of differentiated MYH6/αMHC-positive cardiomyocytes. We used G418 selection to generate an undifferentiated hESC clone carrying the MYH6/αMHC-GFP cassette.

In the second step, we re-infected the undifferentiated cells with another double-cassette lentiviral vector (Figure 17.1A) expressing a puromycin-resistance gene (puromycin *N*-acetyltransferase) from the MYH6/αMHC promoter and a blasticidin-resistance gene from the ZNF42 (REX1) promoter. We used blasticidin selection to generate an undifferentiated hESC clone carrying MYH6/αMHC-Puro as well as MYH6/αMHC-GFP.

The resulting hESC line, MYH6/αMHC-GFP/ MYH6/αMHC-Puro expresses both eGFP and puromycin resistance when the cells differentiate into MYH6/αMHC-expressing cardiomyocytes. This allows for selection of the differentiated cells both by fluorescence-activated cell sorting and by drug resistance.

Embryoid body formation

Maintenance of hESC cultures

- Undifferentiated MYH6/αMHC-GFP/ MYH6/αMHC-Puro hESC (passage >50) is expanded by co-culture with MEFs plated on Matrigel matrix in hESC maintenance medium.
- Medium is changed daily and cells are split 1:3 once a week.

Removal of MEFs

- hESCs must be completely free of MEFs in order to form differentiation-competent EBs.

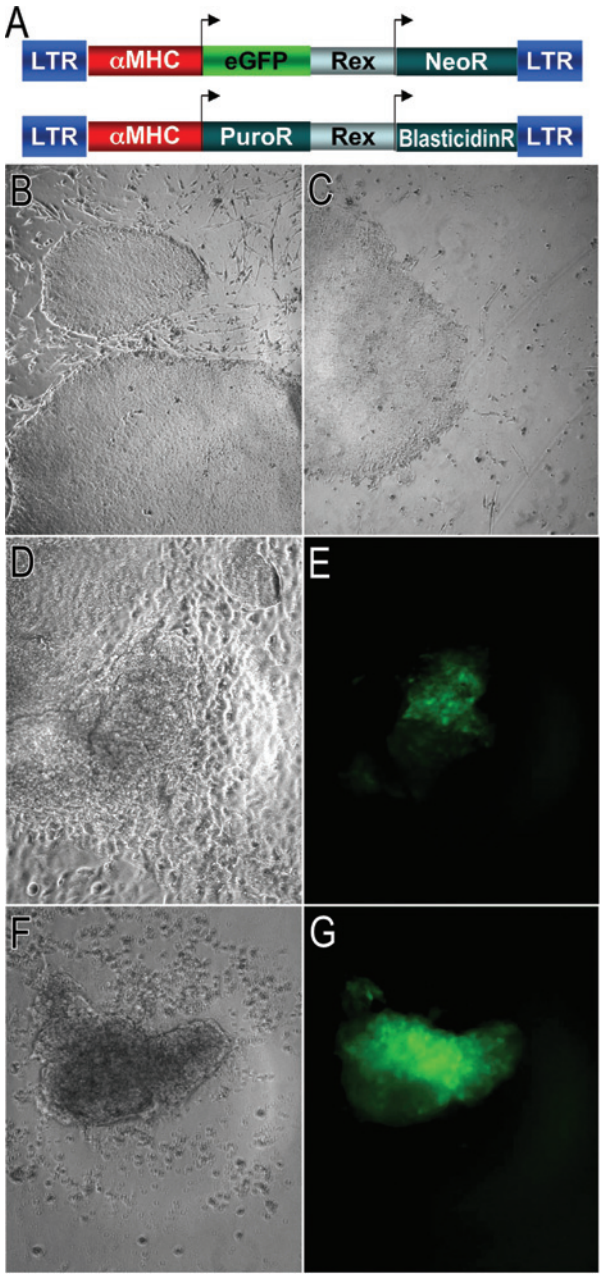

FIGURE 17.1 hESC-derived cardiomyocytes. (A) Upper HIV lentiviral based construct provides eGFP to visualize cardiomyocytes whereas lower construct allows blasticidin resistance selection of stem cells and puromycin resistance selection of cardiomyocytes during the differentiation process. In order to prepare hESC cultures for EB formation, blasticidin S antibiotic is added 6 days after plating (B) and most of the MEFs become detached by the next day (C). MEFs have an antagonizing effect on cardiomyocyte differentiation. Following mild collagenase treatment according to the protocol, hESC clusters are allowed to form EBs that spontaneously differentiate to yield a minor population of cardiomyocytes visible 8 days after initiation of EB differentiation, as shown in bright field (D) and eGFP epifluorescent (E) images. Puromycin-resistance selection is used according to the protocol provided to enrich for MYH6/αMHC-positive cardiomyocyte clusters, shown 10 days after initiation of EB differentiation (F,G).

- MEFs are blasticidin-sensitive, and are eliminated from hESC co-cultures (Figure 17.1B) by adding blasticidin S antibiotic at a final concentration of 5 μg/mL on day 6 after hESC plating.
- Most of the MEFs become detached (Figure 17.1C) by the next day.

NOTE: When hESCs differentiate they usually downregulate expression of ZFP42 (REX1), so in this cell line, blasticidin treatment also serves to clear spontaneously differentiated cells from the undifferentiated hESC cultures.

Formation of embryoid body (EB) spheres

1. Start EB formation on day 7 after hESC plating, or when blasticidin treatment has visibly affected the MEF feeder layer.
2. Prepare fresh collagenase IV solution and pre-warm to 37°C.
3. Aspirate medium from hESCs in six-well plate.
4. Add PBS (Ca^{2+}- and Mg^{2+}-free) to rinse the cells and remove detaching blasticidin-sensitive MEFs. Aspirate PBS.
5. Add 1 mL/well of collagenase IV solution and incubate for 6 min at 37°C. (Longer (10–12 min) incubation period loosens up the colonies into smaller clusters and is used for hESC passaging.)
6. Aspirate collagenase and wash cells by gently adding 4 mL PBS to each well.
7. Aspirate PBS and add 2 mL of EB differentiation medium into each well.
8. Scratch hESC colonies off the plate in cross-hatched fashion by using 2 mL aspirating pipette.
9. Carefully, without further pipetting and dissociating hESC clusters, transfer clusters into 15 mL conical tube.
10. Let hESC clusters sediment to the bottom of the tube for about 5 min. Aspirate supernatant containing residual single cells. This step is important for thorough removal of residual MEFs.
11. Gently resuspend the pellet by adding EB differentiation medium and transfer hESC clusters into ultra-low attachment six-well plates (Corning catalog no. 3471). Plate hESC clusters collected from two wells onto one well of the new plate.
12. Change medium the next day by transferring clusters into a 15 mL conical tube and allowing them to settle to the bottom of the tube for 5 min.
13. Change medium every day as described in previous step.
14. Plate cells on 0.1% gelatin-coated dishes or coverslips after 6 days in suspension.
15. GFP-positive beating areas (Figure 17.1D,E) are first visible on day 8 after induction of EB formation and the number of GFP-positive cells noticeably increases over time. Day 14 after EB formation (8 days after plating on gelatin), the cells are purified by fluorescence activated cell sorting (FACS) or drug resistance.

Enrichment of cardiomyocytes

Percoll gradient centrifugation

Cardiomyocytes can be enriched by dispersal of the cells followed by Percoll gradient centrifugation. This method is often used for enrichment of cardiomyocytes derived from non-genetically modified hESCs. Enrichment of cardiomyocytes to approximately 20% of the total cell population can be expected.

Flow cytometry or FACS isolation of fluorescently labeled cells

Day 14 cardiomyocytes derived from fluorescently labeled cell line (e.g. MYH6/αMHC-GFP line) can be analyzed by standard flow cytometry or enriched by FACS.

Survival of cardiomyocytes is limited following FACS, so isolation by FACS is most useful for when viable cells are not needed, such as for mRNA preparation.

Attached EB outgrowths are dispersed by gentle dissociation during incubation with Accutase™ (Chemicon). Most of the beating areas are dissociated within 15–20 min at 37°C.

Accutase is inactivated by dilution with EB differentiation medium and cells are passed through the 40 μm cell strainer to prepare samples free of cell clusters for FACS.

A negative control is essential to set FACS gates, in particular because cardiomyocytes are expected to be a minor fraction (<1%) of the total cell number despite being readily detectable by immunostaining or visual scanning for beating clusters. We use non-genetically modified day 14 EB outgrowths.

Drug selection of cardiomyocytes

Drug resistance was developed as a straightforward means of isolating populations of mESC-derived cardiomyocytes. We have used this approach effectively in hESCs using the −5.4 kb MYH6/αMHC promoter fragment to direct both eGFP and puromycin-resistance genes.

Antibiotics are added directly to the EB cultures and result in spheres of cardiomyocytes within a few days to a week, depending on the antibiotic.

Unlike FACS or Percoll sedimentation enrichment of cardiomyocytes, antibiotic resistance selection does not require dispersal of the cells. Thus, antibiotic selection can be used at any stages the resistance gene is expressed whereas dispersal can only be used to obtain very immature cardiomyocytes because the cells become quite sensitive to physical manipulation upon withdrawal from the cell cycle and onset of terminal differentiation.

Figure 17.1 F,G show bright field and eGFP fluorescent images of day 13 cardiac spheres derived from an MYH6/αMHC-GFP/ MYH6/αMHC-Puro hESC line treated with puromycin (1.8 μg/mL) for 3 days starting at day 10 post-induction of differentiation. Medium was changed daily during puromycin selection.

Clonogenic analyses

Rhythmically beating areas of cardiomyocytes are readily detectable within cultures of differentiating hESCs approximately 9 days after EB formation. At this stage the cardiomyocytes are still very immature and relatively robust; therefore, it is possible to disperse the beating areas into single cells and generate individual clones consisting of single cardiomyocytes. This allows study of the expression of lineage-specific markers and to perform electrophysiologic analyses in cells derived from the same cardiomyocyte. Using this assay it is also possible to study coupling and integration of individual cells after introduction into a network of bona fide cardiomyocytes or transplantation into an animal model.

Dispersal of cardiomyocytes for flow cytometry or clonogenic assays

EBs are prepared from hESCs as above. Identification of cardiomyocytes is readily apparent if labeled by eGFP expression from a myocardial promoter such as MYH6/αMHC. Differentiation is allowed to proceed for 9 days.

1. Isolate beating areas by microdissection in a cell culture hood. Collect the foci of beating cells in 1.5 mL Eppendorf tubes containing differentiation medium.
2. Wash dissected beating areas twice with PBS.
3. Incubate for 5 min at 37°C with 0.25% trypsin:EDTA.
4. Add 1 volume of differentiation medium with fetal calf serum (FCS) and pipette gently until cells become dispersed.
5. Pass cell suspension through a 50 μm cell strainer (BD Falcon) to remove clusters of cells. Rinse the single cell suspension with media.
6. Collect the cells by centrifugation for 5 min at 250×***g***. At this point, cells can be used directly for flow cytometry if they incorporate a fluorescent reporter protein such as eGFP, or immunostained by conventional methods for flow cytometry.
7. Coat glass coverslips with 1% gelatin:
   - Immerse glass coverslips for several hours in 1% gelatin.
   - Fix gelatin for 10–15 s with 0.5% glutaraldehyde in PBS.
   - Rinse three times with PBS, incubate with medium for 1 h, and rinse again with medium.
8. Resuspend cells in differentiation medium containing 10% FCS and plate them onto 1% gelatin-coated coverslips at low density (10 000–20 000 cells/cm^2). Check dispersed cells under microscope for viability, presence of clusters and plating density. More than 95% of cells should be single cells.
9. Incubate the cultures for several days, during which time the cardiomyocytes will divide a few times and form small colonies. Cells can then be characterized by immunocytochemistry and electrophysiology.

Characterization of cardiomyocytes

For immunological characterization, the cells are fixed under conditions appropriate for the antibody recognition of antigen, and immunostained.

- Characterization of atrial versus ventricular cell types often includes staining with a polyclonal antibody against atrial natriuretic peptide (ANP) and a monoclonal antibody against myosin light chain 2 v (MYL2/MLC2v).
- Atrial cells are characterized by MYL2/MLC2v^{neg} and ANP^{+}, whereas ventricular cells are MYL2/MLC2v^{+}, ANP^{neg} or^{+}, and indeterminate or immature cells are MYL2/MLC2v^{neg}, ANP^{neg}.
- An antibody against the cell cycle antigen MKI67, identified by the antibody Ki-67, can be used to study the proliferative status of cardiomyocytes.
- To study the electrical and mechanical coupling of hESC-derived cardiomyocytes, dispersed cells (or the whole beating area) can be plated over a monolayer of bona fide cardiomyocytes.
- One day after plating, hESC-derived cardiomyocytes beat and show calcium waves synchronous with bona fide cardiomyocytes.
- Antibodies directed against cadherins and connexins 43 and 40 stain junctions between hESC-derived and bona fide cardiomyocytes. Although expression of connexins in early cultures is initially weak, staining intensity increases with time, suggesting that maturation of cardiomyocytes and increased electrical coupling occurs, consistent with acquisition of more mature, atrial and ventricular action potentials.

Equipment

Specific equipment to study cardiomyocyte differentiation

A microelectrode array recording systems is available commercially from Multi Channel Systems (MCS) GmbH, Reutlingen, Germany (http://www.multichannelsystems.com). This system for *in vitro* applications includes a small amplifier for data acquisition, computer for recording and analysis, and can be accompanied by a programmable fluid perfusion temperature device. Specimen chambers with microelectrodes of various sizes arrayed at various distances and configurations are available from MCS as well as from Ayanda Biosystems SA (http://www.ayanda-biosys.com).

Supplies and Reagents

Supplies

Item	Supplier	Catalog no.
0.25% Trypsin:EDTA	Gibco	25200-056
Accutase	Chemicon	SCR005
Cell strainer	BD Falcon	352340

(*Continued*)

Item	Supplier	Catalog no.
Aspiration pipette	BD Falcon	357558
Matrigel	Becton Dickinson	356231
Six-well tissue culture dishes	Corning	3506

Antibodies

Item	Supplier	Catalog no.
ANP: Anti-atrial natriuretic peptide polyclonal antibody	Chemicon	AB5490
Cadherin: Anti-cadherin (PAN) epitope rabbit polyclonal antibody	Spring Bioscience	E2361
Connexin40: Anti-connexin40 rabbit polyclonal antibody	Alpha Diagnostic	CX40-A
Connexin43: Anti-connexin43 mouse monoclonal antibody	Chemicon International	MAB3067
Ki67: Anti- Ki67 rabbit polyclonal antibody	Novus Biologicals	NB 500-170
MF20: Anti-MF20 mouse monoclonal antibody (Donald A Fischman, MD).	Developmental Studies Hybridoma Bank	
MYL2/MLC2v: Anti-myosin light chain 2 mouse monoclonal antibody	Alexis Biochemicals	BC-1150-S
Titin: Anti-titin mouse monoclonal antibody	Chemicon International	MAB1553

PCR primers

Gene	Primer
cTnT	GGCAGCGGAAGAGGATGCTGAA and GAGGCACCAAGTTGGGCATGAACGAC, 150 nt product
hANP	GAACCAGAGGGGAGAGACAGAG and CCCTCAGCTTGCTTTTTAGGAG, 406 nt product
MYH6/αMHC	GTCATTGCTGAAACCGAGAATG and GCAAAGTACTGGATGACACGCT, 413 nt product

Recipes

Matrigel-coated plates

1. Thaw original bottle containing 10 mL Matrigel slowly on ice for 7–8 h or overnight. To make 15× stock, add 10 mL ice-cold knockout D-MEM and aliquot the mixture (1 mL/tube). Store at −20°C.
2. Prepare 1× Matrigel solution by adding 14 mL of ice-cold knockout D-MEM to a 1 mL aliquot thawed on ice. Prepare Matrigel-coated plates by adding 1 mL of 1× Matrigel per well in six-well cell culture plate.
3. Coat plates for either 2 h at room temperature or overnight at 4°C. If the plate is properly coated, it will appear to have a fine granular texture under phase contrast optics.

hESC maintenance medium

Component	Supplier	Catalog no.
Knockout D-MEM	Invitrogen	10829-018
20% KnockOut™ serum replacement (KSR)	Invitrogen	10828-028
Glutamine 1 mM	Invitrogen	11140-050
Non-essential amino acids (1×)	Invitrogen	11140-050
2-Mercaptoethanol (0.1 mM)	Sigma	M7522
Penicillin/streptomycin/amphotericin B (1×)	Omega Scientific	AA-40
FGF2 (8 ng/mL), added immediately before use	Invitrogen	13256-029
	Sigma	F0291

Puromycin solution

Component	Amount	Stock concentration
Puromycin (Sigma catalog no. P7130)	1.8 mg/mL in distilled water	1000×

Filter-sterilize through 0.2 μm filter.

Blasticidin solution

Component	Amount	Stock concentration
Blasticidin (Invitrogen catalog no. R210-01)	1.8 mg/mL in distilled water	1000×

Filter-sterilize through 0.2 μm filter.

Collagenase IV solution

Component	Amount	Stock concentration
Collegenase IV (Invitrogen catalog no. 17104-019)	10 mg in 10 mL knockout D-MEM	1 mg/mL

Filter through 0.22 μm filter unit. Store at 4°C and use within 2 days.

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CHAPTER

18

Hematopoiesis from Human Embryonic Stem Cells

Kausalia Vijayaragavan, Veronica Ramos-Meijia, and Mick Bhatia

INTRODUCTION

Hematopoiesis provides a well-defined system for studying molecular and cellular processes that control growth and development. The hematopoietic system consists of a heterogeneous array of cells ranging from rare primitive hematopoietic stem cells to large numbers of mature cells. Studies from amphibians, chicks, fish, and mammals show that blood cell emergence is established through waves of distinct hematopoietic compartments arising from distinct anatomical sites. In many of these animal embryogenesis models, initial emergence of blood cells is recognized in the extraembryonic yolk sac which principally sustains primitive erythropoiesis.

A second wave of hematopoiesis is then believed to occur within the embryo proper at the aorta-gonad-mesonephoros (AGM) and in the placenta. This supports the definitive erythropoiesis and the multi-potent hematopoietic stem cells (HSCs) that form the basis of the fetal and adult hematopoietic system. Later during development the hematopoietic cells briefly expand in the liver and then stabilize in the thymus and the bone marrow. The waves of hematopoiesis are intermingled with the emergence of endothelial cells – leading to the joint formation of blood and blood vessels.

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Although there is compelling evidence that the developmental phases of human hematopoiesis are analogous to those described for murine system, the scarcity of available human embryos at early stages and the limitations of assays have hampered the study of human embryonic hematopoiesis. Thus, human embryonic stem cells (hESCs) provide a powerful model system to better understand the cellular and molecular basis of human hematopoietic emergence and development. In addition, the generation of HSCs from hESCs could provide a significant source of transplantable cells and circumvent host compatibility for individuals undergoing therapy against cancer or blood-borne diseases.

Methods have been adopted from murine ESC technologies to influence spontaneous hematopoietic differentiation from hESCs, by the formation of embryoid bodies (EBs) or by co-culture with stromal cell lines to mimic potential hematopoietic microenvironments, along with introduction of relevant transcription factors in the hematopoietic cells.

Embryoid bodies are formed from aggregates of ESCs that spontaneously differentiate into multiple derivatives (including the mesoderm/blood lineage), mimicking some early developmental events. Although there is little evidence of spatial regulation of primitive hematopoietic differentiation within the human EBs, there seems to be a temporal sequence of events similar to many model embryo systems. While it is important to keep in mind that there are documented differences in hematopoietic differentiation between human and mouse ESCs, human EB formation may provide a manner to explore spatiotemporal emergence of early developmental hematopoiesis.

Overview

Hematopoietic differentiation

Hematopoietic differentiation can be compartmentalized into different cell types: the bipotential hemogenic and endothelial precursors cells, HSCs, hematopoietic progenitors, and mature hematopoietic cells. The compartments are characterized by differences in surrogate cell surface phenotype, which are correlated retrospectively to their functional properties using a variety of read-out assays (Table 18.1).

The induction and support of these various hematopoietic compartments during human EB development is influenced by different cytokines and growth factors. The use of cytokines, including SCF, Flt3L, IL-3, IL-6, G-CSF, VEGF, and the ventral mesoderm inducer BMP-4, have been shown to promote the differentiation of a highly enriched hematopoietic progenitor population that possess a $CD45^{+}CD34^{+}$ phenotype similar to the first definitive hematopoietic cell detected within the wall of the dorsal aorta of human embryos. Under these growth conditions we have also identified a unique BMP-responsive $CD45^{neg}$PECAM-1^{+}/Flk-1^{+}/VE-cadherin^{+} population (termed $CD45^{neg}$PFV), that gives rise to both hematopoietic and endothelial cells. Such clonogenic bipotential cells have also been characterized in the mouse embryo and murine EBs.

The *in vitro* generation of human multilineage HSCs from hESCs has been challenging. The "gold standard" assays defined by somatic HSCs, such as cord blood and

TABLE 18.1 Surrogate cell surface markers for the various hematopoietic compartments

	Surrogate cell surface phenotype	Cellular expression
Primitive		
	KDR/Flk^{+} VE-Cadherin^{neg}CD45^{neg}	Mesodermal derived cells precursor to hemogenic and endothelial cells?
	CD45^{neg}PECAM-1^{+}/Flk-1^{+}/ VE-cadherin^{+}	Hemogenic and endothelial precursor
	CD34^{+}CD38^{neg}CD45^{+}	Hematopoietic progenitor
	CD34bright	Immature progenitor
	CD34dim	Lineage-committed progenitors
	CD31^{+}, CD45^{neg}, vWF^{neg}, eNOS^{neg}, VE-Cadherin^{+}, Dil-AC-LDL uptake^{+}	Endothelial-like cells
	vWF^{neg}, eNOS^{neg}	Immature endothelial-like cells
	CD31/PECAM-1^{+}	Endothelial, myeloid and B cells
	CD34^{+}	Lympho-hemato progenitor and endothelial cells
	CD38^{+}	Lymphoid and plasma cells
	CD45^{+}/leukocyte common antigen	Hematopoietic cells
	VE-Cadherin^{+}	Endothelial cells
	CD4^{+} and CD8^{+}	Thymocytes
	CD15^{+}	Myeloid lineage marker
Mature	CD19^{+}	Most B cells

bone marrow, require demonstration of long-term repopulating ability *in vivo* in human/mouse (and recently sheep) xenotransplantation models. In these assays the cells we have generated from hESCs show the more limited characteristics of hematopoietic progenitors.

Properties of primitive hematopoietic cells derived from hESCs are systematically analyzed by methods established from somatic HSC studies, such as surrogate *in vitro* colony-forming units (CFUs) and long-term culture initiation cell (LTC-ICs) assays and *in vivo* using xenogenic models.

The CFU assays, which identify prospective hematopoietic progenitors, are quantified and qualified by identification of unipotent progenitor progeny when seeded into semi-solid media such as methylcellulose. These assays include: CFU-granulocyte (CFU-G), CFU-macrophage (CFU-M), CFU-GM and BFU-E (burst-forming units-erythroid) and multipotent progenitor progeny (CFU-GEMM).

The LTC-IC is an *in vitro* stromal cell-based assay that takes advantage of the ability of an infrequent and primitive hematopoietic cell population to produce myeloid progenitor

cells for a prolonged time. The LTC-ICs thus represent precursors to the CFUs and are considered to be the most primitive hematopoietic progenitors detectable *in vitro*.

Xenogenic models are used to prospectively identify hematopoietic cells that are capable of long-term repopulation of the entire hematopoietic system, which is the hallmark of an HSC. In most xenogenic models, hematopoietic cells are transplanted into immune-compromised mice (such as non-obese diabetic severe combined immunodeficiency (NOD-SCID), NOD-SCID2 or NK-depleted NOD-SCID) at various stages of development (fetal to adult) and/or different regions of hematopoietic ontogeny (intravenous, intrafemoral, and intrahepatic). The primitive human engrafting cell is defined as a SCID-repopulating cell (SRC) and is biologically distinct from CFUs and most LTC-ICs. Based on the somatic human SRC studies, the transduction efficiencies are small (1 SRC in 617), but when successful, they are able to produce about 400 000 progeny cells in 6–12 weeks post transplantation. Therefore populations defined *in vitro* cannot be considered HSCs and the SRC provides a "functional" surrogate for the human HSCs.

In this chapter, we provide techniques commonly employed by our laboratory to differentiate and phenotypically and functionally assay the hematopoietic cells derived from human EBs.

Procedures

Hematopoietic differentiation from hESC embryoid bodies

A schematic of hematopoietic differentiation protocol is shown in Figure 18.1.

hESC cultures

1. Approximately one week before forming embryoid bodies, pass hESCs onto six-well plates pre-coated with Matrigel™ at a 1:6 dilution in knockout D-MEM.
2. The day after the passage, change the medium (MEF-conditioned medium supplemented with 8 ng/mL hbFGF) and add 0.5 mL of 1:6 diluted Matrigel to each well to help form thick hESC colonies.
3. By day 7, the hESC culture should contain thick, well-defined healthy colonies with approximately 60/40 ratios between the undifferentiated hESCs and fibroblast-like cells (see Figure 18.1). This culture is ready for EB formation.

Differentiation of hematopoietic precursors

Day 0: Formation of embryoid bodies

1. Aspirate the medium from each well.
2. Add 0.5 mL of pre-warmed collagenase IV (200 units/mL) solution per well.
3. Incubate at 37°C for 5–10 min.
4. Aspirate the collagenase IV.

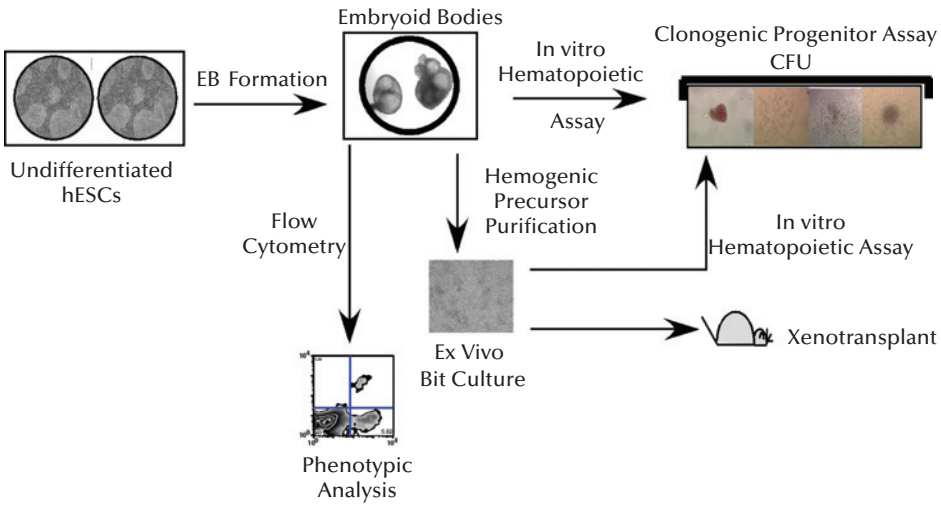

FIGURE 18.1 Schema of hematopoietic differentiation protocol from hESC-derived embryoid bodies.

5. Wash with 2 mL of pre-warmed knockout D-MEM per well and aspirate.
6. Add 2 mL of warm EB medium (80% knockout D-MEM, 20% FBS (non-heat inactivated), 10 mM non-essential amino acids, 1 mM L-glutamine and 0.1 mM 2-mercaptoethanol) to each well.
7. Using a 5 or 10 mL pipette, gently scrape the bottom of each well in "strips."
8. Transfer the content of 1.5 or 2 wells of undifferentiated hESCs to 1 well of an ultra low attachment plate.

CRITICAL STEP: Gently shake the plate left to right 1–2 times and then front to back 1–2 times for 5 s, to prevent agglomeration of EBs.

9. Add EB medium to each well up to a final volume of 4 mL.
10. Culture overnight at 37°C, 5% CO_2 to allow EB formation.

NOTE: The well ratio of hESC/EB must be 1.5/1 or 2/1 to account for the cell death observed the day after human EB formation.

NOTE: Only six-well plates have been tested successfully for human EB formation.

Day 1: Treatment of human EBs with hematopoietic cytokines and BMP-4

1. Collect content of each well into a separate 15 mL centrifuge tube.
2. Centrifuge at 129×*g* (as soon as the speed is reached, stop the centrifuge).

NOTE: Human EBs can be allowed to settle down by gravity as this may help preserve the integrity of the newly formed EBs. However as human EBs start becoming cystic and lighter in density, they should be centrifuged to facilitate change of media.

3. Add up to a total volume of 4 mL of EB medium supplemented with the hematopoietic cytokines (300 ng/mL hSCF, 300 ng/mL hFlt-3 L, 10 ng/mL hIL-3, hIL-6, and 50 ng/mL hG-CSF) and 25 ng/mL BMP-4 to each tube.
4. Transfer the human EBs gently to the same wells.
5. Continue to culture human EBs at 37°C, 5% CO_2.
6. Change the medium with the hematopoietic supplements every 4–5 days for a total of 15–22 days of human EB differentiation (see Figure 18.1).

Day 10, 15 or 22: Human embryoid body dissociation

The human EBs can be dissociated at any time during the course of their development; however, the highest hematopoietic differentiation was observed between days 10 and 22. Hematopoietic precursors ($CD45^{neg}PFV$) emerge around day 7 of human EB differentiation and at days 15–22 more progenitors ($CD34^+CD45^+$) and mature hematopoietic ($CD45^+$) populations emerge.

1. Transfer human EBs into 15 mL centrifuge tube and centrifuge at 129×***g*** for 3 min.
2. Aspirate supernatant and add 2–4 mL (depending on the numbers of human EBs) of pre-warmed collagenase B (0.4 units/mL).
3. Resuspend and return the human EBs to the wells.
4. Incubate in a 37°C, 5% CO_2 incubator for 2 h.
5. Collect the human EBs into a 15 mL centrifuge tube and centrifuge at 450×***g*** for 10 min.
6. Aspirate supernatant and resuspend human EBs in 2 mL of cell dissociation buffer (Invitrogen).
7. Incubate in a 37°C water bath for 10 min.
8. Centrifuge at 450× ***g*** for 10 min.
9. Aspirate supernatant and wash the dissociated EBs in PBS/3% FBS or IMDM.
10. Aspirate the supernatant and, depending on the number of EBs, resuspend in 200–500 μL of:
    (a) PBS/3% FBS for phenotypic characterization by flow cytometry analysis and/or
    (b) IMDM for CFU plating or *in vivo* experiments.
11. To achieve a single-cell suspension, gently triturate (40–50 times) with a 200 μL pipette (set at 100 μL) or a 1000 μL pipette (set at 200–450 μL) depending on the number of cells.
12. Filter through a sterile 40 μm cell strainer.
13. Count live and dead cells by Trypan Blue exclusion using a hemocytometer.

Phenotypic characterization by flow cytometry analysis

Phenotypic characterization of the different hematopoietic cell types derived from the human EBs is carried out by flow cytometry. Standard markers are specific surface cell differentiation (CD) fluorochrome-conjugated antibodies such as CD31, CD34, and CD45. Similar flow cytometry analysis, perhaps in combination with different mature CD antibodies, can also be performed on the 1′and 2′ hematopoietic colony (CFU)-derived cells or $CD45^{neg}$PFV-derived hematopoietic cells.

1. Add single-cell suspension ($0.5–2.5 \times 10^5$ cells/mL), either derived from the human EBs, CFUs, or $CD45^{neg}$PFV cells in PBS/3% FBS per polystyrene fluorescence activated cell sorting (FACS) tube.
2. Add a dilution 1/40 to 1/100 of each fluorochrome-conjugated antibody to the cell suspension in PBS/3% FBS and mix.

Critical Step: Primary antibody-fluorochrome conjugates are co-stained on three separate channels/tube (provided proper compensation is done): FL1 (FITC), FL2 (PE), and FL4 (APC) and leaving FL3 open for 7AAD viability staining.

3. Incubate for 30 min at 4°C in the dark (or 15 min at room temperature if time is limited).
4. Wash cells twice with 3–4 mL PBS/3% FBS.

Note: The antibody dilutions are only indicative; titration should be done for each new antibody.

5. Resuspend cells in 200–300 μL PBS/3% FBS.
6. Add 7AAD viability dye (dilution 1/100) to each tube.

Critical Step: This step is necessary to exclude the dead/dying cells left over from the cell dissociation protocols (up to 50% of cells in a treatment may be dead).

7. Incubate for 10–15 min at room temperature in the dark.

Note: Make sure that all settings have had preliminary four-color compensation carried out. This should be re-checked upon beginning of new experiments and preventative maintenance of the flow cytometer.

Functional analysis

Colony-forming unit assay

The CFU assay is a measure of the clonogenic progenitor capacity of hematopoietic cells. Counts of different types of colonies are carried out based on morphological characteristics.

1. Thaw methylcellulose H4230 supplemented with 3 U/mL hEPO, 50 ng/mL hSCF, 10 ng/mL hGM-CSF and 10 ng/mL hIL-3 at room temperature.
2. In a 1.5 mL sterile Eppendorf tube, add the proper number of live cells for the final number of cells wanted in the well, in a 100 μL volume of pre-warmed IMDM.

TABLE 18.2 Cell dilutions for colony forming unit (CFU) assay

Final cell no./well	Final vol/well (μL)	Cell nos in 100 μL medium	Methylcellulose H4230$^+$ (μL)
100 K	500	140 K	600
75 K	500	105 K	600
50 K	500	70 K	600
25 K	500	35 K	600
15 K	500	21 K	600
10 K	500	14 K	600
5 K	500	7 K	600

3. Using a 1 mL syringe coupled to a 16-gauge needle, add the proper volume of methylcellulose to the cell suspension (Table 18.2).
4. Mix the cells and methylcellulose by vortexing a few seconds.
5. Let the tube stand for 5 min to remove bubbles.
6. Using a 1 mL syringe coupled to a 16-gauge needle, transfer 500 μL of the cell preparation to one well of 12-well non-tissue culture-treated plates. Move the plate around slowly in order to distribute methylcellulose evenly in the well.

CRITICAL STEP: It is important to only transfer 500 μL of the methylcellulose cell mixture so as to make sure equal numbers of cells are plated into each well.

7. Add autoclaved MilliQ water to the surrounding empty wells to maintain moisture within the plate.

CRITICAL STEP: Make sure that the wells do not dry up.

8. Culture the cells in a 37°C, 5% CO_2 incubator.
9. According to the standard morphological criteria, identify and count the different colonies under an inverted microscope at two different time points after the day of plating (between days 7 and 10 and at day 14). Colonies can be examined up to 40 days.

Flow cytometry can be performed on the colonies; preparation of single-cell suspension for flow is described below.

CRITICAL STEP: A colony of 10 000 single cells is usually adequate. Generally 2–3 different antibody combinations can be performed from large colonies, but for smaller colonies it may be best to pool 2–3 colonies in a single treatment.

NOTE: We have gone as low as 4000 cells if numbers are limiting.

Preparation of single-cell suspension of hematopoietic cells from 1′and 2′ CFUs

Cells should be growing as clusters/colonies in methylcellulose (from at least 8–10 days onwards).

1. Under a microscope or stereoscope, pluck individual colonies.
2. Count cells if possible or proceed directly to flow cytometry staining.
3. Due to increased background "noise," always be sure to include an isotype tube for analysis. This should either be a small aliquot from the colony to be analyzed or from a similar colony from the same plate as the colony to be analyzed.

NOTE: If cells are not washed/filtered adequately, residual methylcellulose will increase background "noise" during FACS analysis.

Isolation of individual CFUs from methylcellulose for secondary plating

Secondary plating from primary CFUs is performed to assay for progenitor self-renewal abilities and is measured as the number of secondary colonies arising from individual primary CFUs.

1. Under microscope or stereoscope, pluck individual colonies with a 20 μL pipette in as little methylcellulose as possible, and transfer the colonies into Eppendorf centrifuge tubes containing 300–500 μL of pre-warmed IMDM.

NOTE: Use either large single colonies or pool 3–8 smaller colonies of similar composition.

2. Let sit for 20–30 min then centrifuge for 5 min at 450× *g* in a microcentrifuge.
3. Aspirate the supernatant, leaving behind approximately 60 μL of IMDM.
4. Wash once with 300–500 μL of IMDM to remove methylcellulose.
5. Centrifuge for 5 min at 450× *g* in a microcentrifuge.
6. Aspirate the supernatant, leaving behind approximately 60 μL of IMDM.
7. Count the cells (if possible).
8. Add 300 μL of methylcellulose to the tube and vortex.
9. Plate 250 μL of cells into one well of a 48-well suspension plate.
10. Identify and count the different colonies as described above.

Flow cytometry can be performed on the colonies or on single-cell suspension as described above.

Purification of $CD45^{neg}PFV$ precursors (by FACS)

We have shown that a hemogenic endothelial bipotential population that expresses PECAM-1, Flk-1, and VE-cadherin, but not CD45 (termed $CD45^{neg}PFV$) specified from hESCs during human EB development is uniquely responsible for the hematopoietic cell fate. Purification of this intermediate population offers a powerful clonogenic model system to study lineage relationships in human hematopoietic differentiation.

1. Resuspend single cells dissociated from approximately day 10 human EBs (see method above) in PBS/3% FBS (2–5 μ 10^6 cells/mL).

NOTE: Human EBs may be used between days 7 and 11.

2. Pre-coat 5 mL FACS tubes with 0.5 mL of FBS for 30 min for collection of the sorted cells.
3. Add a dilution of 1/50 of anti-human PECAM-1/CD31 PE-conjugated monoclonal antibody and anti-human CD45-APC monoclonal antibody to the cell suspension.
4. Add a 1/100 dilution of mouse IgG-PE and mouse IgG-APC monoclonal antibodies into isotype control tubes containing the cell suspension.
5. Incubate for 30 min at 4°C.
6. Centrifuge the tubes at 450×***g*** for 5 min then aspirate supernatant.
7. Wash twice with 3 mL of PBS/3% FBS then resuspend cells in PBS/20% FBS at a concentration of 2×10^6/mL.
8. Add a dilution 1/50 of 7-AAD dye to the cell suspension and stain for 10 min at room temperature to exclude dead cells.
9. Filter cell suspension through a sterile 40 μm cell strainer just prior to sorting in order to remove cell clumps that could clog the sorting nozzle.
10. Set sorting gates (purity or enrichment mode will depend on applications), including histogram markers and dot plot quadrants, by use of respective IgG isotype controls. Sort the $CD45^{neg}PECAM\text{-}1^{+}$ subpopulation that is used as a strategy for purification of $CD45^{neg}$PFV cells.
11. Determine purity immediately after sorting using the same sorting gate settings.
12. Wash the cells with 2 mL of IMDM medium and centrifuge at 450×***g*** for 5 min.
13. Count live and dead cells by Trypan Blue exclusion using a hemocytometer.

Derivation of hematopoietic cells from $CD45^{neg}$PFV precursors – HEM-culture

1. After sorting (purity mode), certrifuge the $CD45^{neg}$PFV cells at 450×***g*** for 5 min.
2. Aspirate supernatant, add 2 mL of IMDM medium and centrifuge at 450×***g*** for 5 min.
3. Resuspend cells at a final concentration of 2.5×10^5 per mL in serum-free hematopoietic conducive 1× BIT medium (1× BIT serum substitute, 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol, 300 ng/mL hSCF, 300 ng/mL hFlt-3 L, 10 ng/mL hIL-3, 10 ng/mL hIL-6, 50 ng/mL hG-CSF, in IMDM).

CRITICAL STEP: The hematopoietic and endothelial medium with supplements must be prepared prior to use and can be stored at 4°C only up to 12 h.

4. Seed $CD45^{neg}$PFV cells in fibronectin-coated plates at a final density of 5×10^4 cells/cm^2: add 200 μL/well for a 96-well plate and 1 mL/well for a 24-well plate.

5. At days 3 and 5, gently replace half the volume of medium with freshly prepared serum-free hematopoietic conducive 1× BIT medium. Single bright round hematopoietic cells emerge around day 3.

Critical Step: It is crucial to only remove half the BIT medium to avoid discarding these cells during medium replacement.

6. At day 7, add cell dissociation buffer (Invitrogen) to each well (50 μL for 96-well plate, 200 μL for 24-well plate) and incubate at room temperature to dissociate the adherent cells.
7. Examine the cells under the microscope until they round up (requires 1–5 min).
8. Gently pipette the dissociated cells and transfer them to 15 mL centrifuge tubes.
9. Centrifuge at 450×*g* for 5 min, aspirate supernatant and wash once with IMDM medium.
10. Count live and dead cells by Trypan Blue exclusion using a hemocytometer.
11. Prepare 5000–100 000 viable single cells/tube for flow cytometric analysis, 10 000–50 000 viable cells for CFU plating, and 500 000–1 000 000 viable cells for *in vivo* xenotransplant experiments.

Critical Step: A culture of 10 000 single $CD45^{neg}$PFV-derived cells is adequate for flow cytometry analysis, however, 20 000–50 000 cells/analysis is preferable.

Derivation of endothelial cells from $CD45^{neg}$PFV precursors – ENDO-culture

1. Prepare a single-cell suspension of hematopoietic cells from $CD45^{neg}$PFV-derived cells:
   (a) Dissociate cells according to the appropriate protocols.
   (b) Resuspend cells in PBS/3% FBS.
   (c) Filter through 40 or 70 mm cell strainers to obtain a single-cell suspension.
   (d) Count live and dead cells by Trypan Blue exclusion using a hemocytometer and remove appropriate numbers for flow cytometry analysis.
2. Seed $CD45^{neg}$PFV cells in fibronectin-coated plates at a final density of 5×10^4 cells/cm^2. Add 200 μL/well for a 96-well plate and 1 mL/well for a 24-well plate.
3. Culture the cells for 7 days in Medium-199 supplemented with endothelial growth factors (20% FBS, 50 ng/mL bovine pituitary extract, 10 UI/mL heparin and 5 ng/mL hVEGF-A_{165}).

Critical Step: The hematopoietic and endothelial medium with supplements must be prepared prior to use and can be stored at 4°C only up to 12 h.

4. Change the medium at days 2, 4 and 6 of culture.
5. Use primary human umbilical vein endothelial cells (HUVECs) as a positive control of ENDO-culture conditions.

Supplies and Reagents

Culture media

Item	Supplier	Catalog no.	Alternative
Knockout D-MEM	Invitrogen	10829-018	
BIT serum substitute	Stem Cell Technologies	BIT-9500	
Iscove's modified Dulbecco's medium (IMDM)	Invitrogen	12440-053	
Medium-199	Invitrogen	12340-030	
Fetal bovine serum (FBS)	HyClone	SH-30070	
Methylcellulose H4230	Stem Cell Technologies	H4230	
L-Glutamine	Invitrogen	25030-081	
2-Mercaptoethanol	Sigma	M-7522	
Non-essential amino acids	Invitrogen	11140-050	

Extracellular matrix

Item	Supplier	Catalog no.	Alternative
Matrigel (growth factor reduced)	BD Biosciences	354230	
Heparin (1000 IU/mL ampoule)	Leo Pharma Inc.		

Enzymes

Item	Supplier	Catalog no.	Alternative
Collagenase IV	Invitrogen	17104-019	
Collagenase B	Roche	11088807001	
Cell dissociation buffer – enzyme free	Invitrogen	13151-014	
Trypsin/EDTA (0.05%)	Invitrogen	25300-062	

Growth factors

Item	Supplier	Catalog no.	Alternative
hbFGF	Invitrogen	13256-029	
Stem cell factor (hSCF)	R&D Systems	225-SC	
Flt-3 ligand (hFlt-3 L)	R&D Systems	308-FK	
Interleukin-3 (hIL-3)	R&D Systems	203-IL	
Interleukin-6 (hIL-6)	R&D Systems	206-IL	
Granulocyte colony-stimulating factor (hG-CSF)	R&D Systems	214-CS	

(*Continued*)

Item	Supplier	Catalog no.	Alternative
BMP-4	R&D Systems	314-BP	
Erythropoietin (hEPO)	R&D Systems	286-EP	
Granulocyte monocyte colony-stimulating growth factor (hGM-CSF)	R&D Systems	215-GM	
Bovine pituitary extract	Invitrogen	13028-014	
hVEGF-A165	R&D Systems	293-VE-010	

Flow cytometry and FACS

Item	Supplier	Catalog no.	Alternative
7AAD viability dye	Beckman Coulter	OM3422	
Anti-human PECAM-1/CD31 PE-conjugated monoclonal antibody	Pharmingen-BD Biosciences	555546	
Anti-human CD45-APC monoclonal antibody	BD Biosciences	555485	
Anti-human CD34-FITC monoclonal antibody	BD Biosciences	555821	

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PART

IV

Genetic Manipulation of Stem Cells

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CHAPTER

19

Genetic Manipulation of Human Embryonic Stem Cells: Lentivirus Vectors

Ruchi Bajpai and Alexey Terskikh

Introduction

Genetic modifications are essential for labeling of human embryonic stem cells (hESCs) with expression markers, overexpression/knockdown of genes (corrective or directive) for use in screening and transplantation experiments. However, the most widely used methods for DNA delivery, such as lipid-mediated transfection and electroporation, are not well developed for hESCs. Unlike mouse ESCs, hESCs are difficult to clone, which presents an additional challenge for selecting rare genetically modified events. In the face of these limitations the relatively simple procedure of lentivirus-mediated DNA delivery is an effective method for genetic manipulation of hESC cells. Modification of hESC cells with viruses using fluorescent markers allows for the separation of groups of cells manually under a fluorescence-equipped dissecting microscope or by fluorescence activated cell sorting (FACS).

Overview

A wide variety of lentiviral vectors commonly used for efficiently transducing hESCs are derived from HIV-1. These second- and third-generation vectors, when packaged,

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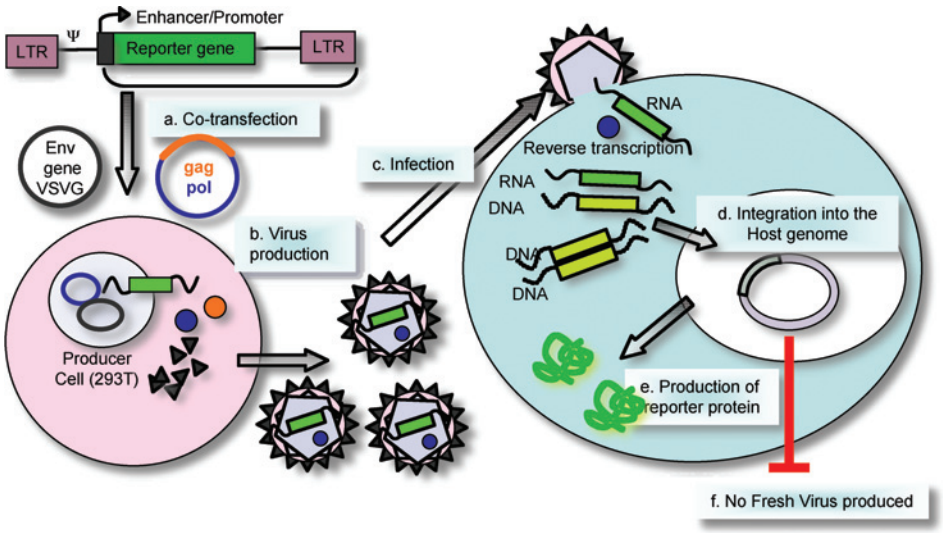

FIGURE 19.1 Vector production process.

result in replication-deficient, self-inactivating viral particles with extremely low probability of formation of replication-competent recombinants (RCRs). Figure 19.1 outlines the process of producing vectors.

The self inactivating process is achieved by eliminating most of the accessory genes that play important roles in the life cycle and virulence of the unmodified wild-type virus. In addition, separating the *cis*-acting elements from the *trans*-acting proteins (the "transfer" vector, comprising the promoters and genes of interest from the "packaging" vectors that separately encode the essential genes for viral production packaging and integration) ensures that the formation of an RCR necessarily requires multiple rare recombination events. These advances in vector design have significantly improved vector safety and virtually eliminated the concern for the formation of pathogenic, replication-competent virus during vector production or target cell infection.

Lentiviruses can transduce post-mitotic cells at high efficiency. They can stably integrate into the genome without incurring cellular toxicity and maintain sustained expression of the transgene during prolonged proliferation and subsequent differentiation. The simple genetic organization of the transfer vector coupled to a cloning capacity of approximately 9 kb makes it an effective tool for delivery of most transgenes to both actively dividing and non-dividing (cycling, quiescent, or terminally differentiated) cells.

Figure 19.2 shows the types of vectors that are typically used to genetically modify hESCs.

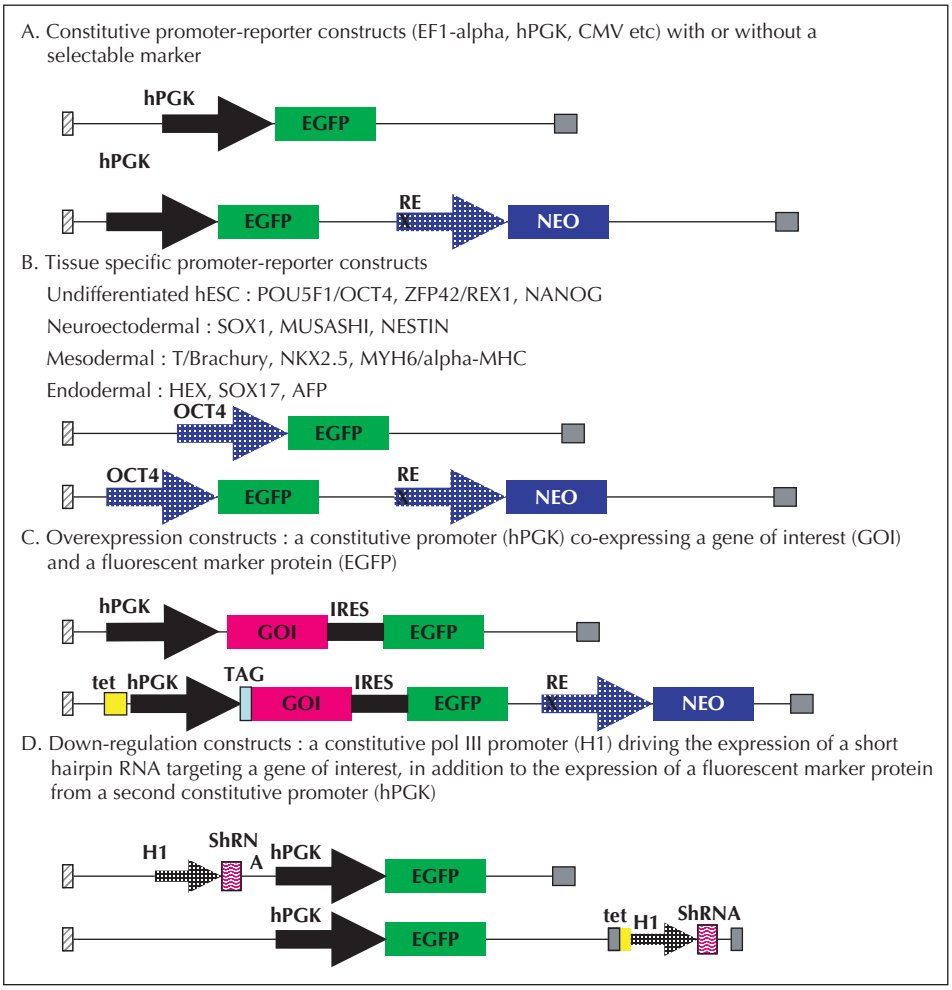

FIGURE 19.2 Design of lentiviral vectors.

PROCEDURES

Selecting and maintaining HEK-293T cells

293T cells, a human kidney epithelial cell line immortalized using SV40 large T antigen, was found to be highly efficient in producing lentivirus and is now routinely used for harvesting lentiviruses.

- HEK-293T cells are simple to culture by standard methods.
- Cells are passaged every 4–5 days at a split ratio of 1:10.
- Low-passage cells are used. The cells seem to lose the ability to be efficiently transfected after about 18–20 passages.

- It is advisable to subclone the line and select a clone that gives the best transfection efficiency and viral titers.
- Cells in exponential growth phase are used for plating prior to transfection. Cells from old or overly confluent cultures do not transfect well.

Production of lentivirus: transient transfection protocol

Viral stocks pseudotyped with the vesicular stomatitis virus G protein (VSV-G) are prepared by transient co-transfection of 293T cells using a three-plasmid system (the transfer vector; the pCMVΔ R8.74 encoding Gag, Pol, Tat, and Rev; and the pMD.G plasmid encoding VSV-G). Infectious lentivirus is harvested at 48 and 72 h post transfection and filtered through 0.22 μm-pore cellulose acetate filters.

The lentiviruses can be concentrated either by ultracentrifugation (2 h at 68 000×***g*** in SW28 rotor) or using Amicon 150 kDa molecular weight cut-off (MWCO) concentrators and subsequently frozen at −80°C in small aliquots. All infections are carried out in the presence of 5–10 μg/mL polybrene at a multiplicity of infection (MOI) of 10–100 for 1–14 h.

NOTE: This protocol can be successfully scaled-up/down as long as the ratios of the individual components are maintained.

The three plasmids used for co-infection are:

1. **Transfer vector:** This is the shuttle plasmid that contains the gene of interest and a selectable marker within the viral long terminal repeats (LTRs). The segment within the LTRs gets packaged in the mature, infective viral capsid. This packaged virus does not contain any of the genes required for viral replication.
2. **Pcmvδ R8.74 plasmid:** This plasmid encodes the essential viral genes (*gag*, *pol*, *tat*, and *rev*) for virus production and integration. All these genes may be present on a single plasmid or two separate plasmids.
3. **pMD.G plasmid:** This encodes the gene for the coat proteins of vesicular somatitis virus G (VSV-G).

One day before transfection of HEK-293T cells for 10 cm (15 cm) plates

1. Plate 293T cells in 10 cm (15 cm) plates 12–24 h before transfection.
2. Cells should be 70–80% confluent on the day of transfection (i.e. 12–15 × 10^6 cells/15 cm plate).

Day 1: transfection of HEK-293T cells

1. Mix the three plasmids (transfer vector, pCMVΔ R8.74, pMD.G) in a ratio of 3:2:1 in a 15 mL Falcon tube:
   - 10 μg (30 μg) transfer vector plasmid.
   - 6.6 μg (20 μg) pCMVΔ R8.74 plasmid.
   - 3.3 μg (10 μg) pMD.G plasmid.
2. Add H_2O (pH 7.0) to a volume of 450 μL (1350 μL) and mix well.
3. Dropwise add 50 μL (150 μL) of 2.5 M $CaCl_2$.
4. Finally add 500 μL (1500 μL) of 2 × HBSS (or HeSS) solution dropwise with constant vortexing.

NOTE: Either of the two buffers can be successfully used for transfection. However, 293T cells selected for efficient virus production using one buffer are invariably not well transfected with the other.

5. Immediately add the transfection mix dropwise to the plate of 293T cells in minimal medium 7–8 mL, 14–16 mL.
6. Incubate the transfected cells in a CO_2 incubator at 37°C for 14–16 h.
7. Replace the transfection media with fresh viral harvesting medium. Incubate at 37°C.
8. Virus can be harvested in serum-containing culture medium or in serum-free medium.

Day 3 or 4: Collect virus

Viral supernatant from the transfected plates is collected every 24 h and replaced with fresh medium. The highest virus recovery is ~28–48 h after transfection is completed. At its best the titer of the virus in the supernatant is usually between 10^5 and 10^6 infectious units per mL. If serum-free medium is used the virus can be harvested for up to 4 days with only slight decrease in yield.

1. Clear the collected viral supernatant by low-speed centrifugation (400×***g*** for 5 min at 4°C) and then filter through a 0.22 μm filter (low protein binding cellulose acetate).
2. Store viral supernatant at 4°C for no more than 2–4 days. For later use it can be stored in aliquots at −80°C. Repeated freezing and thawing is not advisable.

Pooled viral supernatant can be further concentrated over 200-fold by ultracentrifugation or ultrafiltration. The titer of the concentrated virus is estimated by determining the number of transduced cells per unit viral stock solution.

Ultracentrifugation

1. Centrifuge the supernatant in ultra clear tubes and a Beckman Swinging Bucket rotor SW28 at 20 000 rpm for 2–2.5 h at 4°C.

2. Aspirate the supernatant.
3. Resuspend viral pellet in 200–400 μL of remaining medium or PBS with 0.1% BSA by constant shaking at 200 rpm for 2–12 h at 4°C.
4. The concentrated virus can be used fresh or stored in aliquots at −80°C.

Ultrafiltration

Several filtration devices are commercially available and used according to manufacturer's instructions. These two are recommended:

- Apollo Spin concentrators CLP Catalog no. 3505.2 (QMWL-150 kDa)
- Centricon Plus-80 Millipore Catalog no. UFC5BHK02 (NMWL-100 000).

Transduction of hESCs with lentivirus

It is essential to estimate the final titer for calculating the ideal MOI (number of viral particles per unit recipient cells). Statistical methods suggest that if all cells in a population are equally likely to be infected, and if 20% or fewer cells actually get infected then the probability of multiple insertions in each of the cells is very low.

However the MOI required to achieve 20% infection in different cell types varies enormously. For example, for mouse embryonic fibroblasts (MEFs) this can be achieved with an MOI of around 3 but for hESCs an MOI of greater than 20 is required.

- For transduction of hESCs, concentrated virus is added directly to the culture medium of freshly plated hESCs. The cells are incubated with the virus for 24 h, after which the virus is washed off. Since the larger well spread feeders are more susceptible to viral infection, much higher amounts of virus are required to efficiently transduce hESCs cultured on MEFs. It is difficult to get infected hESCs at an MOI much less than 20.
- Alternatively, the hESCs can be effectively transduced in suspension. This method not only eliminates the virus lost to MEFs but also increases the effective concentration of the virus, which results in increased infection of the hESCs for the same MOI.
  1. After splitting, allow the hESC colonies to settle down in a 5 mL polystyrene tube.
  2. Carefully aspirate most of the medium.
  3. Resuspend cells in 400 μL of fresh complete medium containing the concentrated virus.
  4. Incubate the cells in the tube at 37°C for 3–12 h. If viral titers are higher than 10^8 then suspension infection for as little as 1 h is sufficient. Longer incubations may cause the cells to aggregate.
  5. Wash the cells by filling the tube with PBS and allowing the colonies to settle. Plate the infected cell clumps on Matrigel™-coated plates with MEFs in complete hESC medium.

- Transduction efficiency is increased by including 5–10 μg/mL polybrene (hexadimethrine bromide, Sigma H9268) in the transduction mix containing the cells and the virus.
- eGFP expression in hESCs transduced with pSIN18.PGK-EGFP.WPRE vector can be first detected under a fluorescence microscope ~24–48 h after transduction.
- Transduced cells are typically assayed for eGFP expression (or for expression of transgene) by FACS 3–6 days after transduction.

Pitfalls and Advice

Enrichment of infected cells

Since only a small subset of the hESCs are modified they can be selected by a variety of methods, each having its own advantages and disadvantages. Figure 19.3 shows hESC colonies isolated by manual selection and by FACS.

Manual enrichment

This is possible only for hESCs modified with viruses having fluorescent markers. The groups of labeled cells are separated using a fluorescence-equipped dissecting microscope. By using a warming stage, large numbers of labeled cells can be collected in relatively short periods of time without compromising the quality of the cells. However, it may be difficult to get a pure population of only labeled cells.

FACS sorting

FACs can be used to enrich for fluorescently labeled cells or those labeled by antibody staining for cell surface proteins. The biggest drawback of the selection of a group of cells by flow cytometry is the necessity for making a single-cell suspension of the hESC colonies. This is a good method for isolating labeled cells that can be used for differentiation or end point experiments but it may be very difficult to culture them as undifferentiated colonies.

Antibiotic selection

Any of the routinely used antibiotic selection markers can be incorporated within the transfer vector for subsequent selection. Use of promoters of genes expressed only in undifferentiated cells, such as POU5F1/OCT4 or ZFP42/REX1 can serve a dual purpose of selecting transduced, undifferentiated hESCs. However, use of antibiotic markers makes it necessary to use custom MEFs harboring the same resistance genes. Since the length of the RNA packaged within the virus is relatively fixed, incorporating resistance genes limits the amount of DNA of interest that can be transferred. Antibiotic selection works best if initiated 4–5 days post infection or even after the first passage. This allows for the survival of small patches of infected cells as opposed to single cells. This also means that the individual surviving colonies are not necessarily clones of single infected cells.

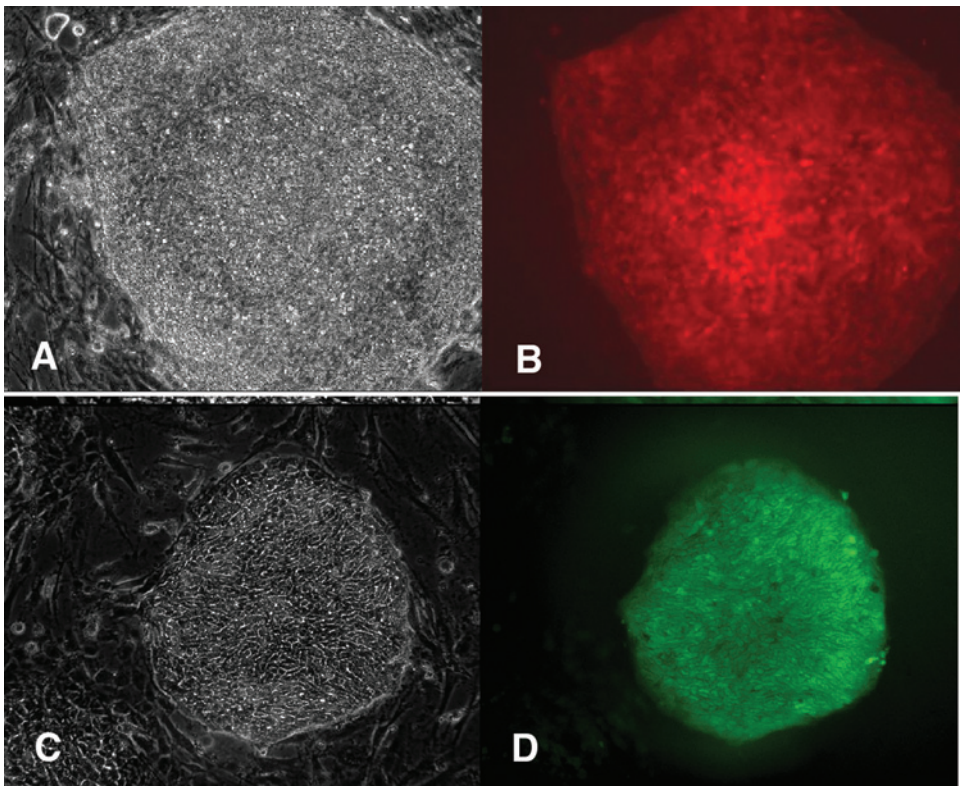

FIGURE 19.3 Cloning of lentiviral-infected hESCs. A,B: Undifferentiated hESCs (WA09) infected in suspension with hPGK-dsRed lentivirus. Colonies expressing the red fluorescent protein were manually enriched by dissection under a fluorescent microscope. C,D: Undifferentiated hESCs (WA09) infected in suspension with POU5F1/OCT4-eGFP lentivirus. Clones were selected by FACS (eight passages after selection).

Using reporter expression to monitor hESCs

Once clones of hESCs expressing fluorescent proteins are established, they can be used to monitor hESC in culture.

For example, cells constitutively expressing eGFP can be monitored after transplantation into animal hosts or when used in co-culture with unlabeled cells or cells labeled with another marker such as dsRed. Differentiated derivatives of such hESC clones continue to express the marker and can be similarly traced.

hESCs expressing a fluorescent marker under the control of a developmentally regulated gene can be monitored for differentiation, either because they begin to express a gene associated with a particular type of differentiation or because they cease expression of a gene associated with pluripotence.

Figure 19.4 shows examples of a clone of hESCs expressing eGFP under control of a POU5F1/OCT4 promoter. Spontaneous differentiation is easy to monitor as the cells cease expression of the fluorescent marker, showing areas of the culture that are not

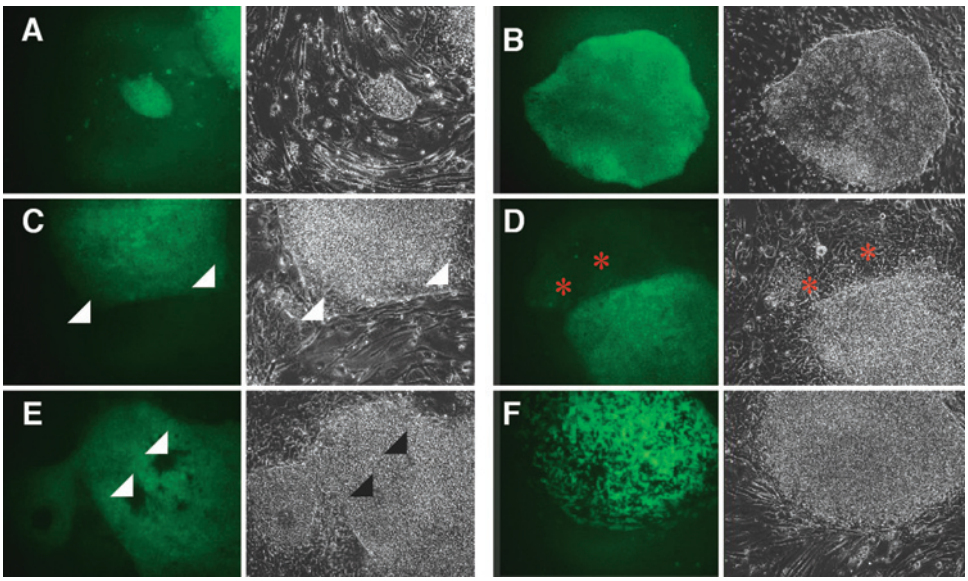

FIGURE 19.4 The good, the bad, and . . . the ghastly. hESC (WA09) colonies expressing GFP under the POU5F1/OCT4 promoter. The good hESC colonies (A,B) are flat, compact, with sharp edges composed of small cells with very high nuclear to cytoplasmic ratio. All cells in a good colony express POU5F1/OCT4. Often colonies begin to differentiate from the corners (arrows in C) or the edges (area around the asterisks in D).The differentiating colonies lose their sharp edges (C–F).The individual differentiating cells become larger with small nuclei and no longer express POU5F1/OCT4.

obviously differentiated by morphological criteria, but clearly have downregulated POU5F1/OCT4.

Methods of infection

Figure 19.5 shows the variation in results obtained when hESCs are infected *in situ* on feeder layers and in suspension without feeder cells. The fibroblastic feeder layers are often infected more easily than the hESCs, indicating that infection in suspension is preferable.

SUPPLIES AND REAGENTS

- Ultraculture medium (Bio-Whittaker catalog no. 12-725F)
- Fetal bovine serum (Invitrogen)
- D-MEM high glucose
- Sodium pyruvate (100×)
- Polybrene (hexadimethrine bromide) (Sigma catalog no. H9268BSA)
- L-Glutamine (100×)

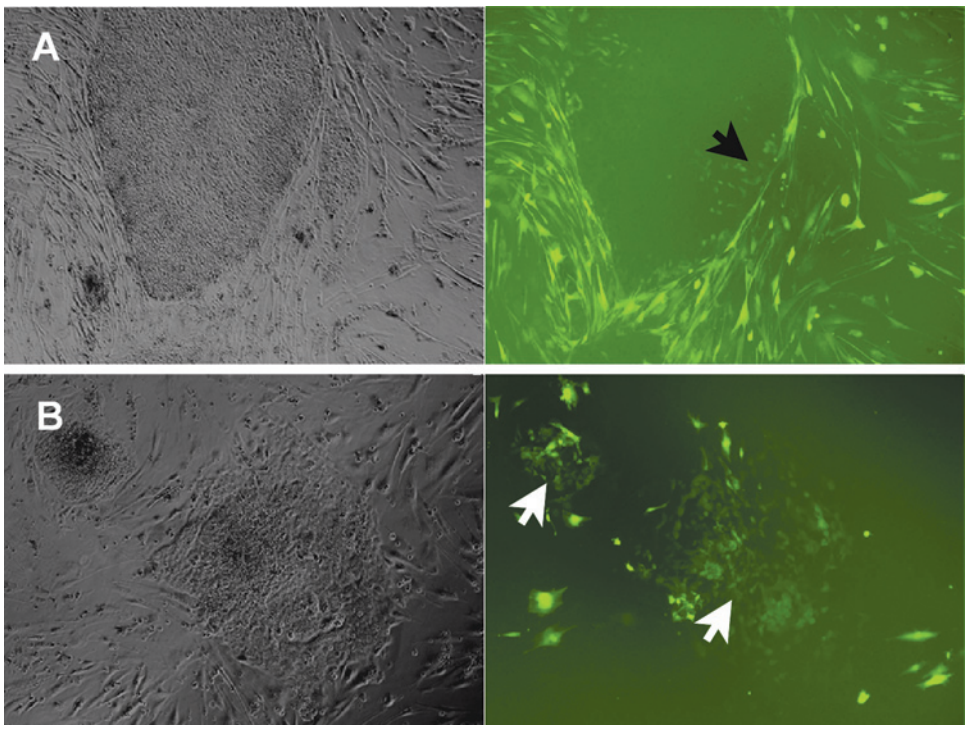

FIGURE 19.5 Comparison of infection efficiency of hESCs infected on the plate and in suspension. (A) Only a small number of cells in ~10% of the hESC colonies were infected (black arrow) when virus was added to cells 12 h post plating. Most of the virus was sequestered by the surrounding feeder cells. (B) Infection of same number of hESCs in suspension with comparable amount of virus resulted in the majority of the colonies with >20% infected cells.

- Pen-strep (100×)
- Apollo Spin concentrators CLP catalog no. 3505.2 (QMWL-150 kDa)
- Centricon Plus-80 Millipore catalog no. UFC5BHK02 (NMWL-100 000).

RECIPES

293T culture medium/virus harvesting medium with serum (500 mL)

Component	Amount	Final concentration
D-MEM high glucose	435 mL	
Sodium pyruvate (100×)	5 mL	1×
L-Glutamine (100×)	5 mL	2 mM
Pen-strep (100×)	5 mL	1×
Fetal bovine serum (Invitrogen)	50 mL	10%

Virus harvesting medium without serum (500 mL)

Component	Amount	Final concentration
Ultraculture medium (Bio-Whittaker catalog no. 12-725F)	485 mL	
L-Glutamine (100×)	10 mL	4 mM
Pen-strep (100×)	5 mL	1×

293T culture medium/virus harvesting medium with serum (500 mL)

Component	Amount	Final concentration
D-MEM high glucose	435 mL	
Sodium pyruvate (100×)	5 mL	
L-Glutamine (100×)	5 mL	2 mM
Pen-strep (100×)	5 mL	
Fetal bovine serum (Invitrogen)	50 mL	10%

2× HBSS (500 mL)

Component	Amount	Final concentration
NaCl	8.0 g	
KCl	0.37 g	
$Na_2HPO_4.7H_2O$	0.19 g	
Dextrose	1.0 g	
HEPES	5.0 g	

Adjust pH to 7.0–7.2 with 10 N NaOH, Make up volume to 500 mL. Filter through a 0.22 μm filter. Aliquot and freeze.

2× HeSS (500 mL)

Component	Amount	Final concentration
NaCl	8.2 g	
HEPES buffer	5.8 g	
$Na_2HPO_4.7H_2O$	0.15 g	

Adjust pH to 7.0–7.2 with 5 N NaOH, Make up volume to 500 mL. Filter through a 0.22 μm filter. Aliquot and freeze.

NOTE: The efficiency of transfection varies significantly between batches of HBSS/HeBS.

Reading List

The following list of books and papers is a selective compilation for investigators not currently using the lentiviral system.

Katkov II, Kim MS, Bajpai R, Altman YS, Mercola M, Loring JF, Terskikh AV, Snyder EY, Levine F (2006). Cryopreservation by slow cooling with DMSO diminished production of *Oct-4* pluripotency marker in human embryonic stem cells. *Cryobiology* 53: 194–205.
The authors describe a clone of hESC that expresses GFP under the tissue specific POU5F1/OCT4 promoter.

Liu YP, Dovzhenko OV, Garthwaite MA, Dambaeva SV, Durning M, Pollastrini LM, Golos TG (2004). Maintenance of pluripotency in human embryonic stem cells stably over-expressing enhanced green fluorescent protein. *Stem Cells Dev* 13: 636–645.
This study uses a dual promoter lentiviral vector for the independent expression of GFP and a selectable marker.

Ma Y, Ramezani A, Lewis R, Hawley RG, Thomson JA (2003). High-level sustained transgene expression in human embryonic stem cells using lentiviral vectors. *Stem Cells* 21: 111–117.
The authors have modified the lentivirus such that there is high level of transgene expression with minimal variation.

Sidhu KS, Tuch BE (2006). Derivation of three clones from human embryonic stem cell lines by FACS sorting and their characterization. *Stem Cells Dev* 15: 61–69.

Trono D (2002). *Lentiviral Vectors*. Berlin, Heidelberg: Springer-Verlag.
A comprehensive yet simple description of the variety of lentiviral vectors, their biology, integration and application, also see http://tronolab.epfl.ch/page58114.html

Xiong C, Tang DQ, Xie CQ, Zhang L, Xu KF, Thompson WE, Chou W, Gibbons GH, Chang LJ, Yang LJ, Chen YE (2005). Genetic engineering of human embryonic stem cells with lentiviral vectors. *Stem Cells Dev* 14: 367–377.
The authors demonstrate the stable expression of a U6 promoter-driven small interfering RNA (siRNA), which was effective in silencing its specific target in hESCs.

Zaehres H, Lensch MW, Daheron L, Stewart SA, Itskovitz-Eldor J, Daley GQ (2005). High-efficiency RNA interference in human embryonic stem cells. *Stem Cells* 23: 299–305.
Authors demonstrate the effect of siRNA-mediated downregulation of pluripotency genes in hESCs.

Zufferey R, Nagy D, Mandel RJ, Naldini L, Trono D (1997). Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. *Nat Biotechnol* 15: 871–875.
The protocol for lentivirus production described here is detailed in this chapter.

CHAPTER

20

Genetic Manipulation of Embryonic Stem Cells

Tobias Raabe and Robin L. Wesselschmidt

INTRODUCTION

Two remarkable discoveries in the early 1980s, embryonic stem cells (ESCs) and mammalian homologous recombination, enabled one of the greatest advances in biomedical research in the last 50 years; in 2001 these advances were rewarded by the presentation of the Lasker Award for Basic Medical Research to Mario Capecchi, Martin Evans, and Oliver Smithies for "the development of a powerful technology for manipulating the mouse genome with exquisite precision, which allows the creation of animal models of human disease."

Mouse ESCs (mESCs), which could be genetically manipulated and then converted to living mice, made it possible for the first time to methodically examine the function of individual mammalian genes. The most common kind of directed mutation in mESCs is called a "null" or "knockout" mutation, in which one of the two alleles of a given gene is completely inactivated by homologous recombination of a carefully designed targeting construct.

Gene targeting in mESCs and generating mutant mice has become an accessible technology for hundreds of laboratories, and thousands of genes in the mouse genome have been knocked out in the last 20 years. While hESCs can never be used to study mutations *in vivo*, they are one of the few diploid human cell lines capable of differentiating into

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derivatives of all three germ layers, and offer a unique opportunity to study *in vitro* many of the earliest stages of human development.

Overview

It would be very useful to be able to apply the sophisticated methods developed for mESCs to genetically manipulate hESCs. But it has not been easy to translate the methods from mouse to human, and many challenges lie ahead. In this chapter we introduce the genetic tools that have been successfully used to modify mESCs and review the recent efforts to genetically modify hESCs. The goal of this chapter is to give a general overview to stimulate thought and discussion on how to best adapt relevant mESC technology to the study and use of hESCs.

Procedures

There are currently two dominant methods for genetic manipulation of mESCs: gene targeting and gene trapping. Gene targeting is used to precisely introduce virtually any desired large or small mutation into any region of the genome by homologous recombination. In contrast, gene trapping is used to establish large libraries of ESC clones containing mutations in genes expressed in ESCs. Table 20.1 summarizes the methods used to genetically modify mESCs.

Gene targeting in mESCs

The term "gene targeting" is commonly used to describe the introduction of virtually any kind of genetic change into a specific gene locus in the genome via the process of homologous recombination. It is the only method that allows for the generation of any desired mutation with single nucleotide accuracy.

The importance of targeted mutagenesis of the mouse for contemporary drug discovery cannot be overstated: 80% of the 100 currently most widely sold drugs act on only one human gene and each of these drugs' effects can be investigated in the mouse by targeting the respective homologous mouse gene. Thus it is very likely that novel drug target candidates will emerge from targeting and detailed phenotypic analysis of all known mouse genes. There are currently three large-scale efforts to systematically target every known mouse gene by international research consortia: KOMP (Knockout Mouse Project, funded by the National Institutes of Health), EUCOMM (European Conditional Mouse Mutagenesis Program, funded by EU Research Framework), and NorCOMM (North American Conditional Mouse Mutagenesis project, funded by Genome Canada).

Although hESCs have not been as amenable as mESCs to the multiple manipulations necessary for efficient gene targeting (see discussion below) the basic cellular machinery of homologous recombination is active in both human and mouse cells, and it should be possible to adapt many of the existing targeting techniques for use with hESCs. Figure 20.1 shows the basic principles of gene targeting. By harnessing the cell's DNA recombination machinery, a sequence that is homologous to the cell's DNA sequence replaces the endogenous sequence.

TABLE 20.1 Methods used to genetically modify mouse ESCs

Date cell line	Observation	Reference
1985 (1) Human bladder carcinoma (EJ) (2) Hu11 hybrid	**Gene targeting/homologous recombination** Human beta-globin Showed that homologous recombination can occur between exogenous DNA and the chromosome of a cultured mammalian cell. Showed that insertion of defined DNA sequences can be stably and predictably introduced into mammalian cells whether or not the target locus was expressed. Frequency of homologous recombination was ~1/1000 G418 resistant clones	Smithies *et al.*, 1985
1987 Mouse ESCs	**Gene targeting/homologous recombination construct design** HPRT locus Showed that a two-fold increase in homology between the targeting vector and target locus resulted in a 20-fold increase in targeting efficiency	Thomas and Capecchi, 1987
1987 Mouse ESCs: E14TG2a	**Gene targeting/homologous recombination** HPRT locus Targeted correction of mutant HPRT gene in mESC showing the general application of the use of exogenous DNA to specifically target a mammalian gene on a chromosome and transmit the targeted modification through the germline of a mouse. E14TG2a cells are HPRT negative	Doetschman *et al.*, 1987
1988 Mouse ESCs	**Positive/negative selection (PNS)** Proto-oncogene int-2 First time shown that it is possible to target a locus other than HPRT in mouse ESCs. Using PNS vector design, a negative selectable marker flanks the homologous targeting arms so that cells expressing this marker will not survive in culture. The goal is to use the negative marker to enrich for the rare HR event. (a) Positive selection neo (G418) to isolate clones that	Mansour *et al.*, 1988

(*Continued*)

TABLE 20.1 (Continued)

Date Cell line	Observation	Reference
	incorporate the vector. (b) Negative selection using HSV-tk (ganciclovir) to select against random integration events. Selection medium contains both G418 and ganciclovir	
1992 Mouse ESCs E14	Isogenic DNA increases targeting efficiency Authors propose a major change in the then current approach to gene targeting away from the generation of elaborate targeting vectors, selection, and screening methods towards the use of isogenic targeting vectors, where the DNA used to construct the vector is isolated from the ESC line used to target the gene. Retinoblastoma susceptibility gene (Rb) locus: (1) isogenic (129-derived) construct: 33 recombinant clones/94 G418 resistant; (2) non-isogenic (Balb/c-derived) construct: 1 recombinant clone/144 G418 resistant	te Riele H *et al.*, 1992
1991 Mouse ESCs	**Gene trap: "beta-geo" reporter** βgeo (β-galactosidase (β-gal)/neomycin phosphotransferase fusion gene) DNA constructs introduced by electroporation or retroviral infection, gene trap events were identified by G418 resistance. When injected into mouse blastocysts, expression could be tracked by staining tissues with X-gal. Identification and mutation of the trapped gene. Reporter cassette: SA *β*-geo pA.	Friedrich and Soriano, 1991
1992 Mouse ESCs D3	**Gene trap: lacZ reporter** Linearized plasmid is electroporated into ESCs and is believed to randomly integrate into the genome. If it lands in genes that are expressed in ESCs, a fusion gene containing 5′ endogenous gene fused to lacZ is produced that simultaneously inactivates the gene and allows for tracking by lacZ staining. Reporter cassette: SA lacZ neo pA	Skarnes *et al.*, 1992

1998 Mouse ESCs	**Gene trap: OmniBank** Large-scale generation of mutant ESC clones by random integration of gene trap coupled to automated identification of sequence tags regardless of whether the trapped gene is expressed in ESCs. Gene trapped ESC clones were stored and catalogued by sequence tag in a library (OmniBank) of mutant mESC clones. Retroviral infection of gene trap vectors (VICTR3 or VICTR20) and selection of puromycin resistant ESC clones. Vector: SA IRES βgeo pA → PGKpuroSD	Zambrowicz *et al.*, 1998
1989 Mouse fibroblast cell line: LMtk–	**Cre-loxP recombinase** Bacteriophage P1 Cre recombinase is 38 kDa protein that specifically promotes intra- and intermolecular recombination at 34 bp loxP sites excise a segment of DNA that is flanked by loxP sites (floxed DNA) in mouse cell line. Cre belongs to the lambda-integrase (Int) family of site-specific recombinases	Sauer and Henderson, 1989
1993 Mouse ESCs E14.1	**Cre-loxP recombinase** IgH gene locus Two-step method to delete selectable marker from gene targeted ESC clones: (1) locus homologously targeted with vector containing loxP sites, (2) transient transfection of Cre enzyme and selection for clones that have recombined at the loxP sites and deleted the DNA between the loxP sites	Hua *et al.*, 1993
1991 Mouse ECs: F9 Monkey kidney: CV-1	**Flp-FRT recombinase** Yeast-derived, *Saccharomyces cerevisiae*, site-specific recombinase system that efficiently and specifically excises a segment of DNA that is flanked by FRT sites. Like Cre recombinase, it is a member of the lambda-integrase (Int) family of site-specific recombinases	O'Gorman *et al.*, 1991
1996 Mouse ESCs: CCE Mouse ECs: P19	**Flp recombinase** Shows that flp/FRT system efficiently promotes site specific DNA recombination in ECC, ESC, and transgenic mice	Dymecki, 1996
1995 HeLa	**Tet repressor-operator-effector system (Tet R)** *E. coli* tetracycline resistance operon used to develop an "on/off" gene expression system. That can be regulated by dosing with Doxycycline (dox). Cells that constitutively express the transactivator and contain an appropriate stably integrated reporter, rapidly induce gene expression	Gossen *et al.*, 1995

(*Continued*)

TABLE 20.1 (Continued)

Date cell line	Observation	Reference
2005 Mouse ESCs: Novel GFP expressing-ESC line	**Inducible gene expression in mouse ESCs** An ESC-based approach to gene functional analysis in a spatio-temporal inducible manner, by coupling Cre-loxP-mediated gene activation with tetracycline-mediated repression. They established a method to control gene expression in differentiating ESCs *in vitro* and embryos *in vivo*	Mao *et al.*, 2005
2000	**Recombinogenic bacteria for chromosome engineering** The first use of defective lambda-prophage for recombineering. The modification of DNA and production of targeting vectors based on the use of recombinogenic *E. coli*. Eliminates the need for many of the traditional steps required to generate targeting vectors. PCR amplification is used to generate targeting vector homology arms, which are electroporated into recombinogenic bacterium that contains the target DNA, which can be a plasmid, P1, or BAC clone. the bacterium recombines the target DNA at engineered PCR homology sites 40–50 bp	Yu *et al.*, 2000
2003	**BAC recombineering** Further simplification of vector construct generation based on recombinogenic *E. coli*. BACs are modified in their host *E. coli* strain by transfecting the recombination system into the bacteria containing the target BAC. Does not require the construction or screening of genomic libraries and permits the generation of most targeting vectors including conditional and knock in vectors within two weeks	Cotta-de-Almeida *et al.*, 2003
2003 RW4 CJ7 a B6J line	**BAC recombineering: VelociGene** High-throughput generation of gene targeted mice. High-speed vector construction via BAC recombineering was coupled with automated screening for gene targeted ESC clones. The use of full-length BAC clones to target genes in mouse ESCs alleviates the need to use isogenic DNA or positive negative selection and still achieve high efficiency gene targeting. BAC targeting efficiency: 3–4%	Valenzuela *et al.*, 2003

ESC, embryonic stem cell; ECC, embryonic carcinoma cell; BAC, bacterial artificial chromosome; PCR, polymerase chain reaction; SA, splice acceptor sequence; SD, splice donor sequence; pA, polyadenylation sequence; PGK, phosphoglycerate kinase-1 promoter; puro, puromycin *N*-acetyl-transferase gene; bgeo, beta-galactosidase/neomycin phosphotransferase fusion gene.

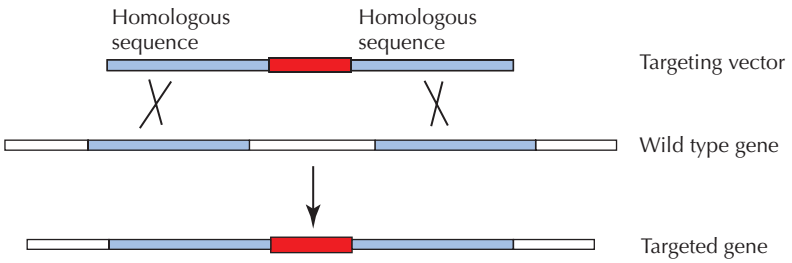

FIGURE 20.1 Principle of gene targeting by homologous recombination.

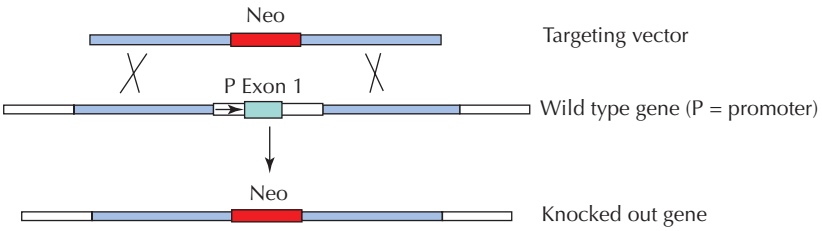

FIGURE 20.2 Elements of a knockout construct.

Knockout strategy

The basic technique for generating a knockout mutation begins with generation of a targeting vector that consists of fragments of the gene flanking the sequence that is to be deleted. Usually, in order to completely inactivate a gene, the targeted region includes the promoter and exons that are essential for mRNA production and the synthesis of functional protein. Deleted genetic regions are replaced with selectable markers such as antibiotic resistance genes like neomycin-resistance (neomycin phosphotransferase II) (Figure 20.2).

A homozygous cell line ("double knockout") can be generated by a second targeting vector (using a different selectable marker), or by selecting for homozygous cells that result from rare double recombination events. The selection for double recombinations is accomplished by taking advantage of the higher expression of the drug resistance marker by the two copies of the cassette. By increasing the concentration of the drug used for selection, the higher expressing cells are the only ones that survive.

If the promoter can be preserved because gene inactivation can be achieved by deleting crucial exons, the knockout mutation can be combined with a reporter gene such as enhanced green fluorescent protein (eGFP) or beta-galactosidase (b-gal) inserted precisely downstream of the endogenous promoter in place of the deleted exons. Analysis of reporter gene activity in the mouse will then reveal the normal tissue and stage-specific expression of the inactivated gene.

Knock-in strategy

A subtler form of gene targeting allows insertion of a sequence that will be driven by the endogenous promoter. Known as a "knock-in" to contrast with a knockout, it is

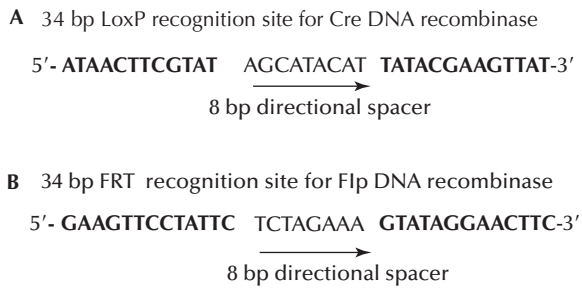

FIGURE 20.3 Palindromic sequences of loxP (A) and FRT (B) recombinase-specific sites.

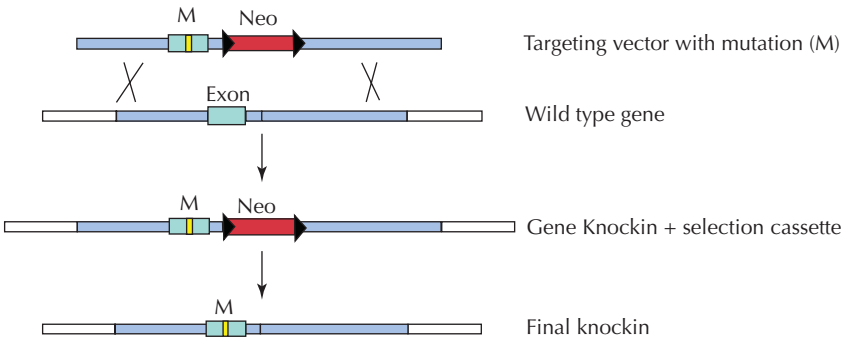

FIGURE 20.4 Elements of a knock-in construct. M = mutation in exon. LoxP sites or FRT sites (black arrowheads) surround neo selectable marker.

often used to introduce a small and precise alteration such as single amino acid changes or to slip in a reporter gene such as GFP or b-gal, while keeping fully intact all other functional parts of the gene so that accurate representation of gene expression is maintained. The selectable marker used to select positive ESC clones can be removed by flanking the selection cassette with sequences recognized by site-specific recombinases to avoid possible interference with gene function. The loxP and FRT sites (Figure 20.3) are the most widely used for excision of sequences. By introducing the appropriate recombinase into the targeted ESCs, using a method such as transient transfection, the selection cassette is excised, leaving the "knocked-in" sequence (Figure 20.4).

By floxing sequences it is also possible to invert pieces of DNA. As shown in Figure 20.5, if the flanking loxP sequences are oriented in opposite directions, Cre recombinase flips the DNA sequence between the sites.

Conditional knockout strategy

One of the shortcomings of a simple knockout of a gene is that many genes are essential for early development and their ubiquitous knockout results in embryonic lethality. In this case only a tissue-specific or stage-specific gene inactivation can allow generation of live animals. Conditional knockouts allow genes to be inactivated in specific tissues or at particular developmental stages.

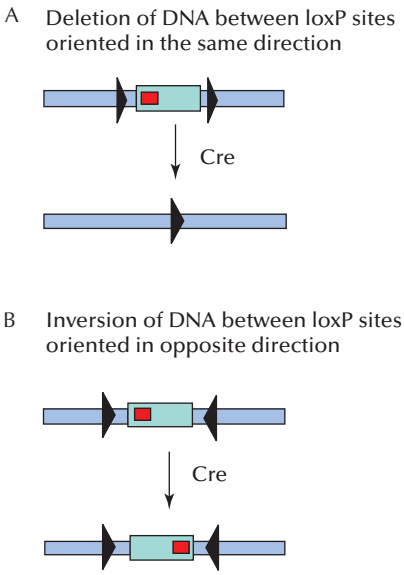

FIGURE 20.5 (A) Deletion of DNA between loxP sites oriented in the same direction. (B) Inversion of DNA between loxP sites oriented in the opposite direction.

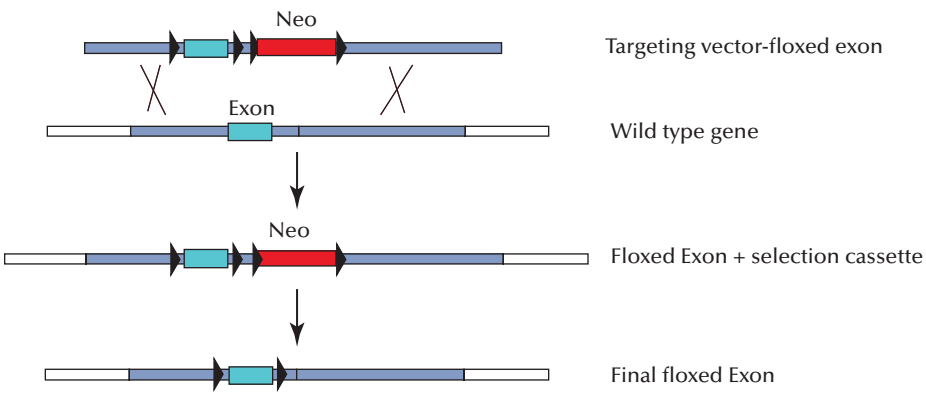

FIGURE 20.6 Conditional knockout strategy. A site-specific recombinase is used to first remove the selection cassette, leaving the "floxed" exon in the ESCs.

The technique involves the introduction of DNA recombinase sites (loxP and FRT) around a region that is to be deleted within a gene. In the example shown in Figure 20.6, the strategy is to remove a single exon in a gene in specific tissues of the mouse. Both the exon and the selection cassette (neo) are surrounded by site-specific recombinase sites. In the example shown, different recombinases are used to excise the selection cassette and the exon. If the selection cassette is flanked by FRT sites, it is removed by introducing flp into the ESCs, leaving the loxP-flanked ("floxed") exon. After a gene-targeted mouse is generated from the ESCs, the floxed exon is removed under specific conditions by mating to a mouse expressing Cre under control of a tissue- or developmental stage-specific promoter.

Gene trapping in mESCs

Gene trapping was developed as a high-throughput alternative to gene targeting to rapidly knock out mouse genes. A single retroviral gene trap vector can be used to quickly establish a whole library of ESC clones; in each of the clones the vector is integrated into a different gene.

The most common type of gene trap vector consists of a splice acceptor site followed by a promoterless selectable marker gene (usually neo or neo/b-gal fusion) and a polyadenylation site. These elements are flanked by retroviral direct repeats. After electroporation of vector DNA into ESCs only those cells that have inserted the vector downstream of an active endogenous promoter will express the selectable marker, which is usually neo, and thus can be selected by adding G418 to the growth medium.

Instead of driving expression of the wild-type endogenous gene the trapped gene promoter now drives expression of a short fusion RNA consisting of the gene upstream of the trap vector spliced via a splice acceptor signal to the vector's neo reporter and polyadenylation signal which terminates the fusion transcript. The strategy is outlined in Figure 20.7.

Promoterless gene traps, while allowing high-throughput genetic manipulation of ESCs, have some drawbacks that should be kept in mind.

First, a "trapped" gene will only be completely inactivated by this process if the splice acceptor site of the gene trap vector is strong enough to completely shut off normal splicing to any exon downstream of the gene trap. This is often not the case, thus resulting in simultaneous production of wild-type and mutant protein.

Second, some insertions of the gene trap vector will still allow production of truncated endogenous protein that may retain some biological activity. Both of these mechanisms lead to "hypomorphic," or partially inactivated, alleles whose phenotypes are often difficult to interpret. Third, promoterless gene traps cannot trap any gene that is not expressed in the host cells. About 20% of genes are not expressed in mouse or human ESCs.

Promoter-containing gene traps, also called polyadenylation traps, have been devised recently to circumvent these problems. These gene traps contain a promoter and a

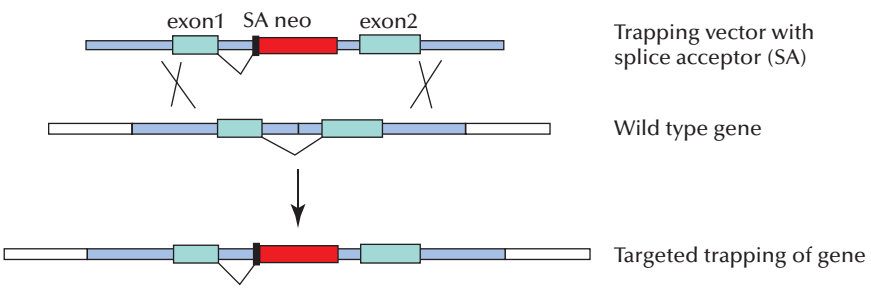

FIGURE 20.7 Gene trap strategy.

reporter gene (typically neo) and a splice donor site instead of a polyadenylation site, and theoretically could confer neo resistance to any cell that has the trap inserted upstream of any endogenous poly(A) site. However, promoter-containing gene traps also suffer from the generation of hypomorphic alleles, as do all mutagenic vectors that solely depend on altered RNA processing as a mechanism of inactivation.

Another concern about trapping approaches is that all types of gene traps favor inactivation of large over small genes, simply because a large gene has a statistically much higher probability of being "hit" by the vector. This means that most one- or two-exon genes are seldom inactivated by gene traps, so they must be mutated using classical gene targeting approaches.

Application of mESC genetic manipulation techniques to hESCs

Even though mouse and human ESCs are similar in many ways, translation of the genetic manipulation methods from mouse to human is not straightforward.

This is because gene targeting is a multi-step process, and for mESCs, each step has been optimized over 20 years through the efforts of many laboratories. Many of the steps in the process must be revised or reinvented to accommodate the peculiarities of hESCs.

In this section we examine each step of the mouse ESC targeting technology, highlighting the differences between mouse and human ESCs and proposing ideas for human-specific alterations in the methods. Where they are available for hESCs, we include detailed methods.

Steps in gene targeting

- Design of gene targeting vectors
- Delivery and integration of DNA
- Selection of antibiotic-resistant clones
- Cloning hESCs
- Confirming targeted clones
- Analysis of targeted cells.

Design of gene targeting vectors

Design of the targeting vector is critical for all of the following steps. Targeting vectors are optimized for homology, selectable markers, and screening strategy.

Homology

Experience with mESCs has demonstrated that increasing homology between the targeting vector and the target in the genome increases the frequency of homologous recombination. For this reason, most researchers design vectors using DNA from the same strain of mouse as the ESC lines in which they intend to target the gene. While using isogenic

DNA increases efficiency of homologous recombination, in the mouse, each hESC line has a unique genomic sequence. But isogenic sequence may not be as important for homologous recombination in human as it is for mouse. The highly inbred mouse strains tend to differ from each other by multiple deletions in the genomic sequence, while the diversity of human genomes is more likely to be at the level of single nucleotide polymorphisms. One way to achieve high targeting efficiency without requiring identical sequences is to use very large targeting constructs made from BAC clones. This approach improves targeting efficiency but introduces new challenges for transfection and analysis strategies, and has not yet been tested in hESCs.

Selectable markers

Positive selection for cells that have integrated the vector is important for limiting the number of clones that need to be further analyzed. The selectable markers that confer resistance to antibiotics (such as neomycin, puromycin, and hygromycin) are available as standard cassettes and can be used for human cells. Some mutated or genetically engineered versions of these markers have been designed to confer different degrees of drug resistance, which allow for subtle modifications of the screening methods. But the concentrations of the drugs used for mESCs may not be appropriate for hESCs. The minimal toxic dose and timing of each drug's effects should be tested for each hESC line individually.

The promoter used to drive expression of the drug resistance genes is critical for the effectiveness of the selection. The following gene promoters have been reported to work in hESCs:

- PGK phosphoglycerate kinase
- SV40 (simian virus 40)
- EL1α (elongation factor 1a)
- ACTB (human beta actin)
- ZPF42 (REX1).

Screening strategy

It is critical that the strategy for screening for a homologous recombination event be carefully designed into the targeting vector so that screening gives an unambiguous result. While PCR screens are easier to perform than Southern blots, they can be misleading, especially when the vector and target are isogenic. Southern blot strategies have been extensively reviewed and will not be discussed further here.

Delivery and integration of DNA

Table 20.2 summarizes the major methods for delivery of homologous recombination vectors. Electroporation is the standard method for transfecting mESCs and has been used successfully to produce thousands of mESC clones that remain pluripotent following electroporation, drug selection, screening, and expansion. An alternative to electroporation, preferred by some labs, is lipid-mediated DNA transfection, also called lipofection. Lipofection is a passive method for the introduction of DNA into cells, using synthetic cationic lipids that interact with DNA to form lipid/DNA complexes,

TABLE 20.2 Methods used to genetically modify hESCs

Date cell line	Observation	Reference
2001 WA09	**Transgenesis: random integration, gain of function** Lipofection: ExGen500 (1) Murine Rex1 promoter-eGFP (2) Murine PGK-eGFP SV40 driving neo resistance (200 μg/mL G-418) 13 days of selection. Different methods of transfection were tested; lipofectamine, electroporation, FuGene, ExGen (ExGen 500 was found to be most efficient in this study)	Eiges *et al.*, 2001
2003 WA09	**Transgenesis: random integration, gain of function HSV-tk via pPNT vector** Lipofection: ExGen500 Expression of HSV-tk renders the cells sensitive to ganciclovir and provides a mechanism to selectively eliminate the population expressing this gene. Tumors in mice could be eliminated by treatment with ganciclovir. Culture: KD-MEM, 20% KSR, 4 ng/mL FGF-2 10^7 cells, 12 μg of plasmid, selected with G418, 10 days after selection Results: 9 colonies, 6 of which were sensitive to ganciclovir	Schuldiner *et al.*, 2003
2003 WA01.1	**Homologous recombination: loss of function** Electroporation (1) Knockout: *HPRT1* (2) Knock in: GPF POU5F1 (OCT4) 12 kb of homology gave 22 *hprt*-negative clones out of 56 stable clones	Zwaka and Thomson, 2003
2004 WA13	**Homologous recombination: loss of function** Lipofection: ExGen 500 Knockout replacement vector: HPRT1 Human ESCs selected hygromycin (100 μg/mL) and 6TG (1 μg/mL) containing medium, clones isolated after 3 weeks	Urbach *et al.*, 2004

(*Continued*)

TABLE 20.2 (Continued)

Date cell line	Observation	Reference
2004 WA09	**Transgenesis: gain- and loss-of-function study: GFP, RFP, and siRNA** Lipofection Lipofectamine 2000 and ExGen 500 (found Lipofectamine 2000 more effective) Produced transient siRNA and stable hairpin-loop siRNA Evaluated five different red and green fluorescent proteins for stable long-term expression and ability to knock down expression with siRNAs	Vallier *et al.*, 2004
2004 WA01 WA09	**Transgenesis: random integration: gain of function** Lipofection FuGene 6 10 μg linearized plasmid, 24 h after transfection, selected with 100 μg/mL G418, after 12 days a single eGFP-expressing colony was observed. It was further propagated in MEF-CM containing G418 for four months. Plasmids: EF1α driving eGPF and EF1α driving neomycin resistance	Liu *et al.*, 2004
2005 HES3	**Transgenesis: random integration** Electroporation "Envy" hβactin-GFP Mapped to chromosome 12 q23.1 between the genes encoding thrombopoietin (TPO) and solute carrier 25 (SC25) Normal, pluripotent, and uniformly expressed GFP in derivatives of all three germ layers in the absence of ongoing selection	Costa *et al.*, 2005
2006 WA09.2	**Transgenesis: two-step generation of Cre-modified human ESC clones** Electroporation to generate stable transgenic clones: 40 μg plasmid; 1–3 × 10^7 cells; 4 mm gap cuvette; 500 μL culture medium + 300 μL PBS; 300 V, 200 μF, 5–20 ms pulse time; 300 μg/mL G418 selection started 48 h after electroporation Lipofection for transient transfection of Cre plasmid	Nolden *et al.*, 2006

which fuse with the cell membrane and deliver the DNA to the cell. Lipofection can be used for transient expression as well as stable expression. Both methods have been used on hESCs, and sample protocols are outlined in the following section. Viral infection techniques have been used extensively for hESC transformation, but the relatively small capacity of these vectors makes it difficult to use them for targeting strategies that require large regions of homologous sequence (see Chapter 19).

Electroporation

Differences between electroporation in mouse and human ESCs have not been carefully examined, but a well-known problem with hESCs is that, unlike mESCs, the cells do not survive well in single-cell suspension. Electroporation causes massive cell death, so it is difficult to separate the effects of the electroporation itself from the effects of cell dissociation. This makes systematic testing of electroporation protocols difficult, and in the techniques that have been used so far, cells are electroporated in multicell clumps rather than as single cells.

Lipofection

Lipid-mediated methods do not require dissociation to single cells, but the cells must be accessible to the lipid vesicles, so this method does not work well for multilayered cultures. Experiments with mESCs show that some dissociation of the cells is required for optimal transfection efficiency, and transfection may work better in suspension cultures than in attached cells. Several manufacturers have lipofection products, and each should be tested on the cells that are to be transfected, varying DNA/lipid ratios, concentrations, and incubation times. Commercially available lipid mixtures include FuGene 6 (Roche Diagnostics), ExGen 500 (Fermentas), and Lipofection 2000 (Invitrogen).

Selection of antibiotic-resistant clones

There has not been extensive testing of drug selection on hESCs and individual lines may have different sensitivities to the drugs used, so it is advisable to perform a "kill curve" in order to determine the lowest effective concentration that will kill untransfected cells in 7–10 days.

Concentrations that have been reported to be effective for neomycin, hygromycin, and puromycin are:

- Neomycin (G418): 50–300 μg/mL
- HygromycinB: 100 μg/mL
- Puromycin: 1 μg/mL.

Cloning hESCs

This is one of the most difficult steps to optimize. Cloning mESCs has always been relatively simple. Single mESCs survive well and predictably have high cloning efficiencies, ranging up to 50%. This is not the case for hESCs, which seem to be exquisitely sensitive to dissociation and typically clone with efficiencies of less than 1%. Methods to improve cloning efficiencies are in active development, and include combinations of growth factors, co-cultures and optimizing other medium components. These are summarized in Table 20.3. For now, the best approach seems to be to keep the hESCs in

TABLE 20.3 Methods of cloning human ESCs without drug selection or manual isolation

Cell line	Description	Reference
WA09 Clones: WA09.1 WA09.2	**Dissociation into single cells and individually plated.** Plate individually in a single well of 96-well plate (2 clones/384 cells plated) Method: Trypsin (0.05%/EDTA) Culture: MEF feeders, KO-DMEM, 20% KSR, 4 ng/mL FGF-2 or MEF feeders, KD-MEM, 20% FBS (improved cloning efficiency in KSR, 4 ng/mL FGF-2 medium)	Amit *et al.*, 2000
HES3 Clones: HES3.1 HES3.2 HES3.3	**Flow cytometry used to sort based on size and forward light scatter.** Cells individually plated into a well of a 96 well plate. Method: Collagenase IV to lift colonies followed by trypsin (0.05%/EDTA) Culture: HEF (p6), KO-DMEM, 20% KSR, 4 ng/mL FGF-2, 1 × ITS. Single cells plated into individual wells of 96-well plates. Efficiency: <0.5% Clones stable in culture for more than 1 year, but differed in gene expression. HES-3.2 expressed GATA-6 as well as pluripotency markers	Sidhu and Tuch, 2006
WA01, WA09 WA01: 4 clones	**Modified growth medium** Clonal isolation of human ESCs by supplementing the growth medium with neurotrophins: BDNF, NT3, NT4 (50 ng/mL each) Trypsin (0.05%/EDTA) individually plated in a well of a 96-well plate on MEFs or Matrigel. Culture: D-MEM, 20% KSR, 4 ng/mL FGF-2 With neurotrophins: 14.6% of clones survived (42/288) Without neurotrophins: 0.4% of clones survived (2/480)	Pyle *et al.*, 2006
WA01, WA09	**Flow cytometry used to clone SSEA-3 positive cells** SSEA-3-positive population was able to survive clonal expansion better than SSEA-3 negative population. Method: Collagenase IV followed by exposure to cell dissociation buffer (Invitrogen). Cells were sorted based on SSEA-3 expression collected in 0.5 mL of medium and plated into six-well dishes and cultured in either MEF-CM and 8 ng/mL FGF-2 or medium with 20% KSR and 36 ng/mL FGF-2 on Matrigel alone or Matrigel and hdF	Stewart *et al.*, 2006

FACS, fluorescence activated cell sorting; SSEA-3, stage specific embryonic antigen-3; hdF, human ESC-derived fibroblasts; HEF, primary human fetal fibroblasts; ITS, insulin-transferrin-selenium; FGF-2, basic fibroblast growth factor; KO-DMEM, knockout Dulbecco's modified Eagle's medium; KSR, KnockOut™ serum replacement; BDNF, brain-derived neurotrophic factors; NT3, neurotrophin 3; NT4, neurotrophin 4; HSV-tk, herpes simplex virus thymidine kinase.

small aggregates rather than single cells, and to serially subculture colonies to separate individual clonal lines.

Confirming targeted clones

The screening strategy is built into the design of the vector, but analysis of hundreds of clones is a separate issue and requires considerable planning. Ideally, before screening for a homologous recombination event, all of the clones should be expanded and subcultured so that the positive clones can be recovered easily for further experiments. Logistically, this is a challenge for hESCs. Techniques have been developed for cryopreserving mESCs in multiwell dishes so that they can be stored during analysis rather than requiring daily maintenance. Cryopreservation techniques for hESCs have not achieved this level of efficiency, so hESC clones must be individually expanded and maintained during analysis. For the moment, the only sure strategy is to isolate and expand as many clones as is practical for the laboratory's resources, hoping that the targeting frequency is high enough so that the task is not unmanageable. After targeted clones are identified, all of the others can be discarded.

Analysis of targeted cells

For mESCs, the phenotypic analysis of targeted mutations is done almost exclusively after germline transmission of the ESCs and, often, mating of the mice to create a null genotype. For human cells, there is no equivalent assay that allows assurance that the cells have remained pluripotent and that the phenotype of the cells is due to the mutation and not to selection of a culture or manipulation artifact.

Artifacts of clonal selection include chromosomal abnormalities and unknown effects on differentiation potential. The best solution for this issue is to always examine multiple independent clones from a targeting procedure. Although this requires a great deal of effort, we recommend at that at least two independently targeted clones should be analyzed before concluding that a phenotype is caused by the mutation.

Another challenge unique to hESCs is that null genotypes are more difficult to achieve, since they will require double targeting to mutate both alleles. While this is achievable by using two rounds of targeting, it requires a great deal of effort. Gene conversion occurs occasionally in individual cells, resulting in both alleles carrying the mutation, but it is a rare event and requires development of screening methods (such as increasing drug concentrations) that have not been tested in hESCs.

Examples of gene targeting in hESC

Table 20.3 summarizes the electroporation and lipofection methods used for genetically modifying hESCs. The protocols outlined below are generalized methods adapted from those publications. Protocols are provided for:

- Electroporation
- Lipofection
- Picking selected clones.

Electroporation

Using this method (adapted from Zwaka and Thomson, 2003 and Nolden *et al.*, 2006, see Table 20.3), the cells are electroporated in clumps in cell culture medium and plated at very high density following electroporation.

- Vector DNA: 40 μg of linearized plasmid.
- Electroporator: The conditions for each manufacturer will need to be worked out, but any electroporator should work. The conditions used for the BioRad gene pulser II are 320 V, 200 μF; 5–20 ms pulse time; 4 mm gap cuvette.
- Cells: 1.5–3 × 10^7 cells.

One week before electroporation
Passage hESCs to Matrigel™-coated dishes and culture in MEF-conditioned medium (MEF-CM) supplemented with 8 ng/mL FGF2.

One day before electroporation
Prepare antibiotic-resistant feeder cells. Inactivate mouse embryonic fibroblasts (MEFs) and plate on 2–10 cm dishes.

The day of electroporation

1. Harvest hESCs by treatment with collagenase IV for 7 min at 37°C.
2. Gently wash the colonies from the dish and resuspend in total of 10 mL of culture medium.
3. Pellet the cells at 800 rpm for 5 min.
4. Wash 1× in 5 mL culture medium.
5. Spin down the cells at 800 rpm for 5 min.
6. Re-suspend the cells in 0.5 mL of medium.
7. Add 0.3 mL of PBS containing 40 μg of linearized vector total volume of 800 μL.
8. Mix all by gently pipetting the solution 3–5 times and put into a 4 mm gap cuvette.
9. Electroporate.
10. Allow the electroporated cells to rest at room temperature for 10 min.
11. Add the 800 μL of electroporated cells to 4 mL of hESC medium.
12. Plate on 2–10 cm dishes plated with antibiotic-resistant MEFs.
13. Start selection 48 h after electroporation.
14. Exchange the medium daily thereafter.

Lipofection with FuGene 6

Method adapted from Liu *et al.* (2004). See Table 20.3.

Two weeks prior to transfection
Passage cells to Matrigel plates in MEF-CM with 4 ng/mL FGF2.

One week prior to transfection
Repeat passage cells to Matrigel plates in MEF-CM with 4 ng/ml FGF2.

One to two days prior to transfection
Plate approximately 5×10^5 cells/35 mm well on Matrigel in MEF-CM.

Day of transfection

1. Wash cells with OPTI-MEM® (Invitrogen).
2. Add 1 mL of hESC medium/well (not MEF-CM) and place in the incubator.
3. Combine: 15 μL FuGene 6, 85 μL of OPTI-MEM and 10 μg of linearized plasmid in 50 μL of water (150 μL volume) and incubate at room temperature for 30 min.
4. Add 150 μL of FuGene 6/plasmid mixture to the hESCs.
5. Place in incubator.
6. After 4 h, remove medium and add 2.5 mL of MEF-CM with 4 ng/mL FGF2.
7. The next day remove medium and begin selection.

Picking clones

Clones should be visible within a week and can be picked from the dish between 7–14 days and further cultured in individual wells of a 24-well plate for clonal expansion.

1. Under a microscope, pick individual colonies and place into 96-well plate containing 100 μL of medium.
2. Mechanically dissociate the colonies by gently pipetting the colonies and put each colony into an individual well of a 24-well plate that has been plated with freshly inactivated feeder cells.
3. After cells have grown for 1–2 weeks in the 24-well plate:
   - Passage half of the cells to a freshly prepared 24- or 6-well plate to prepare master stocks of the clones.
   - Use the other half of the cells for genomic screening.

EQUIPMENT

Standard equipment for tissue culture and molecular biology.

Supplies and Reagents

Transfection reagents

Item	Supplier	Catalog no.	Alternative
Lipofectamine 2000	Invitrogen	11668-019	
FuGene	Roche-Applied Science	11815091001	
ExGen 500	Fermentas (www.fermentas.com)	R0511	
Geneticin (G418) (solution 50 mg/mL)	Invitrogen	10131-035	Sigma
Hygromycin B (50 mg)	Sigma Chemical	H3274	Invitrogen
Puromycin (25 mg)	Sigma Chemical	P8833	Invitrogen

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PART

V

Advanced Methods

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CHAPTER

21

Derivation of Embryonic Stem Cells from Human Blastocysts

Prithi Rajan, David Smotrich, Ric Ross, Louise Laurent, and Jeanne F. Loring

INTRODUCTION

Human embryonic stem cells (hESCs) are pluripotent cells derived from the inner cell mass (ICM) of blastocyst-stage embryos. The ICM is a mass of about 50 cells which gives rise to both embryonic and extraembryonic tissues. More than 200 hESC lines have been reported worldwide, and while the earliest hESC lines were derived in medium containing fetal bovine serum (FBS) and/or on mouse feeder layers, more defined conditions are in development. These include derivation on human rather than animal feeder layers, and use of cocktails of purified growth factors instead of serum. There is still a great deal of room for improvement in derivation methods and recently there have been some important advances. In 2006, hESC lines were derived under GMP (Good Manufacturing Practice) conditions specifically for use in cell therapy. Also in 2006, hESC lines were derived under completely defined conditions without feeder layers, and new methods were reported for derivation of hESC lines from single isolated blastomeres. Table 21.1 summarizes the published reports on embryo-derived cell lines and conditions of their culture.

Human Stem Cell Manual, edited by J. F. Loring, R. L. Wesselschmidt, and P. H. Schwartz.
ISBN: 978-0-1237-0465-8.

TABLE 21.1 Derivation of hESC lines

Reference	Source	Preparation: Efficiency	Feeder layer/ substratum	Culture medium and conditions	Cell lines
Mandal *et al.*, 2006	Fresh surplus embryo, hatched with pronase	Whole embryo: 1 line/1 embryo	Mitomycin C-treated MEFs on 0.2% gelatin	D-MEM/F12 + 15% FBS/5% KSR, bFGF (4 ng/mL), LIF	ReliCell hES1 46XX
Baharvand *et al.*, 2006	Fresh surplus embryos, day 6, hatched with Tyrode's	Immunosurgery: 5 lines	MEFs on gelatin	D-MEM 20% + FBS, LIF, then insulin/selenium/ transferrin; pen-strep	Royan H2 46XX, Royan H3 69XXY, Royan H4 67XXY, Royan H5 46XX, Royan H6 46XY
Lysdahl *et al.*, 2006	Fresh surplus embryos	Immunosurgery: 4 lines/ 198 embryos	Irrad. human foreskin fibroblast	Knockout D-MEM + KSR, bFGF, LIF	CLS1, CLS2, CLS3, CLS4
Ludwig *et al.*, 2006	Blastocysts from frozen human pre-embryos, cultured 7 days after thawing and hatched with pronase	Immunosurgery: 2 lines/5 embryos	Collagen IV, fibronectin, laminin, vitronectin. No feeder cells	D-MEM/F12 + no serum, bFGF, LiCl, GABA, pipecolic acid, TGF, human serum albumin, vitamins, antioxidants, trace minerals,specific lipids; 10% CO_2/5% O_2, pH 7.2	WA15 46XY to tri 12, WA16 XXY
Mateizel *et al.*, 2006	Normal IVF (40 fresh/15 frozen) or PGD for monogeneic disorders (14 fresh)	Immunosurgery: 5 lines/55 embryos	Irrad. MEFs on 0.1% gelatin	Knockout D-MEM + 20% FBS (KSR in later cell lines), bFGF (4 ng/mL), LIF (omitted in later cell lines)	All normal karyotypes, VUB01, VUB02, VUB03_ DM1 + Myotonic Dystrophy 1 expan- sion (470 repeats), VUB04_CF + F508d el/5T compound

					heterozygote, VUB05_ HD + HD expansion (44 repeats)
Kim *et al.*, 2005	Blastocysts cultured 5–7 days from frozen human 2PN, hatched with pronase	Immunosurgery 6 lines/22 embryos; Partial embryo culture: 6 lines/16 embryos; Whole embryoculture: 1 line/9 embryos	Mitomycin C-treated mouse STO on 0.1% gelatin	Medium I: Knockout D-MEM, 10% FBS or 20% KSR Medium II: D-MEM/F12 + 20% KSR, 4 ng/mL bFGF, 2000 U/mL LIF; pen-strep	Not further described
Wang *et al.*, 2005	Surplus IVF blastocysts, hatched with pronase	ICM mechanically isolated: 1 line	Irrad. hESC-derived fibroblasts	D-MEM/F12 + 20% KSR, 8 ng/mL bFGF	SH7
Chen *et al.*, 2005	Day 3 embryos with low morphologic scores (<16), cultured to blastocyst stage	Immunosurgery2 lines/130 embryos	Mitomycin C-treated MEFs	D-MEM + 20% FBS, 4 ng/mL bFGF; pen-strep	hES-8 46XX, hES-18 46XY
Klimanskaya *et al.*, 2005	Cryopreserved embryos grown to blastocyst stage	Immunosurgery:1 line/5 embryos	ECM from MEFs; no feeder cells	Knockout D-MEM + 8% KSR, plasmanate, LIF, 16 ng/mL bFGF; pen-strep	ACT-14 46XX
Genbacev *et al.*, 2005	Cryopreserved embryos grown to blastocyst stage, hatched with Tyrode's. 321 fresh / 56blast/1 line, 192 frozen/55 blast/1 line	Whole embryo culture: 2 lines	Irrad. human placental fibroblasts	Knockout D-MEM, 12 ng/mL bFGF, 20% KSR	UCSF-1 46XX, UCSF-2 46XY
Inzunza *et al.*, 2005	3 fresh, 7 frozen, hatched with pronase	Immunosurgery: 2 lines/8 embryos; whole embryo: 0 lines/2 embryos	Irrad. human foreskin fibroblast	Knockout D-MEM, 8 ng/mL bFGF, 1% insulin/ selenium/ transferrin, 20% KSR; pen-strep	HS293 46XY, HS306 46XX

(*Continued*)

TABLE 21.1 (Continued)

Reference	Source	Preparation: Efficiency	Feeder layer/ substratum	Culture medium and conditions	Cell lines
Oh *et al.*, 2005	Cryopreserved embryos grown to blastocyst stage, hatched with pronase	Immunosurgery: 3 lines/73 embryos	Mitomycin C-treated mouse STO on gelatin	Knockout D-MEM or D-MEM/F12, 0.4 ng/mL bFGF, 20% KSR; pen-strep	SNUhES1 46 XY, SNUhES2 46XX, SNUhES3 46XY
Strelchenko *et al.*, 2004	Morula	8 lines			No further information
Simon *et al.*, 2005	Cryopreserved embryos grown to blastocyst stage, hatched with Tyrode's	Whole embryo culture 2 lines/40 embryos	Irrad. human placental fibroblasts	Knockout D-MEM, 12 ng/mL bFGF, 20% KSR	VAL-1 46XX, VAL-2 46XX
Stojkovic *et al.*, 2004	Fresh surplus embryos, grown to day 8 blastocysts	Immunosurgery: 1 line/11 embryos	Irrad. MEFs	D-MEM + 10% FBS × 17 days, then with knockout D-MEM + 10% KSR, 4 ng/mL bFGF; pen-strep	hES-NCL1 46XX
Baharvand *et al.*, 2004	Fresh surplus embryo, hatched with Tyrode's	Whole embryo: 1 line/1 embryo	Mitomycin C-treated MEFs on gelatin	Knockout D-MEM + 20% FBS + LIF, then insulin/ selenium/transferrin; pen-strep	Royan H1 46 XX
Heins *et al.*, 2004	Fresh surplus embryos, grown to day 6–7 blastocysts, hatched with pronase	Whole embryo: 4 lines; immunosurgery: 2 lines	Mitomycin C-treated MEFs	VitroHES + 4 ng/mL bFGF; 125 μg/mL hyaluronic acid	SA002 47XX tri 13, FC018 69XXY, AS034 46XY, AS038 46XY, SA121 46XY, SA181 46XY

Cowan *et al.*, 2004	Cryopreserved embryos grown to blastocyst stage, hatched with Tyrode's	Immunosurgery: 17 lines/58 embryos	Mitotically-inactivated MEFs	D-MEM + serum replacement + bFGF, LIF, plasmanate	HUES1/2/5/6/9/12/14/15 46 XX initially, HUES3/4/7/8/10/11/13/16/17 46XY initially, HUES3 and HUES4 developed tri 12, HUES1 developed additions to chromosome 2
Suss-Toby *et al.*, 2004	Fresh abnormal embryos (7 3PN, 2 1PN)	Whole embryo: 1 line/9 embryos	Mitotically-inactivated MEFs		I9 46 XX, heterozygous for MLD (PGD done for this)
Mitalipora *et al.*, 2003	Fresh discarded blastocysts	Immunosurgery: 4 lines/19 embryos	Mitotically-activated MEFs	Knockout D-MEM + 20% FBS, 4 ng/mL bFGF	BG01, 02, 03, 04 (BG0IV)
Hovatta *et al.*, 2003	Fresh surplus embryos (5), only one made it to expanded blastocyst, hatched with pronase	Immunosurgery: 2 lines/5 embryos	Irrad. human foreskin fibroblast	Knockout D-MEM + 20% FBS + LIF	HS181 46XX, HS207 not tested
Richards *et al.*, 2002	Cryopreserved embryo grown to blastocyst stage, hatched with pronase	Immunosurgery: 1 line/1 embryo	Mitomycin C-treated human fetal muscle	D-MEM + 20% human serum, human insulin/transferrin/selenium; pen-strep	46XY
Reubinoff *et al.*, 2000	Fresh surplus embryos, hatched with pronase	Immunosurgery: 2 lines/4 embryos	Mitomycin C-treated MEFs on gelatin	D-MEM LIF + 20% FBS (found not to be necessary); pen-strep	HES-1, HES-2
Thomson *et al.*, 1998	Fresh or cryopreserved	Immunosurgery: 5 lines/36 embryos	Irrad. MEFs	D-MEM + 20% FBS	H1/13/14 46 XY, H7/9 46XX

Overview

In this chapter we describe a method by which we have derived several hESC lines from human blastocysts. Our goal is to optimize conditions that promote survival and proliferation of the cells isolated from the ICM. We describe the procedures by which human embryos/blastocysts are obtained from *in vitro* fertilization (IVF) clinics and banks, culture of embryos *in vitro* to the blastocyst stage if necessary, dissection of the ICM from the blastocyst using microdissection techniques, transfer of the ICM to a culture dish and *in vitro* propagation of these cells, generation of a hESC line where sufficient numbers of cells are generated for further characterization and experimentation. The culture conditions described here are defined, with the exception of a commercial formulation of murine extracellular matrix (Matrigel™) and the factors provided by the human feeder cells. All culture components are human-derived, recombinant, or synthesized compounds, except for the insulin and bovine serum albumin in the commercial product KnockOut™ serum replacement (Invitrogen) (KSR).

Procedures

Embryos

The Stem Cell Resource

IVF clinics and "banks" of donated embryos provide human embryos for research. The Stem Cell Resource (SCR) is a non-profit affiliation of La Jolla IVF clinic and the Burnham Institute for Medical Research which offers an option for patients who have had successful *in vitro* fertilization procedures and who have completed their families to voluntarily donate the remaining embryos for research. The clinical arm of the SCR receives embryos from donors' IVF clinics and codes them so that there is no identifying information provided to the researchers who will work with the embryos. The SCR works under Institutional Review Board (IRB)-approved informed consent and standard operating procedures, and provides all of the documentation materials, shipping services and storage at no cost to the donor. Researchers who wish to use the donated embryos must have IRB and Embryonic Stem Cell Research Oversight Committee (ESCRO) approval to conduct human embryo research, and must apply to a scientific review committee that judges the quality of the proposed research project. Details about the SCR are provided in Appendix 21.1.

Sources of embryos

Most embryos donated for research are those that are frozen, in excess of a patient's reproductive needs, and are donated after successful pregnancies. Frozen embryos vary considerably in quality. The techniques for cryopreservation differ from clinic to clinic and have evolved over time, so embryos may have been frozen by different methods and at different stages. Embryos may be frozen at the two-pronuclei (2PN) stage, on day 3 (eight-cell) stage, or as blastocysts. We strongly recommend that a trained IVF embryologist thaw the embryos and culture them to blastocyst stage using conventional embryo culturing techniques.

Although most embryo donations are made after they are cryopreserved, embryos are sometimes discarded (and can be donated) before they are frozen. The most likely reason for discarding embryos is because they have chromosomal abnormalities or disease-associated mutations. Pre-implantation genetic diagnosis (PGD) is a procedure performed when there is a concern that the embryos may be abnormal; it is often performed when the chances of trisomy 21 are increased because the oocytes are provided by a woman older than 40. PGD can also be used to detect specific genetic diseases, such as cystic fibrosis, Tay Sachs disease, and hereditary cancers. PGD is generally performed by extracting one cell (blastomere) from the eight-cell embryo 3 days after fertilization, and while the remaining embryo develops for 2–3 more days to the blastocyst stage, the blastomere is analyzed by PCR to detect specific mutations or fluorescent *in situ* hybridization (FISH) for chromosomal analysis of the interphase nucleus.

Culture of embryos

Preparation of embryos for hESC derivation

Thawing and culture of embryos

Frozen 2PN and day 3 embryos are rapidly thawed and cultured in blastocyst culture media until they develop into blastocysts (Figure 21.1). Unless they are to be manually dissected (see below), the blastocysts are allowed to hatch, or induced to hatch by applying Acid Tyrode's solution with a micropipette on day 5 or 6. Alternatively, laser-assisted hatching can be used.

Thaw and culture of blastocysts

Embryos that were frozen at the blastocyst stage are thawed and cultured overnight in blastocyst culture medium for re-expansion and hatching.

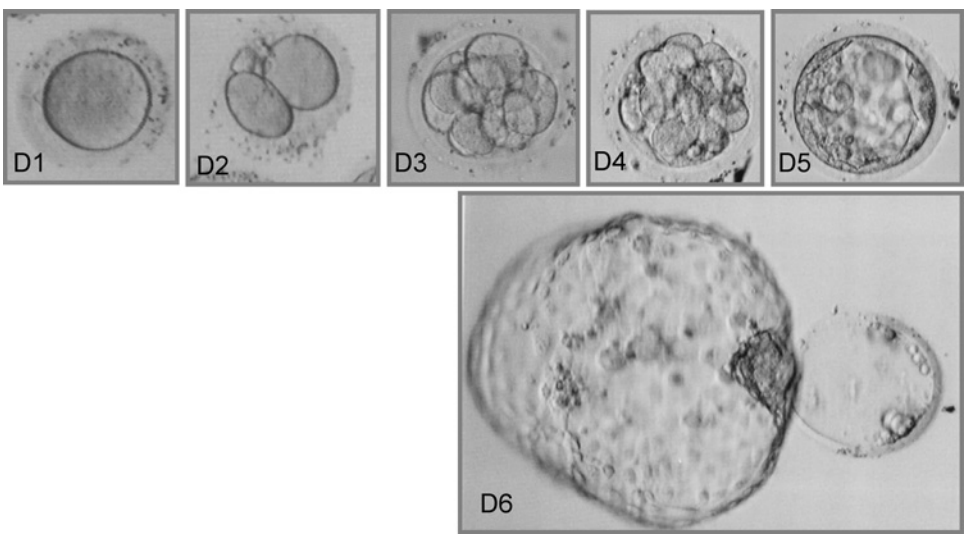

Figure 21.1 D1: Thawed one-cell embryo (day 1). D2: 2–4 cells (day 2). D3: 8 cells (day 3). D4: Morula (day 4). D5: Blastocyst (day 5). D6: Hatching (day 6).

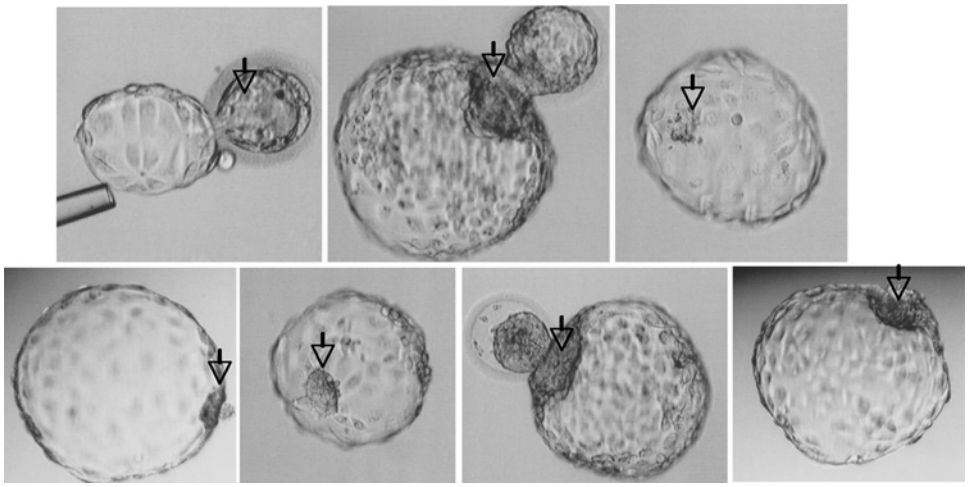

FIGURE 21.2 A variety of hatched blastocysts. The ICM is indicated by the arrow in each photo.

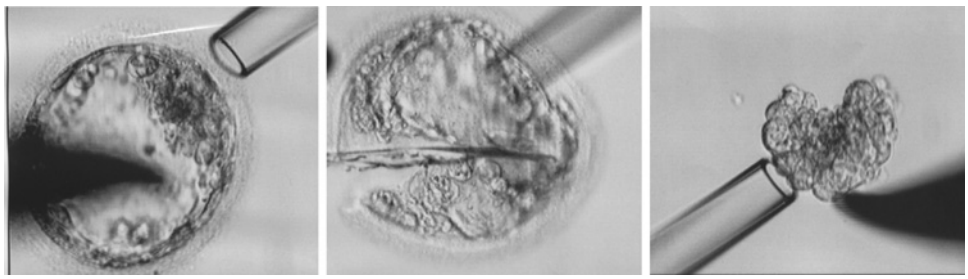

FIGURE 21.3 Dissection of single blastocysts. The embryo is cut with a microblade (left and middle) and the ICM is separated from the rest of the blastocyst (right).

Preparation of blastocysts for culture

There are several approaches to initiating culture of ICMs. The blastocysts can be cultured directly after hatching, subjected to immunosurgery (see Alternative procedures, below) or manually dissected. We favor a manual dissection, which appears to improve the viability and attachment in the initial stages of culture. Figure 21.2 shows a variety of hatched blastocysts.

Dissection of blastocysts to isolate the inner cell mass (ICM)

Embryos are placed in Splitting Medium and orientated such that the ICM is towards the biopsy pipette with a biopsy blade adjacent to it (Figure 21.3). The ICM can be partially or completely pulled into the biopsy pipette. The biopsy blade is then used to carve away the trophectoderm cells from the ICM, releasing the ICM cells into the pipette.

Preparation of culture dishes

Organ culture dishes, 60 mm dishes with a 10 mm well in the center (otherwise called IVF dishes) are used for the derivations because of the small volume (1 mL) and good

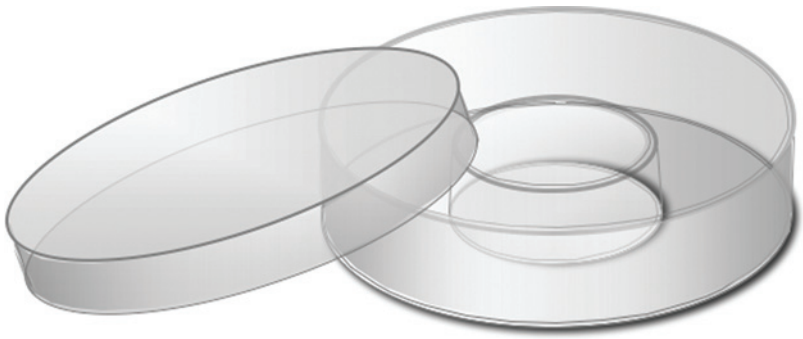

FIGURE 21.4 Sixty millimeter organ culture dish with 10 mm center well.

optical properties (Figure 21.4). Small colonies can be visualized in the limited volume of medium, and the shape of the wells makes it possible to dissect colonies with micro tools.

IVF dishes are coated with Matrigel (Becton-Dickinson; growth factor reduced).

1. Thaw Matrigel on ice to prevent gelling then dilute 1:30 in knockout D-MEM.
2. Coat center well with 0.5 mL Matrigel solution for 1 h at room temperature or overnight at 4°C.
3. Aspirate Matrigel from IVF dishes and add 1 mL of medium to the center well.
4. Equilibrate the medium in the incubator.

Culture procedures

Day 1: Embryo is placed into culture

1. Release the embryo or dissected ICM from the pipette and place it into the Matrigel-coated IVF dish containing 1 mL of medium.
2. Return the dish to the incubator.

Day 2: Feeder cells are added to the dishes in which the ICM was plated

1. Add feeder cells (mitotically inactivated human fibroblasts) to the culture dish without disturbing the embryo. Calculate the number of cells added so that the feeder cells are the same density as used for normal hESC culture.

NOTE: We use HS27 (human foreskin fibroblasts from ATCC) or hESC-derived fibroblastic primitive endoderm cells at a concentration of 50 000–100 000 per IVF dish.

2. Suspend the appropriate number of cells in about 250 μL of medium and gently add it to the culture dish. If the volume in the dish is already near capacity, remove 250 μL of culture medium from the dish before adding the cells.
3. Add bFGF to the medium to a final concentration of 20 ng/mL.

Day 3 and forward: Feeding and passaging of embryo/hESC cultures

Feeding

1. Growth factors are refreshed every day by adding the same amounts that were present at the original concentrations given in the recipe section.
2. The remaining factors are added only when fresh medium is added.
3. Approximately 40% of the culture medium is removed every alternate day and replaced with 50% of fresh culture medium. This discrepancy in volumes is due to the fact that there is always some loss of medium due to evaporation.
4. Always maintain the final concentrations of growth factors by replenishing them every day.
5. Typically healthy ICMs should attach within 3 days Replacing medium for ICMs which have not attached immediately can be done carefully, and is discretionary.

Passaging

1. Passage the cultures every 7th day.
2. Replace medium in the dish with 1 mL fresh complete medium.
3. Mechanically scrape the attached ICM or subcolonies off the IVF dish with sterile insulin syringes while viewing under a 10× or 20× objective with an inverted phase contrast microscope. The lower magnification afforded by dissection microscopes may not be adequate to view the smaller colonies.
4. Transfer the colonies suspended in the fresh medium into a new IVF dish ('current dish') with an established feeder layer.
5. Retain the old dish ('previous dish') – add 1 mL of fresh complete medium. Frequently colonies remain in the dish that housed the previous passage.
6. Replace both dishes in the incubator.

NOTE: For subsequent passages colonies from both the previous and current dish are pooled and the previous dish discarded.

Establishing an hESC line

- Establishing a line is a slow process, and it may be several months before a line is stable. In order for the culture to be designated as a cell line it must be successfully frozen and recovered from a frozen stock.
- When the population has expanded to at least 20 moderate-sized colonies, cryopreserve 8–10 colonies. We use a standard hESC freezing protocol, but others have had success with vitrification.
- Maintain the frozen vial for at least a week, then thaw and culture the cells.
- If you obtain about 80% recovery from freezing, there is a good chance that a stable line is established.
- Continue to expand the cells until several vials can be cryopreserved, then characterize the cells for hESC phenotype.

- We recommend that new hESCs be tested for the presence of diagnostic markers (SSEA4 and POU5F1/OCT4) by immunofluorescence, and karyotyped as soon as possible.
- The differentiation capacity should be tested *in vitro* and *in vivo*, and compared with a well-characterized hESC line to obtain a basic comparative profile.

Alternative Procedures

Immunosurgery

The microdissection technique described to isolate the ICM from the blastocyst can be substituted with an immunosurgery technique modified from a procedure developed for derivation of mouse ESCs. Briefly, after hatching, the blastocysts are incubated with an anti-human cell surface antibody that binds to the external layer, the trophoblast. After rinsing away excess antibody the embryos are treated with guinea-pig complement. The complement–antibody binding initiates lysis of the trophoblast. The ICM is protected because it was not accessible to the antibody. For further information see Mitalipova et al. (2003).

Pitfalls and Advice

- Maintain a daily log of the appearance of the colonies by photographing them each day and keeping a detailed written impression of the cultures.
- The details of the culture conditions may have to be modified based on the appearance of the colonies. For example, rapidly growing or differentiating colonies may need to be passaged more frequently.
- Feeder layers should have consistent plating density of plating and viability upon thawing. If feeder layers begin to degrade or lift in the hESC cultures, immediately passage the cells to a fresh feeder layer.
- The hESC colonies themselves should look phase-bright and healthy without dark centers. However, colonies with dark, dying cells can be recovered by passaging.

Equipment

- Biosafety hood: Sterile tissue culture hood with a provision to place a microscope inside for performing the passaging.
- Incubator: Water-jacketed incubator with carbon dioxide inlet for maintaining an atmosphere of 5% carbon dioxide.
- Centrifuge: Low-speed, swing-out buckets required, with no-brake option.
- Microscope: Good-quality inverted light microscope for observing and passaging colonies.
- Camera: CCD camera attached to the microscope for keeping records of the colonies particularly before and after passage.

- Software: Software to interpret and manipulate images from the camera. The free program ImageJ available from the NIH website serves very well.
- Micromanipulators/stereo microscope: These are required for microdissection of the ICM from the blastocyst, and will be available as infrastructure in an IVF clinic. (Examples are the Leica MZ75 (stereo microscope) and the Leica DM-IRB (inverted microscope).

Supplies and Reagents

Culture supplies

Item	Supplier	Catalog no.	Alternative
Center well organ dishes, 60 mm	BD/Falcon	353037	
Insulin syringes 28G$^{1/2}$	BD	329461	
Matrigel (growth factor reduced, phenol-free)	BD Biosciences	356231	
Knockout D-MEM	Invitrogen	10829-018	
D-MEM/F12 (with Glutamax)	Invitrogen	10565-018	
55 mM 2-Mercaptoethanol	Invitrogen	21985-023	
KnockOut serum replacement (KSR)	Invitrogen	10828-028	
Non-essential amino acids	Invitrogen	11140-050	
Gentamycin	Invitrogen	15750-060	
FGF2	Chemicon	GF003	Invitrogen
Bovine insulin	Sigma	I1882	
Ascorbic acid	Sigma	A4544	
Linoleic acid	Sigma	L9530	

Embryo (IVF) supplies

Item	Supplier	Catalog no	Alternative
1006 Biopsy plate	Falcon	1006	
Splitting media	Irvine Scientific	90103	
Cleavage culture media	Sage	ART-1526	
Blastocyst culture media	Sage	ART-1529	
Embryo thaw media	Irvine Scientific	90124	
Blastocyst thaw media	Irvine Scientific	90110	
Stripper	MidAtlantic Diagnostics	MXL3-STR	
Stripper tips	MidAtlantic Diagnostics	MXL3-203-275	
Biopsy pipette	Humagen	MBB-FPS30	
Hatching pipette	Humagen	MAH-SM-30	
Biopsy blade	AB Technologies	ESE-20	
Holding pipette	Humagen	MPH-MD-30	
Acid tyrodes	Irvine Scientific	99252	
Culture plates	Falcon	FAL3002	
Culture oil	Conception Technologies	OTC-100-F	
Four-well plates	Nunc	176740	

Recipes

Culture medium

Component	Final concentration	Stock concentration
2-Mercaptoethanol	0.1 mM	55 mM
KnockOut serum replacement (KSR)	20%	100%
Non-essential amino acids	0.1 mM	10 mM
Gentamicin	10 μg/mL	50 mg/mL
bFGF	20 ng/mL	10 μg/mL in PBS with 0.1% BSA
Bovine insulin	25 μg/mL	2.5 mg/mL in mildly alkaline water or PBS
Ascorbic acid	0.1 μM	0.1 mM in water
Linoleic acid	1×	100×
Matrigel	1×	30×

Quality Control Methods

In the protocol given above, the only component which is undefined is Matrigel. While this has not been a problem in our laboratory, it is possible that some batches of Matrigel may be less efficient than others. This will have to be dealt with on a case-by-case basis. The other complex, but defined, ingredient is KSR. Batches are quality controlled by Invitrogen and one assumes that all batches will be equally efficient in supporting this culture.

Reading List

Human ESC derivation

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Appendix 21.1 The Stem Cell Resource Bank of Donated Embryos

The following information is available on the Stem Cell Community website: www.stemcellcommunity.org.

What is the Stem Cell Resource?

The Stem Cell Resource (SCR) is an independent not-for-profit organization that was founded in 2003 by a group of reproductive medicine professionals and academic researchers for the purpose of furthering knowledge in the field of human embryology and cell therapy. The SCR maintains a repository and registry of IVF embryos that are not of clinical use and have been designated for medical and basic research.

What does the Stem Cell Resource do?

The SCR provides a no-cost service to physicians who have patients seeking to donate for research surplus embryos that are otherwise destined for destruction. The SCR provides information for patients to ensure that they understand the ethical and scientific issues surrounding such donations, and supplies Informed Consent documents for the donors to sign. The SCR protects the donor's confidentiality by having researcher-independent blinding of all tissues and samples. If a patient wishes to donate, the SCR provides the IVF physician with transport equipment and ensures the safe delivery of the material to the research facility.

How does it work?

Designated embryos are initially transferred from their current storage facility to the SCR, where all the information that identifies the embryos (e.g., donor name, address or other identifying information) is removed from the material itself. This information is stored off-site, and is accessible only by SCR under special circumstances. The embryos are stored at the SCR repository, identified by a registry code that is the only information that will be given to researchers.

The SCR transfers embryos to research scientists who have completed a detailed research proposal that is evaluated by a scientific committee at the SCR. The recipient must agree to adhere to guidelines that ensure that safe, ethical, and meritorious research practices are in place and that federal funds will not be used. The NIH is informed of all transfers.

Information for the potential embryo donor

If you are interested in donating embryos please contact us by email or ask your IVF physician to contact us. We will answer your questions and if requested, we will provide your IVF physician with all of the information required to complete the donation.

Information for the reproductive specialist

The SCR will provide an information packet for your patients explaining the use of embryos for research, and an Informed Consent Document that they must sign if they decide to donate. The SCR will arrange for transportation of the donated material to their repository at no charge to the patient or the physician.

Information for the stem cell researcher

The SCR's goal is to facilitate the research needed to help cure disease. If you are interested in obtaining donated embryos and/or stem cell lineages please contact SCR to request a research application. Biological materials are provided at no cost to the researcher. Only those costs associated with shipping and handling of tissues will be incurred by the applicant.

The researcher will not receive any identifying information about the donors. Only pertinent genomic or genetic data (e.g., aneuploidy or mutation) will be provided to the research group. If clinical correlation is warranted, the researcher may apply to the SCR for additional clinical information, but under no circumstances will information be provides that specifically identifies the donors.

Appendix 21.2 Development of a Cell Line

Figure 21.5 and Table 21.2 summarize the development of the cell line derived from embryo number 06-27-01. While there is no typical case of cell line development, this particular cell line started slowly, and took about five passages to start dividing well. Figure 21.5 shows the large increase in cell numbers starting from the clumps of isolated ICM. The hESC colonies formed as phase bright clumps of cells which were dissected into smaller colonies while passaging, using the bevel at the tip of the needle as a scalpel. The dissected colonies then increased in size over the week in culture. The feeder layer used in this experiment was an hESC-derived fibroblastic primitive endoderm-like cell type.

Table 21.2 shows the progression of passaging regimen, and the increasing number of colonies. Colonies may be frozen as the culture expands, this will give rise to stocks at very early passages.

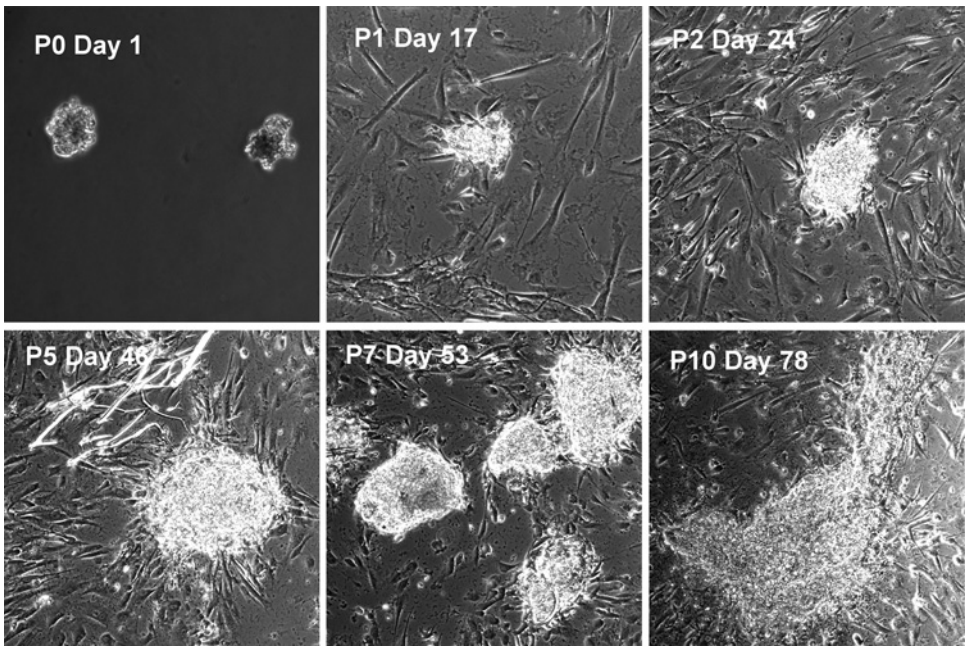

FIGURE 21.5 Summary of development of the cell line derived from embryo number 06-27-01.

TABLE 21.2 Excerpts from daily log of hESC line development

Day	Observation	Passage
day 1	dissected blastocyst into ICM and trophoblast	
day 2	2 clumps: both floating. Added feeder cells	
day 11		Passaged P1
day 17	P0: nothing. P1: 2 clumps	Passaged P2
day 24		Passaged P3
day 30	P2: 1 colony. P3: 2 colonies	
day 31		Passaged P4
day 37	P3: 1 colony. P4: 3 colonies, 2 possible colonies	
day 38		Passaged P5
day 42	P4: 1 colony. P5: 7 colonies	
day 45		Passaged P6
day 52	P5: 4 colonies. P6: ~10 colonies	Passaged P7
day 54	P6: 3 colonies. P7: 20 colonies	
day 59		Passaged P8
day 65	P7: 5 colonies, 1 maybe. P8: 23 colonies	
day 66	Froze 2 vials with ~10 colonies each	Passaged P9
day 74		Passaged P10
day 79	P9: 2–3 colonies. P10: 9 colonies	
day 81		Passaged P11
day 86	P10: 4 colonies. P11: 20 colonies	

CHAPTER

22

Neural Progenitor Cell Culture

Hubert Nethercott, Heather Maxwell, and Philip H. Schwartz

INTRODUCTION

Cultured neural progenitor cells hold considerable promise, both in terms of their application to a wide variety of research projects, and their use in development of therapeutic modalities. In the case of human neural progenitor cells (hNPCs), the primary source has been donated fetal tissue. However, the post-mortem brain could be a source of a vast supply of hNPCs that could reduce or eliminate reliance on fetal or embryonic sources. The practicality of such an approach is supported by recent work demonstrating the viability of hNPCs obtained from cadaveric donors, even after post-mortem intervals exceeding 20 h. The number of hNPCs with high proliferative and differentiation potentials, is greatest in the youngest brains, so brains harvested from premature and neonatal infants provide the best available post-natal, so post-mortem source of hNPCs. For example, well over 10 000 neonatal deaths with no neurological involvement occur annually in the USA (National Center for Health Statistics); thus, cells harvested from these patients may ultimately open up major new options for the prevention or repair of neurological disease or injury.

hNPCs can serve as a tool with which to address many questions concerning both the development and pathology of the human central nervous system, and they may provide

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answers not easily obtainable from studies in animals. There are many potential uses of these cells. One example is elucidation of the genetic signals involved in the differentiation of immature neural progenitor cells into the wide variety of fully developed cells that make up the nervous system. Another example is discovery of the effects of genetic disease on the structure and function of neural cells and the tissues into which they develop. This knowledge may provide a better understanding of CNS pathophysiology, as well as potentially useful strategies for intervention. A third example is to provide an *in vitro* system with which to efficiently screen pharmacological compounds of interest. Finally, undifferentiated human neural progenitors cells may provide a source of donor material for future therapies directed at developmental, degenerative, traumatic, ischemic, infectious, or neoplastic disease of the CNS.

Overview

In this chapter we will describe the basic procedures to be used for the successful culture of human neural progenitor cells, including establishment of primary cultures, passaging, differentiation, and cryopreservation. We also describe the establishment of glial cultures which are used to generate conditioned medium for differentiation of the neural progenitor cultures.

It should be noted that human neural progenitor cells appear to have a preference for high cell density conditions. As a result, common terms for defining percent confluence are difficult to apply. Cells are passaged when they are superconfluent and into no more than twice the surface area from which they are lifted. To make calculations easier, we have provided, in Appendix 22.1, a table of surface areas for the most commonly used tissue culture flasks, dishes, plates and slides.

Procedures

Establishing primary cultures

Tissue harvest

The basic techniques for autopsy and brain dissection are described in Schwartz et al. (2003) and will not be covered further here.

Figure 22.1 shows cells one week after beginning the culture.

Tissue digestion

1. Thaw PPD (papain, protease, DNase) solution and add ~100 mg finely minced fresh tissue to 10 mL PPD. (Alternatively, quickly thaw cryopreserved tissue, dilute 10-fold with DGA, pellet 1–2 min at 400 ×*g*, and transfer to PPD as above.)
2. Incubate at 37°C for 30–90 min with frequent mixing. Cell viability is better if tissue is incompletely digested. Adult brain tissues usually take 30–45 min. Fetal tissues will dissociate in as little as 10 min.

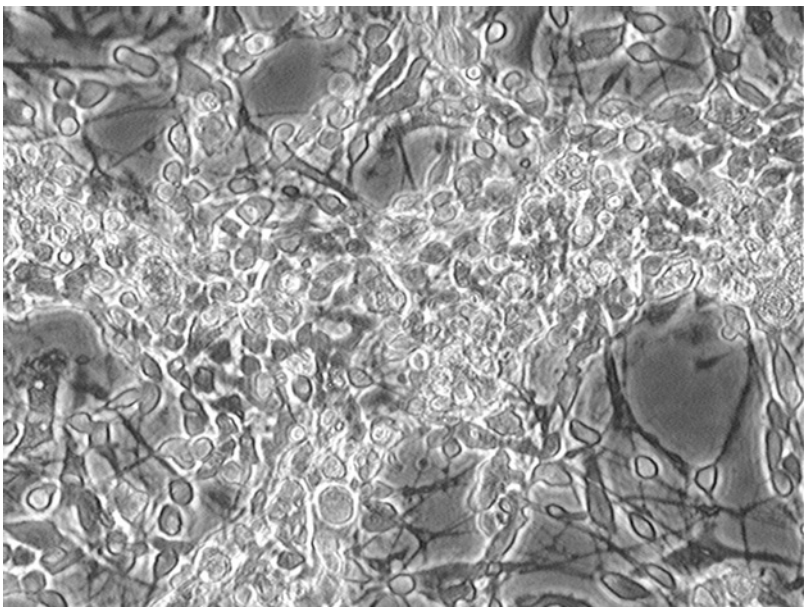

FIGURE 22.1 Phase contrast photomicrograph (20×) showing numerous very small phase bright cells on top of an adherent primary culture of human neural progenitor cells, one week after beginning the culture.

3. Add an equal volume of DGA and pellet dissociated tissues (400 ×*g* × 5 min). Discard supernatant, bang pellet free, and wash three times with 10 mL DGF (pellet 5 min at 400 ×*g*); triturate 2–3 times with the 10 mL pipette at each rinse. Resuspend in 10 mL primary medium to culture progenitors or 30 mL DGF to culture glia (or a fraction each way to culture both).

Figure 22.2 shows a recently plated primary culture.

Initial plating – glial fraction

1. Plate 15 mL of the DGF suspension into each 100 mm plastic Petri dishes.
2. Incubate at 37°C, 5% CO_2.
3. Feed every 4 days with a complete exchange of medium (DGF).
4. When cells are near confluence, feed cells with DGFB to prepare them for collection of glial-conditioned medium (GCM).
5. Feed every 4 days or more often as necessary.
6. Passage at 80–90% confluence by lifting with ATV and splitting 1:4.

Figure 22.3 shows a recently plated primary culture at higher power.

Glial cell conditioned medium (GCM)

- To collect GCM, the medium from confluent glial cultures (DGFB) is first aspirated and the cultures are then washed once with DGAB.

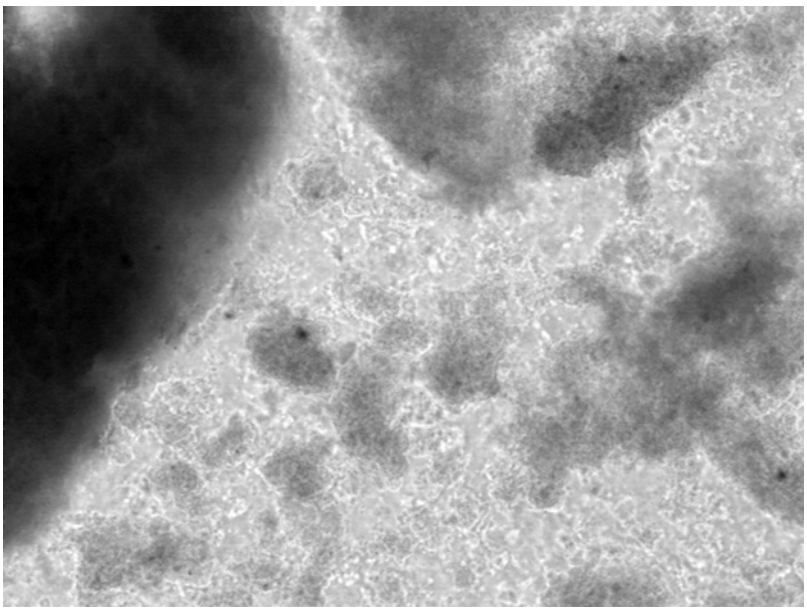

Figure 22.2 Low-power (4×) phase contrast photomicrograph of a recently plated primary culture. Note the density of dissociated tissue and the presence of clumps of undissociated tissue (phase dark clumps).

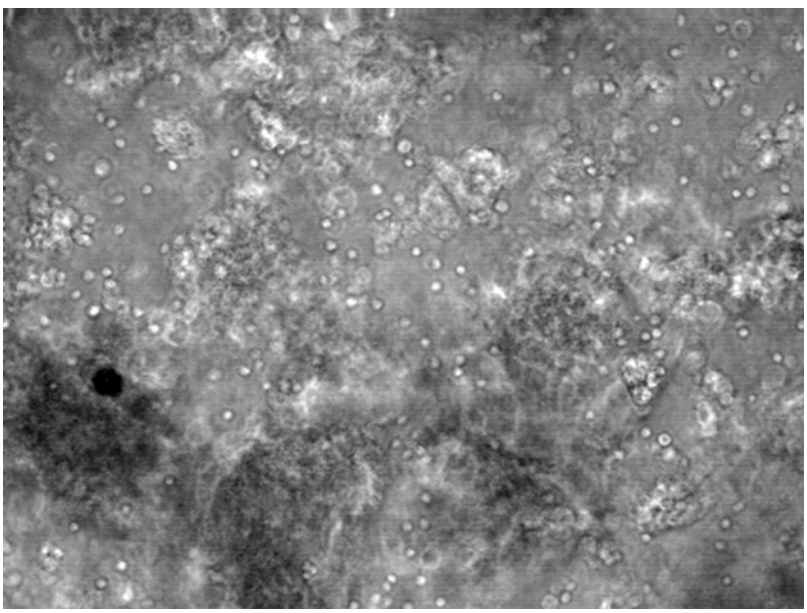

Figure 22.3 Higher power (10×) phase contrast photomicrograph of recently plated primary culture, focal plane at level of fibronectin-coated plastic surface. Note the presence of numerous, small, phase bright cells.

- The cultures are then fed with DGAB and the medium is collected 24 h later as GCM. Medium is replaced with DGFB for 24 h and the process is repeated the next day.
- This can go on for 7–10 cycles before the culture is considered expended and then discarded. GCM should be sterile filtered and stored at −20°C.

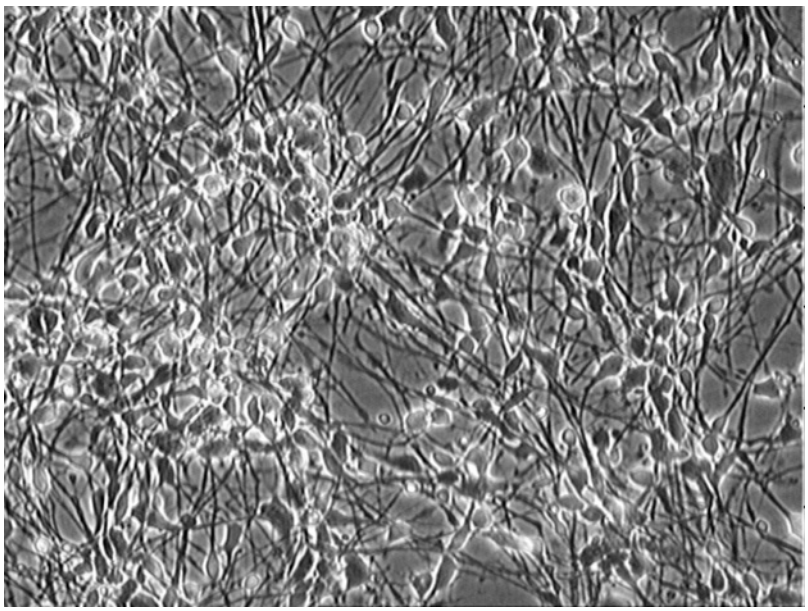

FIGURE 22.4 After 2–3 weeks, a robust culture is established.

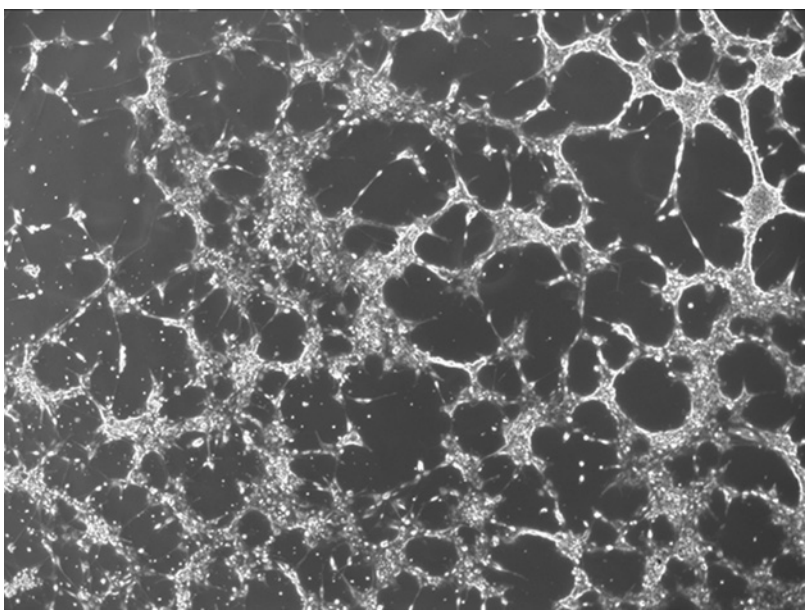

FIGURE 22.5 After 4–6 weeks, the cultures are becoming so dense that they tend to coalesce into areas of tightly compacted cells.

Figures 22.4 and 22.5 show cultures after 2–3 weeks and 4–6 weeks respectively.

NOTE: Different media are used for for the glial cultures: DGF for an expanding culture, DGFB for a culture transitioning for conditioned medium collection, and DGAB for conditioned medium generation.

Initial plating – progenitor fraction

1. Plate 5 mL each of the primary medium suspension into two 60 mm plastic, fibronectin-coated (see below) Petri dishes. The progression of the microscopic appearance of the cultures from initial plating to maturity is shown in Figures 22.1–22.8.
2. Use two 60 mm dishes (5 mL each) per 100 mg tissue.
3. Feed every 2 days with growth medium (GM), replacing 50% of the medium each time. The cultures can be fed on Monday, Wednesday, and Friday. However, if the culture is confluent (or more) on Friday, remove 50% of the medium and add back twice the normal feeding volume to ensure the cells do not run out of nutrients over the weekend.
4. When feeding, if any non-adherent cells are visible, remove half of the medium, spin at 200 $\times g$ for 5 min, carefully aspirate the supernatant, resuspend the pellet in fresh GM and put this back into the culture dish.

Progenitor cell conditioned medium

Progenitor cell conditioned medium (CM) may be collected at every feeding but it is really only necessary to do this when cryopreserving cells as you will need CM for

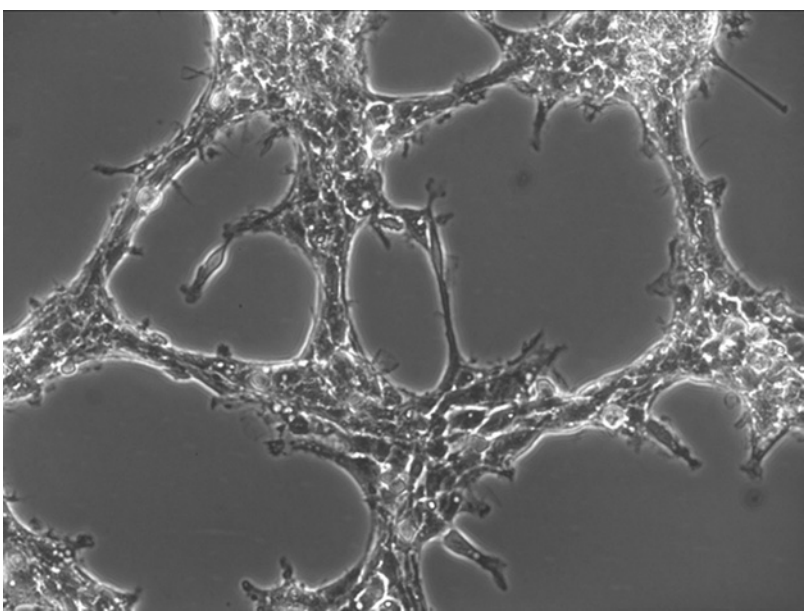

FIGURE 22.6 Higher power magnification of Figure 22.5 showing densely clustered cells with occasional cells migrating out onto the fibronectin coating.

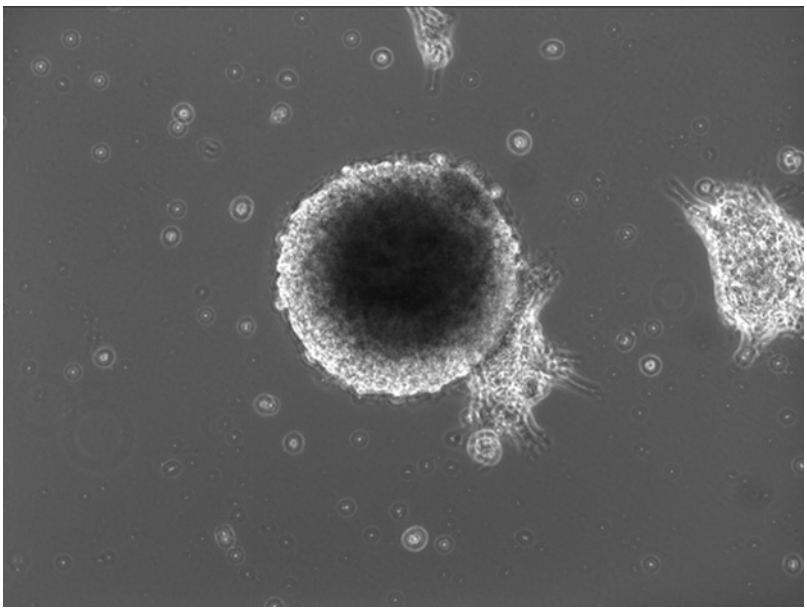

FIGURE 22.7 If the cells of Figure 22.5 are lifted and replated without the fibronectin coating, they tend to form neurospheres rather than attached clusters although some clusters will still attach, particularly if the density is very high.

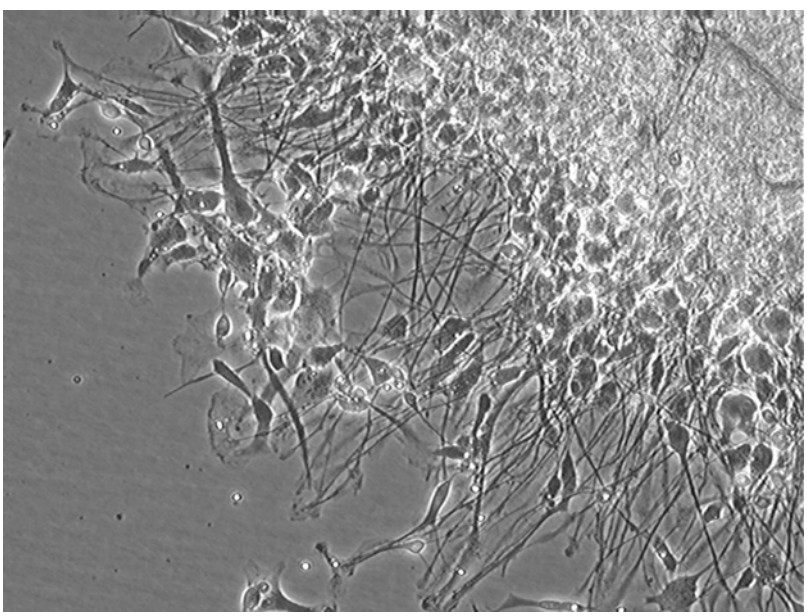

FIGURE 22.8 Spheres such as the one shown in Figure 22.7, replated onto a fibronectin-coated dish. The sphere attaches and cells begin to stream out onto the surface of the dish.

re-establishment of the culture after thawing. When passaging the cells as a 1:2 split, use half of the CM for each new culture.

Thawing and plating protocol

1. Wear eye protection. Obtain cryovial from the dewar and make sure vial cap is screwed down well.
2. Thaw by hand, with constant shaking, under warm tap water. This should take between 60 and 75 s.
3. As soon as the contents of the vial are thawed, rinse the vial with 70% ethanol and place in hood.
4. Transfer the entire vial contents to a 15 mL Falcon tube using a sterile transfer pipette.
5. Add BIT 9500 one drop at a time with constant agitation until volume of tube contents has doubled; then add 10 mL of D-MEM/F12, 0.1 volume at a time.
6. Spin at 200 $\times g$, at room temperature, for 5 min.
7. With tilting, gently aspirate supernatant, leaving behind about 100 μL to avoid aspirating the pellet.
8. Add an appropriate amount of medium (GM:CM, 1:1), to achieve 200% density when plating, suspend with gentle trituration, and transfer to an uncoated plate (i.e. plate cells as neurospheres, see below). A density of 200% is defined as half the surface area from which the frozen cells were lifted.
9. Feed by exchanging 50% of the medium with fresh GM every 2 days; save the removed CM as needed.
10. When the neurospheres reach about 1 mm in diameter, dissociate the cells with Cell Dissociation Buffer (Gibco-BRL) and passage cells into fibronectin-coated dishes of an area twice the original area from which the culture was lifted, in GM:CM (1:1).
11. When confluent, lift and replate the cells into the same area, on fibronectin, in GM:CM (1:1).
12. Feed by exchanging 50% of the spent medium with fresh GM every 2 days; save CM if required.
13. Expand as described below.

NOTE: If the cells are obtained from the National Human Neural Stem Cell Resource, they will be accompanied by an adequate amount of CM for step 8 above. The amount of culture medium and area into which to culture the original vial of cells in step 8 above will be indicated on the packing slip but usually will be 2 mL and 10 cm^2, respectively.

NOTE: To store CM, filter and store at −20°C. It will come in useful on a regular basis.

Preparation of laminin-coated plates

Day 1

1. Coat dish with poly-L-ornithine, using standard plating volumes:
   - 10 μg/mL for polystyrene.
   - 50 μg/mL for glass.
2. Incubate overnight at 37°C.

Day 2

1. Dilute laminin in sterile PBS to a concentration of 5 μg/mL.
2. Aspirate the poly-L-ornithine and wash twice with sterile H_2O (i.e. filtered MilliQ water).
3. Add a standard plating volume of laminin to the tissue culture plate/dish, completely covering the plate.
4. Incubate overnight at 37°C.

Day 3

1. Aspirate the laminin.
2. Rinse the plate once with 5 mL of PBS, aspirate the PBS and plate the neural progenitor cells evenly throughout the dish area.

Preparation of fibronectin-coated plates

1. Prepare fibronectin solution at 10 μg/mL.
2. Completely cover surface of plate/flask with a standard plating volume of fibronectin solution.
3. Incubate at 37°C for at least 1 h, up to 24 h.
4. Aspirate fibronectin.
5. Let plate/dish completely air dry, uncovered, in tissue culture hood before adding cells.

NOTE: Fibronectin solutions can be saved and re-used once. Store 1× -used fibronectin at 4°C for up to two weeks.

Lifting of an established adherent culture

1. From a confluent vessel, remove and save medium (CM), rinse culture with Ca/Mg-free Hanks' Buffered Salt Solution (HBSS), keeping rinse and CM separate.
2. Lift cells with Cell Dissociation Buffer (CDB) by adding a standard plating volume and incubating at 37°C for 5–15 min or until cells have drawn in most of their processes. Do not overincubate.

3. Dislodge cells that remain adherent by sharply rapping culture dish. A Sharpie marker works well for this.
4. Combine cell suspension with rinse (in step 1) and spin it and the CM (separately) at 200×*g*, at room temperature, for 5 min.
5. Resuspend the pellets in GM:CM (1:1) and combine. Remove CM from the pellet (if any) and remove the rinse/CDB from its pellet. Resuspend one pellet, transfer to the other and resuspend it as well in the same medium.

Passaging and expanding an established adherent culture

1. When the cells are confluent (or more), lift with CDB, as described above, and resuspend in twice the volume of GM:CM (1:1).
2. Passage cells into twice the original area from which the culture was lifted. Passage onto fibronectin-coated plates/dishes.

Differentiation

- Differentiation will usually be done when the cells are on glass coverslips or in chamber slides (see protocol below).
- Feed cultures daily, removing half of the medium and replacing with an equal volume of differentiation medium.
- Differentiate for 1–6 weeks (or longer).

NOTE: Differentiation of dense cultures may be difficult and may require feeding twice daily (as determined by the color of the medium). RA must be added to the differentiation medium just before feeding. A stock of differentiation medium, less the RA and enough to last for two weeks, may be made and stored at 4°C but the RA must be added fresh.

Passaging and expansion of a neurosphere culture

1. When the spheres are 1–2 mm in diameter, remove medium and spheres and spin at 200×*g* for 5 min.
2. Remove half of the supernatant and replace with an equal volume of fresh GM.
3. Resuspend the pellet and place into a fibronectin-coated dish of the same size as the dish from which the spheres were removed.
4. Let the spheres attach and spread for one week (or so), feeding as usual.
5. Lift as described for an adherent culture.
6. Plate into an uncoated plate of twice the surface area.

Cryopreservation

1. Cool freezing medium on ice.
2. Lift cells from a confluent flask/dish.

3. Spin at 200×*g*, at room temperature, for 5 min.
4. Resuspend pellet in freezing medium at a cell concentration of 1×10^6 cells/mL.
5. Aliquot into cryovials (4 vials/75 cm^2 dense culture) and slow–freeze to −80°C in a freezing container insulated in isopropyl alcohol for 24 h.
6. After 24 h and up to one week, transfer cryovials to $N_{2(l)}$.

Preparation of cells for gene product analysis

1. Follow lifting protocol, but resuspend pellet in 1 mL HBSS.
2. Transfer resuspension to sterile centrifuge tube.
3. Place centrifuge tube containing cells on ice.
4. Follow protocol for RNA extraction.

Plating chamber slides or coverslips

1. Plate slides/coverslips when passaging or cryopreserving cells (i.e. at confluence). This example assumes the plating of three eight-well slides from a T-75 culture flask during a passage and assumes a dense culture in the T-75.
2. Prepare eight-well slides with extracellular matrix substrate (fibronectin for proliferating cells or laminin for differentiating cells).
3. Calculate the area, using the surface area table in Appendix 22.1. T-75: Area is 75 cm^2 (1a).
4. Eight-well slide: 0.8 cm^2/well × 8 wells/slide × 3 slides = 19.2 cm^2 (1b). For coverslips, use the surface areas of the wells in which the coverslips are placed.
5. Calculate the volume (1b):volume (1a) ratio. 19.2 cm^2:75 cm^2 = 25.6%.
6. Calculate volume of media needed, using the surface area table in Appendix 22.1. Three eight-well slides: 250 μL/well × 8 wells/slide × 3 slides = 6 mL.
7. Make 6 mL of 1:1 GM:CM.
8. Lift the cells from T-75 (see protocol); spin at 1300×*g* for 8 min.
9. Resuspend pellet in 1 mL CM.
10. Take 256 μL of the 1 mL resuspension (25.6%) and add to 6 mL of 1:1 GM:CM.
11. Add 250 μL of the suspension from step 10 to each well of the eight-well slides.
12. The rest of the cells can be passaged or cryopreserved as usual.
13. Feed as usual, being careful not to be too vigorous when adding medium to the wells.

Slide fixation

1. Aspirate medium.
2. Wash each well once, gently, with PBS.
3. Add a standard plating volume of paraformaldehyde (PFA), gently, to each well (see Chapter 9 for details).
4. Incubate for 10 min at room temperature.
5. Aspirate PFA.
6. Wash once, gently, with PBS and perform immunocytochemistry immediately.
7. To store, add 0.05% (w:v) sodium azide to PBS, and fill each well.
8. If storing long-term, wrap slide tightly in parafilm and keep at 4°C.

Alternative Procedures

Plating primary cultures without enzymatic digestion

It is possible to plate a primary culture without using any enzymatic digestion of the tissue. With this method, finely mince the tissue in culture medium, triturate it several times in through a 5 mL pipette, and plate as usual. For each of the following 2–5 days, remove the medium and tissue chunks from the plate, feed the plate with half standard plating volume GM, triturate the removed tissue suspension several times through a 5 mL pipette, and spin down as usual. Then remove half the supernatant, resuspend the pellet in the remaining supernatant, triturating it several times with a 5 mL pipette, and put the resuspension back into the plate. Repeat this daily until the tissue is almost fully disaggregated.

Recovery from/prevention of microbial contamination of primary and early secondary cultures

Removal of brains at autopsy is necessarily a non-aseptic technique and although pains are taken to be as clean as possible, contamination is common. For this reason the primary cultures are established with the cohort of antibiotics described. Generally, the antibiotics may be removed after several passages. Occasionally, however, contamination may recur and because these cultures may represent very rare neurogenetic diseases, efforts to eliminate the contamination must be taken. One thing to remember is that all the antibiotics have a limited half-life in culture, rapidly degrading over just a few days. Thus, feeding regularly is essential, not only to keep nutrient and growth factor levels at their optimum but also the antibiotics. For fungal contamination, use a sterile Pasteur pipette connected to a vacuum source to aspirate the fungal colony before feeding. For this type of contamination and all others, the levels of antibiotics may be doubled or tripled. Especially useful (for an adherent culture) is removing the medium, washing the culture several times with HBSS, and replacing the medium after it has been sterile-filtered. For a neurosphere culture, the spheres can be "pelleted" with a low-speed spin (100–200×*g* for 1–2 min), before the medium is removed and filtered. The pellet may be washed several times with HBSS before putting back into a fresh plate.

Pitfalls and Advice

Do not passage at low density

Although some cells become contact inhibited at high density, that is not the case for these cultures. The proliferation rate of low-density cultures is considerably slower than that of high-density cultures and there is also a tendency for differentiation and apoptosis. Always maintain NPCs at high density.

All tissue culture plastic is not the same

The growth characteristics, morphology, viability, migration, and differentiation of hNPCs are highly dependent on the nature of the surface on which the cells reside. While there is much literature comparing the effects of different extracellular matrices on these properties, it is not well-appreciated that different plastics, even though they may be called by the same name, also have widely different effects on hNPC properties. As a result, we have listed the exact part numbers of the plastic dishes, flasks, and plates that we find work well with these cells. Use of different products may result in unpredictable outcomes.

Start cryopreserved cultures as neurospheres

Since one cannot be absolutely sure what the viable cell count will be after thawing, the safest thing to do is to start cultures as neurospheres. That is, plate the thawed cultures without fibronectin and grow them for 1–2 weeks until you can be sure how many viable cells there actually are before plating them as an adherent culture.

Do not passage with enzymes

Although many other cultures are typically lifted with enzymes for passaging, resist the temptation to do so with these cultures as there is a high risk of high rates of cell death, lack of adherence, or differentiation if you use enzymes. The calcium/magnesium free dissociation buffer works adequately, with patience, but on a rare occasion the cells may not come up (either because they have partially differentiated or because of the use of culture plates other than those recommended). In this case, and while the cells are still in the cell dissociation buffer, add 0.1 volume of ATV to facilitate lifting. Incubate in the tissue culture hood (or under the microscope) and monitor the cells closely, adding 1 volume of DGAB when the cells begin to lift.

Fixing cells for immunocytochemistry

Although this is dealt with in greater detail in Chapter 9, it is of sufficient importance to reiterate it here. Paraformaldehyde (or polyoxyethylene) is a polymer of formaldehyde and is used to make a high-purity formaldehyde-based fixative. Commercial formalin (formaldehyde in neutral buffered salt solution) is stabilized with methanol because formaldehyde, over time, generates a number of chemical by-products that can interfere with antibody binding or can lead to very high background fluorescence. Despite this stabilization, however, commercial formaldehyde solutions still break down over time and, thus, it is desirable to generate a high purity product in the laboratory that can be used shortly after it is made, minimizing the problems

mentioned. This high-purity formaldehyde fixative can be directly produced by depolymerizing PFA, in solution. This is accomplished with heat (<50°C) and alkalinity (<pH 10). The resulting solution can be aliquoted and stored at −20°C for at least six months with satisfactory results in most applications. Some applications may still require fresh solutions to be used.

NOTE: Weighing and dissolution of paraformaldehyde must be done in the fume hood.

EQUIPMENT

Common tissue culture equipment (see Chapter 26).

SUPPLIES

Disposables

Item	Supplier	Catalog no.
Centrifuge tube, disposable Fisherbrand 15 mL	Fisher Scientific	05-539-12
Centrifuge tube, disposable Fisherbrand 50 mL	Fisher Scientific	05-539-8
Chamber slide, Lab-Tek four-well	Nalge Nunc International	177399
Chamber slide, Lab-Tek eight-well	Nalge Nunc International	177402
Filter unit, VacuCap 60 W/0.2 μm Supor Membrane	Pall Corporation	4632
Filter, Acrodisco Syringe 0.2 μm	Pall Corporation	4433
Filters, 50 mL tube top	Corning Incorporated	430320
Freezing container, Cryo 1°C	Nalgene	5100-0001
Membrane, Stericup GP Express Plus 0.22 μm	Millipore Corporation	SCGPU02RE
Pipettes, Fisherbrand 10 mL disposable	Fisher Scientific	13-678-11E
Pipettes, Fisherbrand 2 mL disposable	Fisher Scientific	13-678-11C
Pipettes, Fisherbrand 25 mL disposable	Fisher Scientific	13-678-11
Pipettes, Fisherbrand 5 mL disposable	Fisher Scientific	13-678-11D
Pipettes, Fisherbrand 50 mL disposable	Fisher Scientific	13-678-11F
Plate, Microtest 96-well	Becton Dickinson Labware	35-3072
Scalpels, protected disposable	BD Bard Parker	372611
Tissue culture dishes, CellStar 60 × 15 mm	Greiner Bio-One	N/A
Tissue culture dishes, TPP 100 × 20 mm	TPP	93100
Tissue culture flasks, 250 mL CellStar (T-75)	Greiner Bio-One	658-175
Tissue culture flasks, 50 mL CellStar (T-25)	Greiner Bio-One	690-175
Tissue culture flasks, 650 mL CellStar (T-175)	Greiner Bio-One	661-195
Tissue culture plate, Falcon Multiwell 12-well	Becton Dickinson Labware	35-3043
Tissue culture plate, Falcon Multiwell 24-well	Becton Dickinson Labware	35-3047
Tissue culture plate, Falcon Multiwell 48-well	Becton Dickinson Labware	35-3078
Tissue culture plate, Falcon Multiwell 6-well	Becton Dickinson Labware	35-3046

Chemicals and reagents

Item	Supplier	Catalog no.
All-*trans* retinoic acid, 50 mg	Sigma-Aldrich	R2625
BDNF, 10 μg	Chemicon	GF029
BIT 9500, 100 mL	Stem Cell Technologies	9500
Cell dissociation buffer, 100 mL	Gibco	13150-016
Ciprofloxacin, 400 mg	Bayer	851640
Custom ATV, 100 mL	Irvine Scientific	9920
Dispase II, 5 g	Roche	165859
D-MEM/F12, 500 mL	Irvine Scientific	9052
DMSO, 100 mL	Sigma-Aldrich	D2650
DNase, 25 mg	Worthington	2138
EGF, 100 μg	Invitrogen	13247-051
FBS, 500 mL	HyClone	Sh30070.03
FGF2, 10 μg	Invitrogen	13256-029
Fibronectin, 1% (w:v) 10 mL	Sigma-Aldrich	F0895
Fungizone, 20 mL	Gibco	15290-018
Gentamicin, 10 mL	Sigma-Aldrich	G1397
Glutamine, 100 mL	Irvine Scientific	9317
HBSS, Ca/Mg-free, 500 mL	Irvine Scientific	9228
Laminin, 1 mg	Invitrogen	23017-015
NT-3, 10 μg	Chemicon	GF031
Papain, 100 mg	Worthington	3126
Paraformaldehyde, 500 g	Sigma-Aldrich	158127
PBS, 500 mL	Irvine Scientific	9236
PBS, 10×, 500 mL	Invitrogen	70013-032
PDGFa/b, 200 μg	Peprotech	100-00AB
Pen-strep, 100 mL	Irvine Scientific	15140-122
Sodium azide, 25 g	Sigma-Aldrich	S-8032
Sodium hydroxide, 500 g	Sigma-Aldrich	S-0899

RECIPES

Stock solutions

All-Trans Retinoic Acid stock solution (10 mL)

Component	Amount	Stock concentration
RA	1000 nmol	100 μM
DMSO	10 mL	–

Make this solution with most lights off and freeze 100 μL aliquots at −20°C covered in foil.

ATV stock solution (100 mL)

Component	Amount	Stock concentration
Trypsin	As supplied	0.5 g/L
Na_4EDTA	As supplied	0.2 g/L

BDNF stock solution (1.0 mL)

Component	Amount	Stock concentration
BDNF	10 μg	10 μg/mL
DGAB	1.0 mL	–

Freeze 100 μL aliquots at −20°C.

BIT supplement stock solution (1.0 mL)

Component	Amount	Stock concentration
Bovine serum albumin (buffered with $NaHCO_3$)	As supplied	50 mg/mL
Rh insulin	As supplied	50 μg/mL
Human transferrin (iron-saturated)	As supplied	1 mg/mL

Ciprofloxacin stock solution (40 mL)

Component	Amount	Stock concentration
Ciprofloxacin	As supplied	10 mg/mL

D-MEM/F12 (500 mL)

Component	Amount	Stock concentration
D-MEM/F12	As supplied	As supplied

EGF stock solution (10.0 mL)

Component	Amount	Stock concentration
EGF	100 μg	10 μg/mL
DGAB	10.0 mL	–

Freeze 100 μL aliquots at −20°C.

FBS, Hyclone defined, non-heat-inactivated

Component	Amount	Stock concentration
FBS	As supplied	As supplied

FGF2 (bFGF) stock solution (1.0 mL)

Component	Amount	Stock concentration
FGF2	10 μg	10 μg/mL
DGAB	1.0 mL	–

Freeze 100 μL aliquots at −20°C.

Fibronectin stock solution (1.0 mL)

Component	Amount	Stock concentration
Fibronectin	As supplied	0.1% (1 mg/mL)

Fungizone stock solution (1.0 mL)

Component	Amount	Stock concentration
Amphotericin B	As supplied	250 μg/mL

Gentamicin stock solution (10 mL)

Component	Amount	Stock concentration
Gentamicin sulfate	As supplied	10 mg/mL

Glutamine stock solution (100 mL)

Component	Amount	Stock concentration
Glutamine	As supplied	29.2 mg/mL

NT-3 stock solution (1.0 mL)

Component	Amount	Stock concentration
NT-3	10 μg	10 μg/mL
DGAB	1.0 mL	–

Freeze 100 μL aliquots at −20°C.

PDGF stock solution (20.0 mL)

Component	Amount	Stock concentration
hPDGFa/b	200 μg	10 μg/mL
DGAB	20 mL	–

Freeze 100 μL aliquots at −20C.

Penicillin/streptomycin stock solution (1.0 mL)

Component	Amount	Stock concentration
Penicillin G	As supplied	10 000 U/mL
Streptomycin sulfate	As supplied	10 000 μg/mL

Working solutions

DGA (500 mL)

Component	Amount	Final concentration
Stock gentamicin	0.5 mL	10 μg/mL
Stock ciprofloxacin	0.5 mL	10 μg/mL
Stock fungizone	5 mL	2.5 μg/mL
Stock penicillin/streptomycin	5 mL	100 μg/mL
Stock glutamine	5 mL	292 μg/mL
D-MEM/F12	500 mL	–

Store at 4°C and use within two weeks.

DGAB (500 mL)

Component	Amount	Final concentration
Stock BIT supplement	50 mL	10%
DGA	450 mL	–

Store at 4°C and use within two weeks.

DGF (500 mL)

Component	Amount	Final concentration
FBS	50 mL	10%
DGA	450 mL	–

Store at 4°C and use within two weeks.

DGFB (500 mL)

Component	Amount	Final concentration
Stock BIT supplement	50 mL	10%
DGF	450 mL	–

Store at 4°C and use within two weeks.

Differentiation medium (10 mL)

Component	Amount	Final concentration
GCM	5 mL	50%
DGAB	5 mL	50%
FBS	100 µL	1%
Stock NT3	400 µL	40 ng/mL
Stock BDNF	400 µL	40 ng/mL
Stock ATRA	100 µL	100 nM

Store at 4°C and use within two weeks.

Because ATRA is light- and oxygen-sensitive, it must be added fresh. It is preferable, therefore, to have appropriate aliquots of 1000× on hand for this purpose.

Fibronectin solution (100 mL)

Component	Amount	Final concentration
Fibronectin	1.0 mL	10 µg/mL
D-MEM/F12	99 mL	–

Freezing medium for progenitors/glia (100 mL)

Component	Amount	Final concentration
CM or GCM	45 mL	45%
GM or DGF	45 mL	45%
DMSO	10 mL	10%

Growth medium (GM) (100 mL)

Component	Amount	Final concentration
Stock hFGF2	200 μL	20 ng/mL
Stock hPDGFa/b	100 μL	10 ng/mL
Stock hEGF	200 μL	20 ng/mL
Stock BIT supplement	10 mL	10%
DGA	90 mL	–

Store at 4°C and use within two weeks.

Laminin solution (10 mL)

Component	Amount	Final concentration
Laminin	100 μg	10 μg/mL
PBS	10 mL	–

Paraformaldehyde solution (1000 mL)

Component	Amount	Final concentration
Paraformaldehyde	40 g	4%
Purified water	900 mL	–
Sodium hydroxide	2–3 pellets	–
PBS, 10×	100 mL	–

Into a glass 1000 mL beaker on a hot plate/stirrer are added, in the fume hood, 40 g PFA, 900 mL water and one stir bar. With vigorous stirring, 2–3 NaOH pellets are added and the solution is heated to 50–55°C (and no more). At that time the solution should be clear and colorless. 100 mL 10× PBS is then added to the beaker and the beaker is removed from the hotplate and allowed to cool to room temperature. The pH is then checked and, if necessary, adjusted downward with the dropwise addition of 1 N phosphoric acid. The solution is then filtered, aliquoted, and stored at −20°C.

PPD solution (papain, protease, DNase) (100 mL)

Component	Amount	Final concentration
Papain	250 U	2.5 U/mL
DNase I	25 000 U	250 U/mL
Dispase II (neutral protease)	100 U	1 U/mL
D-MEM/F12	100 mL	–

Add reagents to D-MEM/F12 and warm to room temperature to dissolve. The papain is supplied as a solid suspended in water. Make sure that the papain is well mixed

prior to removing the amount needed. When reagents are completely dissolved (liquid will be completely clear), sterile filter. Store aliquots at −20°C.

Primary medium (100 mL)

Component	Amount	Final concentration
Stock hFGF2	200 μL	20 ng/mL
Stock hPDGFa/b	100 μL	10 ng/mL
Stock hEGF	200 μL	20 ng/mL
Stock BIT supplement	10 mL	10%
DGF	90 mL	–

Store at 4°C and use within two weeks.

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Appendix 22.1 Surface Areas for Commonly Used Cell Culture Containers

Container	Area (cm^2/well)	Standard plating volume
Dish		
20 mm	3	600 μL
35 mm	8	1
60 mm	25	5 mL
100 mm	78.5	15 mL
145 mm	165	30 mL
Plate		
6-well	9.6	2 mL
12-well	3.8	1 mL
24-well	2	500 μL
48-well	0.75	200 μL
96-well	0.32	100 μL
Slide		
1-well	9.4	2.3 mL
2-well	4.2	1.5 mL
4-well	1.8	450 μL
8-well	0.8	250 μL
Flask		
T-25	25	5 mL
T-50	50	10 mL
T-75	75	15 mL
T-175	175	35 mL

CHAPTER

23

Stem Cell Transplantation in the Brain

Jean-Pyo Lee, Nils Ole Schmidt, Paul Christian Baier, Franz-Josef Mueller, and Evan Y. Snyder

INTRODUCTION

Neural stem cells (NSCs) are operationally defined by their capacity for self-renewal and differentiation into multiple cell types of the brain, including, at a minimum, neurons and glial cells. NSCs have been isolated from several species, including mice, rats, and humans. Over the years, various methods for the *in vitro* culture of NSCs have been developed, but there is no general agreement as to what should be considered to be an NSC *in vitro*. Some preparations are called NSCs when they would more properly be called neural progenitors, because of their limited potential of making neurons or glia but not both. For the purpose of this chapter, we will use an inclusive view, assuming that cells that are called NSCs by individual investigators do have common features that allow generalization. However, we add the caveats that all claims made for one particular NSC line or preparation might not apply to all of the other populations, and that it is common to have mixtures of NSC and neural progenitors.

When NSCs are transplanted into normal or diseased brains they often migrate, integrate and differentiate in the host brain. Transplanted NSCs appear to be attracted to areas of pathological changes processes that result from disease, such as inflammation,

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tumor formation, trauma, and ischemia. Some transplanted NSCs differentiate after transplantation in a similar fashion as they do *in vitro* and all their differentiated progeny – oligodendroglia, astroglia, and neurons – can be found after transplantation.

Currently, it is not clear whether the transplanted cells and their differentiated progeny are able to restore functionality to the injured brain and/or if they provide a suitable environment for healing and regeneration of the host tissue. The positive effects of NSC transplantation in a variety of disease models may often be attributed to complex interactions between the grafted NSCs and the host cells rather than a simple replacement function. Because of their extensive motility and specific affinity for pathological areas within the CNS, NSCs are also useful tools as delivery vehicles for gene therapy. The cells can be transfected *ex vivo* with vectors encoding desirable proteins, and then used to deliver the therapeutic protein or its products to the lesions.

Recently, researchers have also been able to differentiate NSCs from human embryonic stem cells (hESCs). Numerous differentiation protocols have been developed so it will be an important challenge in the future to characterize these preparations *in vitro* and *in vivo*. The key to successful *in vivo* characterization of any stem cell preparation is demonstration of the cells' ability to reliable and reproducible engraft into the CNS of laboratory animals. This is an important assay for characterizing the differentiation and migratory capabilities of a newly developed NSC preparation.

Overview

Transplantation of NSCs is an important tool in preclinical research focusing on cell-based therapies of the nervous system. Although many NSC transplantation studies have been published, transplantation remains a technically challenging procedure.

Here we provide a collection of protocols and explanatory sections for establishing a CNS transplantation procedure or for improving and/or troubleshooting an existing transplantation procedure.

Transplanting NSCs into the CNS can be divided into five main components:

1. *In vitro* cell preparation for transplantation
2. Introduction of the cells into the host CNS
3. Labeling and tracing the cells
4. Troubleshooting
5. Consideration of the immunology of transplanted cells.

Specific research interests determine the animal models, the route and location of cell administration, and the timing of transplantation in relation to the disease process or the developmental processes of interest. Sometimes it is clear that the NSCs have to be injected into a certain CNS region at a certain time. For example, if a scientist wants to study the effects of transplanted NSCs on spinal cord affected by an amyotrophic

lateral sclerosis-like pathology, the cells may have to be injected into the spinal cord at, before, or during the time that the pathology develops. An overview of transplantation approaches for specific applications is given in Table 23.1.

We advise anyone who wants to establish a cell CNS transplantation procedure to start with a cell line that is easy to culture and maintain. The cell line should be easily expandable, so the methods can be developed without having to be frugal with the cells, and they should be a line that is expected to integrate stably after transplantation. A cell line that can be cultured as monolayer simplifies dissociation of the cells prior to transplantation. The specific examples provided here were developed using the immortalized mouse NSC line C17.2, which was isolated from neonatal mouse cerebellum. This cell line has been extensively used in cell transplantation studies since the early 1990s. One of the useful features of C17.2 is their stable expressing of LacZ, which makes it possible to track their integration and movement in brain after transplantation.

In this chapter we will describe a method to transplant NSCs into the lateral ventricle of the neonatal mouse brain. The procedure is relatively simple, since the cells can be delivered manually into the ventricles of newborn mice with a glass needle. This approach is appropriate for studies of differentiation potential *in vivo*, as well as testing methods for cell and gene therapy in animal models of genetic disease, neonatal brain diseases, and injuries.

Procedures

Preparation of cultured cells

Approximately 10^6 cells from homogeneous cultures should be prepared for transplantation. Cells that have been in continuous extended culture should *not* be used. Cells should be expanded at early passage and cryopreserved, then transplanted after the first or second passage after thawing. Cultures should not be allowed to become more than 90% confluent, because they start elaborating an extracellular matrix which will make the cells very clumpy and resistant to dissociation. Cells should only be transplanted if they are in a freely flowing single-cell suspension.

1. Remove culture medium and briefly rinse the cell layer twice with PBS to remove all traces of culture medium.
2. Add 2 mL of 0.05% Trypsin-EDTA solution and incubate at 37°C for 2–5 min, then observe cells under an inverted microscope until the cell layer is dispersed.

Note: The dish can be gently tapped to help the cells to detach. Cells that are difficult to detach may be placed at 37°C for an additional 1–2 min.

3. Add 3.0–5.0 mL of 10% FBS in DMEM and aspirate cells by gently pipetting. Adding more medium makes it easier to triturate the cells without introducing any air or bubbles. (The protein in the medium deactivates the trypsin.)
4. Pipette cells into 15 mL tube Falcon centrifuge tube.
5. Centrifuge for 1 min at 300×*g*.

TABLE 23.1 Routes of NSC transplantation – rodents

Time point/route	Application	Advantages	Disadvantages	Special considerations
In utero – intracerebral	Differentiation analysis *in vivo* Preclinical studies of *in utero* transplantations in disorders that can be diagnosed prenatally	Distribution into most regions of the tele- di-, and mesencephalon	Technically challenging, many mice needed Large variation in engraftment Foster mice necessary for raising of pups after P0	CD1 mice are better recipients for grafts than C57/B6 Modern ultrasound guided injection techniques can improve the success rate considerably
Neonatal – intraventricular	Differentiation analysis *in vivo* Preclinical transplantation studies in inborn errors of metabolism (e.g. lysosomal storage diseases)	Easy to perform Distribution into most regions of the tel-, di- and mesencephalon	Cerebellar pathologies cannot be targeted by this technique The system does not mimic the non-neurogenic, adult environment	CD1-mice are better recipients for grafts than C57/B6
Neonatal specific locations (hippocampal, cerebellar)	Preclinical transplantation studies in inborn errors of metabolism (e.g. lysosomal storage diseases)	Cerebellar injection necessary for demyelinating disorder as complementary procedure to neonatal intraventricular injection	Stereotactic procedures often necessary Stereotactic coordinates have to determined empirically	Cerebellar injections can be performed similarly to neonatal intraventricular transplantations
Adult – hippocampal Adult – subventricular zone (SVZ)	Differentiation studies in an adult neurogenic environment Migration studies	Allows analysis of the neurogenic region	Stereotactic procedures often necessary Stereotactic coordinates have to determined empirically	Cells may proliferate longer in this environment

(*Continued*)

TABLE 23.1 (Continued)

Time point/route	Application	Advantages	Disadvantages	Special considerations
Adult – striatal	Most common transplantation model for dopamine deficiency	Functional readout of transplantation success in behavioral models	Results are sometimes hard to interpret since trophic effects might contribute to functional improvement	Cells have to differentiate into dopaminergic phenotype to become functional
Adult – nucleus basalis magnocellularis (Meynert)	Most common transplantation model for acetylcholine deficiency	Functional readout of transplantation success in behavioral models	Results sometimes hard to interpret since trophic effects might contribute to functional improvement	Cells have to differentiate into cholinergic phenotype to become functional
Adult – intraspinal	Relevant for models of motoneuron degeneration (e.g. ALS), multiple sclerosis (EAE) and spinal cord injury	Functional readout of transplantation success in behavioral models	Results sometimes hard to interpret since trophic effects might contribute to functional improvement	Cells have to differentiate into specific phenotypes to become functional
Adult – intrathecal or intraventricular	Widespread pathologies, e.g. in transgenic mouse models of Alzheimer's disease	Distribution into most regions of the tele- , di-, and mesencephalon	Differentiation into neurons rarely observed Only reliably described in cases of CNS pathologies	Cells may have to differentiate into several phenotypes to become functional
Adult – intravenous	Widespread pathologies, e.g. In transgenic mouse models of Alzheimer's disease	Potential specific lesion targeting in all regions of the CNS	Blood–brain barrier may have to be broken down for NSC transmigration Distribution to other sites (lung, liver, spleen)	Cells may have to differentiate into several phenotypes to become functional

6. Remove supernatant by aspiration, but do not disturb the pellet. Wash cells by resuspending in 10 mL sterile PBS and triturate cells gently but well (20 times). This suspension should become very dispersed single cells with absolutely no clumps or aggregations of cells.
7. Centrifuge again for 1 min at 300×***g***.
8. Remove supernatant by aspiration, but do not disturb the pellet. Wash cells by resuspending in 10 mL sterile PBS and triturate cells gently but well (20 times). This suspension should become very dispersed single cells with absolutely no clumps or aggregations of cells.
9. Centrifuge again for 1 min at 300×***g***.
10. Remove supernatant by aspiration, but do not disturb the pellet. Wash cells by resuspending in 100–300 μL sterile PBS and triturate cells gently but well (20 times). This suspension should become single cells with no clumps or aggregations of cells.
11. Remove 9 μL of cell solution and put into a separate microcentrifuge tube.
12. Add 0.04% Trypan Blue dye (~1 μL) to microcentrifuge tube and triturate well.
13. Wait 1 min before counting Trypan Blue-positive and negative cells. The population should have >90% viable cells as identified by Trypan Blue exclusion.
14. Resuspend the pellet in the appropriate volume of sterile PBS to a concentration of 4×10^4 cells/μL and place on ice.

Additional information

- Add a volume of PBS that is equal to the size of the pellet. For example, if pellet occupies ~0.3 mL, then add an additional 0.3 mL of PBS.
- The cell concentration is important: concentrations less than 2×10^4 cells/mL and greater than 5×10^4 cells/mL do not work well.
- If your cellular suspension has any bubbles in it or has any clumps or aggregates at all – as seen with the naked eye or microscopically, then do not proceed. Air bubbles introduce shear stress that can impair cell survival and make it difficult to pipette a reproducible volume.
- Add enough Trypan Blue to the cellular suspension so that you can visualize it clearly enough to guide your transplant and observe where the cells are going.
- The cells tend to settle down within seconds of the vial being held vertically, so gentle trituration is necessary prior to each injection. If you do not triturate constantly, you will be fooled into thinking an animal has received cells when, in fact, it has received only vehicle.
- An alternative way to deal with cell settling is to separate the initial cell suspension into microtubes containing 10–15 μL of cell suspension each. Each microtube can be used for one or two animals depending on the volume of injection. Advantages of this approach are that 10 μL volume is easily titurated ensuring a homogeneous cell suspension and, importantly, if air bubbles are introduced only that vial need be discarded.

- The cell transplantation should be finished within 2 h after cell preparation since the cells will start to die in PBS. An alternative is to use serum-free medium without any supplements or to get freshly prepared cell suspensions every 2 h (which is useful when larger numbers of animals will be injected).

Transplantation into neonatal mice

In newborns, the skull is easy to penetrate and the relative translucence of the head makes it easier to determine where the transplanted cells have been placed (e.g. in the lateral ventricle). Moreover, when placed into newborn brain, transplanted cells often show better survival and more extensive migration since they are placed into a developing environment.

NOTES: No systematic study has examined different mouse strains as cell transplantation recipients, but we have found that CD1 mice are a good choice: they have large litter sizes and are good dams. C57/B6 mice are difficult as transplantation recipients: not only are their litter sizes considerably smaller but also their T1-weighted immune system seems to more aggressively reject cell grafts. For some transplantation studies, outbred strains have been used to minimize genetic effects that might be present in one single inbred strain.

1. Cryo-anesthetize mice less than 3 days old (P0–P3) for 3 min on wet ice. The animals should be monitored very closely and immediately receive their transplantations after they fail to react to toe-pinch.
2. Always handle the pups with examination gloves to avoid changing their smell; otherwise the dam will cannibalize them.
3. Transilluminate the heads (Figure 23.1) and insert the tip of a drawn glass micropipette into each ventricle (gaining access to the subventricular germinal zone [SVZ] that lines the ventricles along the length of the neuraxis). The tip of the micropipette should be colored black with a marker so that you can visualize it.
4. Using a mouth insufflator through a hand-held and hand-guided micropipette (Figure 23.1), gently inject 1–2 μL of the cell suspension. The transplantation procedure should be performed within 10–20 s.
5. Return the pups to maternal care after they achieve normal body temperature (usually within 5 min); they should be actively moving again. One trick is to bury them in one corner of the cage under a layer of bedding. A warming pad or heating light also assists in returning them to normal body temperature.

Labeling and tracing transplanted cells

Many approaches have been developed to unequivocally track and identify cells after they are introduced *in vivo*. Each method has advantages and disadvantages (Tables 23.2 and 23.3).

Bromodeoxyuridine labeling

For bromodeoxyuridine (BrdU) labeling prior to transplantation, 20 μM of BrdU solution (e.g. Sigma) is added to the normal culture medium of neural stem cell cultures and incubated for 48 h.

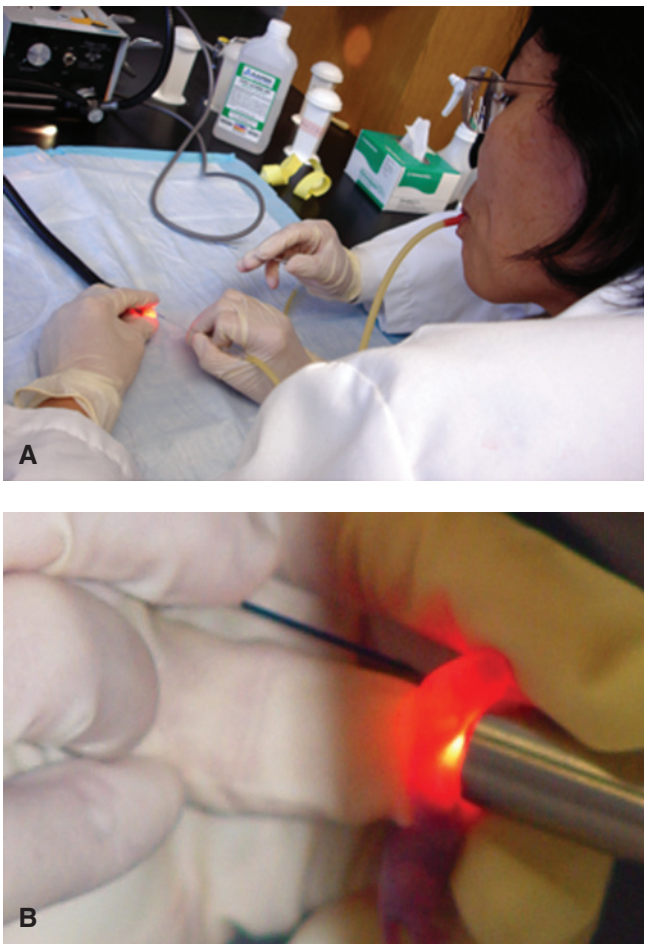

FIGURE 23.1 Manual injection of human NSCs into the lateral ventricle of the newborn mouse. The head is transilluminated allowing visualization of the ventricles and filling with cell suspension in Trypan Blue solution.

Engrafted BrdU-labeled donor cells are detected by an anti-BrdU fluorescent-tagged antibody included in a BrdU detection kit (Jackon Labs). BrdU prelabeling of donor cells is especially useful for identifying human NSCs engrafted in primates.

DiI Labeling

This procedure is recommended for easy-to-handle NSC types growing as monolayers. A very simple procedure is to add DiI stock solution directly to the normal cell culture at a final concentration of 10 μg/mL. Cells should be at least 80% confluent; DiI may impair cell growth and survival. After incubation overnight under normal cell culture conditions, the cells are homogeneously and intensely labeled. The cells should be washed extensively prior to transplantation (at least 3× with PBS) to minimize transfer of dye to the host. It should be noted that DiI is transferred to host cells when transplanted cells die.

TABLE 23.2 Cell labeling techniques – non-genetic labels

Technique	Common application	Advantages	Disadvantages
Lipophilic dyes, e.g. DiI	Short-term migration studies NSC – experimental brain tumor tracking studies	Inexpensive Easy to implement No additional stains necessary after sectioning	Lipophilic dyes can leak out of transplanted cells In most cases only suitable for short-term studies (7–14 days) Dyes are being diluted when cells divide Additional staining is problematic
Amine reactive cell tracers, e.g. CFSE	Short-term migration studies NSC – experimental brain tumor tracking studies	Inexpensive Easy to implement No additional stains necessary after sectioning	Dye might alter the cells' properties Dyes might leak out of transplanted cells Dyes are being diluted when cells divide In most cases only suitable for short-term studies (7–14 days) Additional staining is problematic
BrdU labeling	Additional ("back up") label	Inexpensive Easy to implement Suitable for long-term studies if cells are not dividing	BrdU can be released by dying cells *in vivo* and taken up by phagocytic host cells BrdU is being diluted when cells divide Detection in sections needs harsh treatment which is not always compatible with co-stains
Fluorescent nano-particles	*In vivo* tracking of cells with multiphoton *in vivo* microscopy Macroscopic *in vivo* tracking of cells with *in vivo* imaging systems (e.g. IVIS 200)	Easy to use Relatively inexpensive Suitable for *in vivo* tracking on a microscopic and macroscopic level Fewer animals necessary for time series experiments	Expensive equipment needed for *in vivo* studies Particles leak out of cells after tissue fixation
Magnetic nanobeads/ small animal Magnetic Resonance Imaging	*In vivo* tracking of cells with a small animal MRI device	Sensitive *in vivo* tracking of cells Fewer animals necessary for time series experiments Ferromagnetic beads can be easily detected in tissue section with Prussian Blue stains	Very expensive, small animal MRI set-up necessary Technically challenging Nanoparticles might leak out of dead cells Only clusters of cells can be traced Several protocols using different Fe preparations are available

TABLE 23.3 Cell labeling techniques – genetic labels

Technique	Common application	Advantages	Disadvantages
Viral transduction of cells with vectors encoding fluorescent proteins (FP)	Cell detection in tissue sections *in vivo* tracking of cells with multiphoton *in vivo* microscopy Tracking of cells with certain phenotype, i.e. Certain gene turned on if FP under specific promotor	Extremely versatile tool Currently state of the art (especially lentiviral vectors) Can also serve as model for experimental cell based gene therapies	Frequently problems with transgene inactivation *in vivo* Cell properties may be changed by transformation/ viral integration Lab has to be set up to be able to handle viruses
Viral transduction of cells with vectors encoding LacZ	Cell detection in tissue sections Suitable for establishing transplantation procedures with quick feedback (see protocols in this chapter)	Long standing tool in developmental biology Signal amplification when enzymatic reaction is applied Can also be detected with antibody against β-gal	LacZ antigen needs special fixation method to be detectable with an antibody See also viral transduction of cells with genes encoding fluorescent proteins *In vivo* tracking is not yet possible
Viral transduction of cells with vectors encoding luciferase	Macroscopic *in vivo* tracking of cells with *in vivo* imaging systems (e.g. IVIS 200)	Fewer animals necessary for time series experiments Detected cells must be alive	Only clusters of cells can be traced For histological studies co-label (e.g. FP) necessary See also viral transduction of cell with viral transduction of cells with genes encoding fluorescent proteins
Preparation of cells from FP-transgenic animals	*In vivo* tracking of cells with multiphoton *in vivo* microscopy Tracking of cells with certain phenotype, i.e. certain gene turned on if FP under specific promotor	Stable integration of the transcript Specific expression in certain cells if FP under the control of a specific promotor Selection of altered clones less likely than in viral transduction of cells	Only applicable for cell preparations from some animals, i.e. rat, mouse, pig
Sex – mismatch (male cells in female animals)	Additional ("back up") label Difficult transplantation studies, e.g. human cells into monkeys	"Last resort" if no other method reliable enough or feasible	Difficult detection method that is not established in many labs (*in situ* hybridization) Difficult co-stains
Xenotransplantation, e.g. human cells in mouse	Differentiation studies of human cells transplanted into rodents Additional ("back-up") label	Relatively cheap No additional labeling procedures necessary	Immunological rejection Antibody based detection has to be established first Most of the time, antigen retrieval is necessary Sometimes inconsistent staining results, appropriate controls are necessary

Detecting DiI-labeled cells in cryosections:

1. Let the cryosections air-dry for 30–60 min at room temperature. During the procedure protect the slides from direct light.
2. Hydrate the slides in PBS for 5 min at room temperature.
3. Incubate in DAPI (4′,6-diamidino-2-phenylindole, e.g. Sigma D9542) at a concentration of 1 μg/mL in dd H_2O for 3–5 min at room temperature.
4. Wash twice in PBS for 5 min each.
5. Coverslip the slides with fluorescent mounting medium (e.g. DAKO).

Histological analysis should be performed within the next 3 days.

LacZ labeling

NSCs can be transfected or transduced with vectors coding for markers such as fluorescent proteins or LacZ, so that they can be distinguished from the host cells.

Expression of LacZ must be confirmed in the cells designated for transplant action, since transgenes can be downregulated during extended culture. During the last split before transplantation, an extra dish of cells should be set aside for X-gal processing. This dish should exhibit *at least* 75–90% blue cells. If the percentage is less than this, it is best to abort the transplant and thaw another earlier passage of the cell line.

LacZ detection by X-gal staining:

1. Euthanize transplanted animals by an overdose of pentobarbital (100 mg/kg) and prepare by transcardiac perfusion with 4% buffered paraformaldehyde (PFA).
2. Dissect brain.
3. Fix with 4% PFA overnight.
4. Incubate in 10% sucrose overnight.
5. Incubate in 30% sucrose overnight.
6. Blot the excess solution with Kimwipe.
7. Quick-freeze brains on dry ice.
8. Cut brains with razor blade and put slices onto microscope slides. Alternatively, brains can be cryosectioned at 20 μm.
9. Wash slices with 1× PBS at room temperature for 3 min.
10. Rinse with Solution A twice at room temperature for 10 min.
11. Permeabilize tissue in Solution C twice at room temperature for 10 min.
12. Finish preparation of Solution B: add 50 μL X-gal to 2.5 mL, add to tissue, keep covered in aluminum foil.
13. Incubate at 37°C overnight in box covered with aluminum foil (keep moist).
14. Visualize blue color (Figure 23.2) with bright field microscope.

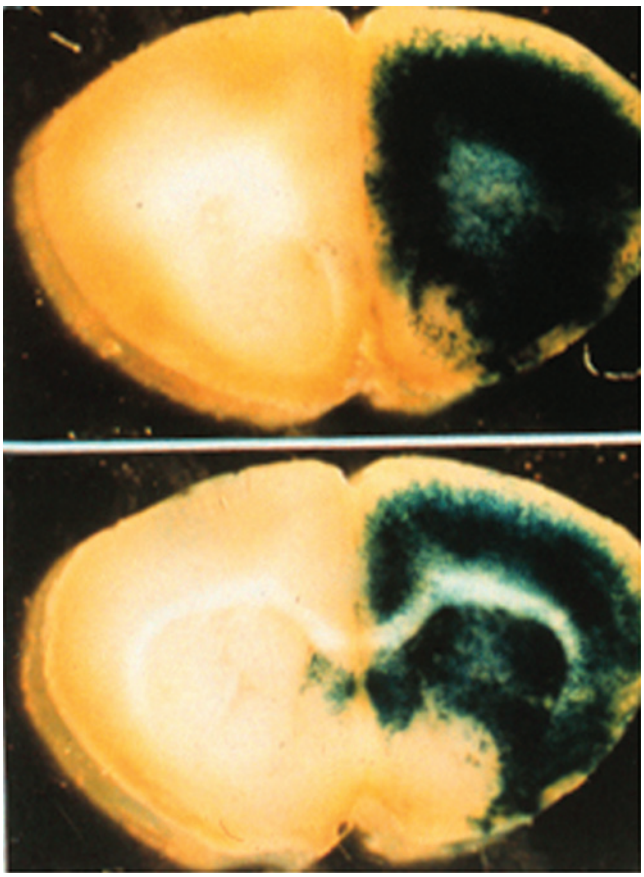

FIGURE 23.2 LacZ-expressing neural progenitors that have migrated throughout the brain after injection into a lateral ventricle.

Species-specific antigens

NSCs of human origin transplanted in a murine model can be detected by using antibodies against human-specific antigens such as nuclear antigen (NuMA) or human mitochondria.

NuMA immunohistology, DAB chromagen:

1. Cryosection the brain, and let the cryosections air-dry for 30–60 min at room temperature.
2. Hydrate the slides 3× in PBS for 5 min at room temperature.
3. Fix the slides in 2% PFA/PBS for 10 min at room temperature.
4. Wash 3× in PBS for 5 min each.
5. Block endogenous peroxidase by incubation of slides in 0.3% H_2O_2 in PBS.
6. Wash 3× in PBS for 5 min each.

7. Incubate the slides in blocking solution (3% horse serum + 0.3% Triton-X100 in PBS) for 30–90 min at room temperature.
8. Incubate the slides with the primary monoclonal mouse antibody against human nuclear antigen NuMA (Ab-2, Oncogene NA09L) at 1:400 in blocking solution overnight at 4°C.
9. Wash 3× in PBS for 10 min each.
10. Incubate the slides with the secondary antibody, biotinylated horse anti-mouse IgG (Vector BA-2001), at 1:250 in blocking solution for 75 min at room temperature.
11. Wash 3× in PBS for 10 min each.
12. Follow the instructions for the Vectastain Elite ABC kit (Vector Laboratories, Burlingham, CA, USA). Incubate slides in freshly prepared ABC solution for 60 min at room temperature.
13. Wash 3× in PBS for 10 min each.
14. Incubate with freshly prepared DAB solution for 5–15 min at room temperature. Monitor the reaction closely and stop by rinsing with water when brown staining is visible while background is still low. The optimal time-point has to be determined for each staining procedure.
15. Wash 3× in PBS for 5 min each and cover slides with mounting medium.

Alternative Procedures

Immunology of transplanted NSCs

We have found that neonatally transplanted NSCs survive for a long time in the recipient mouse brain without immunosuppressant treatment. However, immunosuppression is recommended when NSCs are transplanted into adult mouse brain. In general, transplant recipients can be administered cyclosporin, 10 mg/kg intraperitoneally daily, beginning on day of transplant.

In utero transplantation

This technique can be used to increase the degree of neural replacement of transplanted NSCs. Intervening in the disease progression as early in cerebrogenesis as possible may be even more effective in arresting disease progression and therefore more effective in minimizing or preventing irreversible CNS alterations. NSCs can be transplanted *in utero* at mouse embryonic day 13.5 (E13.5).

1. For timed pregnancies, mate female mice on the day of pro-estrus; the morning following conception is designated E0.5.
2. At E13.5, anethetize pregnant females with isofluorane and perform a 2–3 cm ventral laparotomy incision aseptically.
3. Expose the uterine horns and locate the embryos.

4. Rapidly and aseptically inject 2 μL of a suspension of NSCs (4–5 × 10^4 cells/μL in PBS with 0.05% Trypan Blue) into each embryonic telencephalic ventricle via a single transcutaneous insertion of the tip of a drawn glass micropipette through the transilluminated uterine wall. The entire operation should last <30 min.
5. Irrigate the peritoneal cavity with Ringer's lactate saline throughout the procedure. Suture the skin incision and cover with a topical antibiotic.
6. Allow the fetuses to come to term, the pups are delivered naturally.

Striatal stem cell transplantation (rat)

Parkinson's disease is a common neurological disorder characterized by a dopaminergic deficit in the striatum, principally caused by the degeneration of the substantia nigra. This relatively focal pathology offers the option to treat the disorder with cell replacement therapy. Since the 1980s preclinical and clinical experiments with transplantation of fetal mesencephalic progenitor cells gathered evidence for possible efficacy. However, the use of grafts derived from fetal sources remains problematic and there is a need to find alternative sources for grafts.

Depending on the questions to be answered by an experiment, either naïve animals or suitable models for Parkinson's disease may serve as graft recipients.

1. Anesthetize animal with 100 mg/kg ketamine + 10 mg/kg xylazine. Redosing with xylazine is not recommended; if necessary, redose with ketamine alone.
2. Mount animal in stereotaxic frame.
3. Make a medial skin incision of approx. 15 mm length (Figure 23.3).
4. Use the four bulldog-type clamps to retract the skin from the operation area.

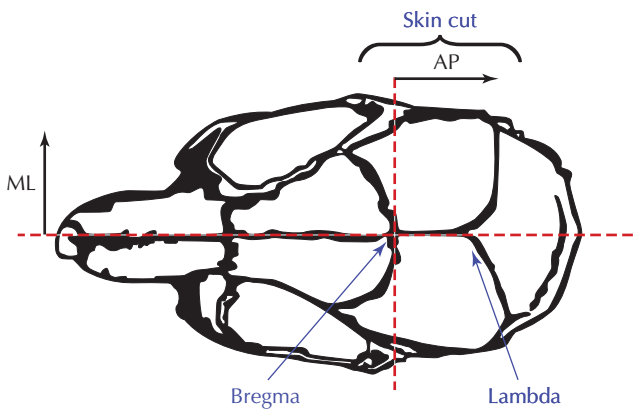

FIGURE 23.3 Rodent skull (mouse). The landmarks bregma and lambda are marked. AP, anteroposterior coordinates; ML, mediolateral coordinates. Ventrodorsal coordinates are orthogonal to the AP–ML plane.

5. Carefully remove periosteum with bone scraper, without applying strong pressure.
6. Clean with disinfectant (i.e. povidone-iodine).
7. Identify bregma (Figure 23.3), measure coordinates [anteroposterior (AP), mediolateral (ML) and ventrodorsal (VD)] with tip of the injection needle. Be careful to only slightly touch the skull with the needle, in order to avoid blunting and/or bending the tip.
8. Identify lambda (Figure 23.3), and adjust incisor bar so that lambda and bregma are at same height.
9. Mark the burr hole position with a surgical skin marker:
   - AP: 0.6 mm posterior to bregma
   - ML: 2.0 mm lateral to bregma.

NOTE: These coordinates are an example for a single graft in the striatum. Depending on the purpose of a study one might perform multiple micro-grafts or graft in different areas of the CNS. Coordinates can be derived from published materials (e.g. Paxinos and Watson, 2004). When starting a new experiment, some pilot experiments with injection of dye or ink at the calculated coordinates should be performed in order to correct for differences in strain, age, and gender.

10. Drill hole. Remove bone fragments and clean skull with disinfectant.
11. Load 10 μL syringe with cell suspension.
12. Lower injection needle to 6.0 mm below the bregma at the burr hole position.
13. Wait 5 min.
14. Inject 2 μL at a rate of 0.5 μL/min.

NOTE: The injected volume is an example. Depending on the cell concentration in the graft suspension, one might want to vary the injected volume. It is not recommended, however, to drastically increase the injected volume at one site. If higher volumes are necessary, we would recommend transplanting to more than one site. In order to minimize damage through transplantation, one can, for example, put several deposits at different heights on one needle tract.

15. Wait 5 min.
16. Slowly retract needle.
17. Suture skin.
18. Remove animal from stereotactic frame and return it to its cage.
19. Keep animals under observation until they are fully awake.

EQUIPMENT AND MATERIALS

- Needle puller (Sutter Instrument Co. Model P-87)
- Fiber-optic light

- Heating pad
- Microscope with bright field objectives (4×, 10×, 20×)
- Pipettors
- Tissue culture incubator, 5% CO_2, 37°C
- Tissue culture hood, Class II
- Stereotaxic frame with rat adapter and ear bars
- Injection pump fixed to manipulator
- 10 μL glass syringe with 26G bevelled needle fixed to injection pump
- Scalpel
- Forceps (anatomical tip)
- Forceps (surgical tip)
- Four bulldog type clamps
- Bone scraper
- Scissors
- Cotton buds
- Surgical skin marker
- Electric drill with 0.5 mm diameter burr
- Needle holder and suture material.

Supplies and Reagents

Item	Supplier	Catalog no.	Alternative
Pipettes, 5 mL, 10 mL, 25 mL	Corning	4487/4488/4489	Fisher, VWR
10 cm tissue culture dishes	Corning	430167	Fisher, VWR
Cryogenic vials 2 mL	Nalgene (Sigma)	V5007	Corning
D-MEM/F12	HyClone	SH30622.01	Invitrogen
Glutamine/PenStrep	Invitrogen	10378-016	Irvine Scientific
FBS	HyClone	SH30070.03	Invitrogen
Horse serum 500 mL	HyClone	SH30074.03	Invitrogen
D-PBS (1×) (without calcium or magnesium)	Invitrogen	14190-144	Many
Xylazine (Rompun 2 mg/mL)	Victor Medical	1216500	Veterinary Supply
Ketamine (Ketavet 10 mg/mL)	Victor Medical	1082725	Veterinary Supply
NaCl solution, 0.9%	Victor Medical	1264325	
Povidone-iodine	Victor Medical	0178317	Veterinary Supply
Trypsin/EDTA	HyClone	SH30236.01	Invitrogen

(*Continued*)

Item	Supplier	Catalog no.	Alternative
DMSO	Sigma Aldrich	D2650	
Potassium ferrocyanide	Sigma Aldrich	P-9387	
Potassium ferricyanide	Sigma Aldrich	P-3667	
Deoxycholic acid	Sigma Aldrich	D-6750	
NP-40	Sigma Aldrich	NP40-S	
10× PBS	Sigma Aldrich	P5493-1L	
X-gal	Promega, Inc.	V-3941 (100 mg, 50 mg/mL store at –20°C)	

Supplies for transplantation

Item	Supplier	Catalog no.	Alternative
Needle: borosilicate glass	Sutter Instrument Co.	B100-75-15	
Microcapillary pipettes, calibrated	Sigma Aldrich Co.	A5177-5EA	

RECIPES

Stock solutions

Component	Amount	Stock concentration
PBS, pH 7.6	500 mL	1×
EGTA	100 mL	0.5 M
Magnesium chloride ($MgCl_2$)	100 mL	1 M
Deoxycholic acid	100 mL	10%
NP-40	100 mL	10%
X-gal	10 mL	1 mg/mL
Paraformaldehyde	1000 mL	4%
Sodium hydroxide (NaOH)	500 mL	1 N

4% Paraformaldehyde (1000 mL)

Component	Amount	Final concentration
Paraformaldehyde	40×g	4%
dH_2O	900 mL	
10× PBS	100 mL	1×

Under hood: Add 40 g of PFA to 900 mL of dH_2O, heat to 55°C with stirring (do not exceed this temperature), and stir until well suspended and partially dissolved. Add a

few drops of 1 N NaOH until solution is clear (will not fully dissolve without the addition of NaOH).

When solution clears add 100 mL of 10× PBS. Adjust pH to 7.4 and store at 4°C.

NOTE: The addition of 1 N NaOH to solution will change the pH, it is important to make sure that the pH is in back to 7.4 before use.

NOTE: It is best to prepare this solution fresh; therefore, modify volumes to make appropriate amounts.

X-gal solution A: rinse solution (2 L)

Component	Amount	Final concentration
PBS pH 7.6	2000 mL	
EGTA (0.5 M stock solution)	8 mL	2 mM
$MgCl_2$ (1.0 M stock)	4 mL	2 mM

Split into 500 mL rinse solution and 500 mL (stock A) for reaction solution.

X-gal solution B: reaction solution (500 mL)

Component	Amount	Final concentration
X-gal "Solution A"	500 mL	
Potassium ferrocyanide	1.06×g	5 mM
Potassium ferricyanide	0.82×g	5 mM

Wrap container in aluminum foil and store at 4°C.

Just before reaction add 1 mg/mL X-gal (50 μL/2.5 mL).

X-gal solution C: detergent solution (500 mL)

Component	Amount	Final concentration
X-gal "Solution A"	500 mL	
Deoxycholic acid (10% stock solution)	0.5 mL	0.01%
NP-40 (10% stock solution)	1 mL	0.02%

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CHAPTER

24

In Vitro Fertilization

Antoine La and David Diaz

INTRODUCTION

"The World's First Test Tube Baby is Born" was the newspaper headline when Louise Joy Brown was born on July 25, 1978, at England's Oldham General Hospital. She was a blonde, blue-eyed baby who arrived at 11.47 p.m. by a planned cesarean birth. Her parents, Leslie and John Brown from Bristol, England, had struggled with infertility for 9 years as a result of fallopian tube obstruction. They had visited doctor after doctor but their efforts were futile until they were referred to the physician and scientist who would alter their lives and change the world of reproductive medicine forever.

The Brown family's hero was Dr Patrick Steptoe, a gynecologist whose medical practice was located near Oldham General Hospital in north-west England. Steptoe was one of the world's first surgeons to adopt the use of a new technique called laparoscopy to peer inside the human body using the prototype of today's modern, slender laparoscopes.

In parallel, Robert Edwards, a physiologist at Cambridge University, had dedicated his career to discovering the secrets of human conception. Building on research reports dating back to the late nineteenth century, Edwards demonstrated that Angora rabbit embryos transferred to the uterus of a pregnant Belgian hare would come to term, showing that pre-implantation embryos could develop in the uterus of a surrogate. In the 1950s MC Chang at the Worcester Foundation had developed methods for *in vitro* fertilization (IVF) of mammalian oocytes, and in 1966 Edwards began perfecting the culture techniques needed for human IVF.

Human Stem Cell Manual, edited by J. F. Loring, R. L. Wesselschmidt, and P. H. Schwartz.
ISBN: 978-0-1237-0465-8.

In 1968, Edwards, the physiologist, and Steptoe, the gynecologist, began to lay the groundwork for achieving one of the milestones of medical science.

When Edwards learned of Patrick Steptoe's pioneering use of laparoscopy, the two agreed to collaborate with the goal of demonstrating the feasibility of human birth through IVF. Their work was often conducted secretly because of the public concern about tampering with the natural order of reproduction. The social and political atmosphere of that time presaged the controversy that surrounds human embryonic stem cell research today.

The plan seemed simple. They set up shop in Bourn Hall, a small clinic outside Cambridge, England, where Steptoe would harvest mature oocytes by laparoscopy and Edwards would then add sperm to fertilize the egg in a Petri dish. But the goal proved to be considerably more elusive than they had anticipated. The methods for confirming a pregnancy were still unreliable. The methods used for ovarian stimulation disrupted the endometrium, leading to implantation defects and early pregnancy losses.

Steptoe and Edwards persevered through more than a hundred failures, and in 1976 they achieved a modest milestone. They reported in the medical journal *Lancet* that a human pregnancy had occurred following the transfer of a morula-stage embryo. Unfortunately the pregnancy did not come to term, because the embryo implanted in the fallopian tube, resulting in an ectopic pregnancy which had to be removed surgically.

Learning from their own failures, they devised a novel approach. They decided to monitor a patient's natural cycle using quantitative methods, so that without ovarian stimulation, they were able to harvest a single oocyte by laparoscopy. Edwards fertilized and cultured the oocyte to the eight-cell stage and on November 10, 1977 Steptoe transferred it into the patient's uterus. Louise Brown was born the following summer.

The public was initially shocked by the news, but over the years grew to accept IVF; the widespread concern about tampering with reproduction was overcome by the prospect of help for infertile couples. Steptoe and Edwards' feat was quickly replicated in Australia and America's first baby conceived *in vitro* was born on December 28, 1981.

Currently, more than 90 000 *in vitro* fertilization cycles are performed each year in the USA alone. A recent Rand Institute survey estimated that there are more than 400 000 frozen embryos in cryostorage located in fertility clinics across the USA. This large quantity of surplus embryos exists because multiple eggs are retrieved and numerous embryos are created to make the *in vitro* process most efficient. Since most couples must fund their own IVF treatment cycle, freezing the embryos allows patients to store and subsequently thaw their embryos for a second or even third pregnancy attempt.

Any unused, surplus, fresh embryos can be processed in five ways:

- Discard the embryos
- Cryopreserve the embryos for later use by the patient

- Donate the embryos to another couple
- Donate the embryos for research
- Donate the embryos for human embryonic stem cell research.

Fertility clinics vary in how embryos are selected for fresh uterine transfer and which embryos are destined for cryostorage and subsequent thawing. If cleavage stage embryos on day 2 or 3 are of equivocal quality, we advise culturing them for two additional days to the morula or blastocyst stage. This type of scrutiny helps to avoid freezing unsuitable embryos that most likely will not survive the thaw process.

It is known that with advancing maternal age greater than 35 years, the percentage of embryos with aneuploidy and polyploidy can be as high as 60%. Currently, preimplantation genetic diagnosis (PGD) can be performed on embryos containing 6–8 cells by hybridizing a fluorescent DNA probe to the centromere of specific chromosome pairs. PGD can identify numerical chromosomal abnormalities like trisomy and monosomy in an embryo before it is transferred to the uterus. Presently, up to 10 chromosome pairs are routinely analyzed from a single blastomere while culturing the embryo for two additional days. It is important to recognize that numerical chromosomal abnormalities may still occur, but more rarely, in the remaining 13 pairs. Thus, morphologically and chromosomally normal embryos can be selected with 95% accuracy. Single gene mutation, as occurs in some metabolic disorders, can also be identified using polymerase chain reaction (PCR) or fluorescence *in situ* hybridization (FISH) probes that hybridize to a specific abnormal gene sequence.

Whether or not it is ethical to use excess and abnormal embryos for research is a topic best left to the individual. From the perspective of the fertility clinics, we should at the very least offer our patients the option of voluntary donation of unused embryos toward stem cell research. Providing appropriate informed consent to those interested in donating should be encouraged in lieu of discarding embryos or abandoning them to an uncertain future in cryostorage.

Overview

The procedures outlined in this chapter are performed by a professional embryologist in an IVF clinic. They are included here to give an overview of the processes that are used to generate embryos for pregnancy. Excess embryos from these procedures may be donated for embryonic stem cell research. The embryologist is involved in the management of each IVF case from the time that the treatment cycle is initiated, and there is a system that ensures that all members of the laboratory staff can be familiar with the treatment plan for each patient. The laboratory staff should also ensure that all appropriate consent forms have been signed by both partners, including consent for special procedures and storage of cryopreserved embryos. The details of any previous assisted conception treatment, including response to stimulation, number and quality of oocytes, timing of insemination, fertilization rate, embryo quality, and embryo transfer procedure, are studied to judge whether any parameters at any stage could be altered or improved in the present cycle. The laboratory case notes, media, culture vessels and tubes for sperm preparation are prepared during the afternoon prior to each case, with clear and adequate labeling.

PROCEDURES

One day prior to oocyte retrieval

Prepare media

1. Prepare HTFM-HEPES (HH)-5% human serum albumin (HSA).
2. Label HH-5% HSA tubes.
3. Label tubes to be used the following day with patient's (donor) name and place into patient-labeled foam test tube rack (approx. 2–3 tubes per patient).
4. Place foam rack into refrigerator until the following day when the labeled tubes will be placed into the water bath (37°C) rack to warm until time of use.
5. Prepare QB XI-5% HSA.

Prepare Nunc four-well culture dishes.

1. Label all dishes with patient's last name and first initial (lid top and bottom edge of dishes) and type of media. The number of dishes is dependent on the number of follicles per patient.

NOTE: Example: If patient has 10 follicles measured, prepare two dishes with 0.5 mL of medium into each well. Each well will hold 3–4 oocytes (always prepare an extra dish per patient).

2. Place 2.0 mL of medium into the center well of the dish and layer 0.25 mL of mineral oil into each outer well. Place all dishes into the incubator (37°C, 5% CO_2) to equilibrate overnight.
3. Place one new bottle of PBS 1× into the water bath (37°C) to equilibrate overnight. The amount of PBS 1× is dependent on the number of follicles (i.e. if patient has 20 follicles you will need approximately 1–2 bottles, approximately 500 mL each).

Day of oocyte retrieval

Media preparation

Egg wash and sperm washing medium: Remove patient's labeled foam rack of medium from refrigerator and place tightly capped labeled centrifuge tubes into a water bath rack until time of use.

Semen sample

Raw ejaculate

1. For an IVF case samples will be collected after transvaginal aspiration (TVA) of the female partner, unless it is known that couple must be together to collect the sample, then the sample will be collected before TVA of the female partner. For donor oocyte TVAs, the recipient's male partner will collect the sample during donor TVA to prevent paths from crossing.

2. Perform routine semen analysis for complete sperm wash. Evaluate morphology on sample (pre- and post-isolate preparation).
3. Perform a complete sperm wash on semen sample.

Cryopreserved semen sample

1. Perform routine semen analysis on thawed vial(s). Evaluate morphology on all samples (pre- and post-isolate preparation).
2. Perform a complete sperm wash on semen sample.

Semen preparation

1. Perform routine semen analysis on all samples for complete sperm wash.
2. Evaluate morphology on all samples (pre- and post-isolate preparation).

Isolate gradient preparation
Discontinuous gradients are prepared by layering 95%, 70%, and finally 50% isolate into conical centrifuge tubes. Volume of the layers depends on the semen parameters:

- *Long gradient*: Each of the three layers is 1 mL in volume. Use for good-quality specimen with count, motility, progression much greater than outlined in "normal values".
- *Regular gradient*: Each of the three layers is 0.5 mL in volume. Use for sample with "normal" semen parameters.
- *Mini-gradient*: Each of the three layers is 0.3 mL in volume. Use for poor-quality semen (low volume, low count, poor motility, etc.).

To prepare gradient, slowly layer 70% isolate on top of 95% fraction in a centrifuge tube using automatic pipette and appropriate sized sterile serological pipette. The line between the two layers must be sharp (definition of a discontinuous gradient). Next, slowly layer 50% isolate on top of the 70% fraction according to the above criteria.

Preparation of ejaculate

1. If the volume is 1–2 mL and the viscosity is normal, layer directly onto a long or medium gradient. If greater than 2 mL, layer directly on more than one gradient or wash the sample and reconstitute in a smaller (2 mL) volume of HH-5% HSA before layering on a gradient.
2. If the sample is viscous, work with a transfer pipette or syringe fitted with an 18 G1 1/2″ needle until viscosity appears normal. If necessary add small volume of HH-5% HSA to the ejaculate and work with a pipette. Layer sample with reduced viscosity on to top of the 50% isolate layer.
3. If the sample continues to be viscous, transfer sample to a labeled (patient's name and date) centrifuge tube and allow the sample to sit for 10–15 min in the water bath at 37°C. Repeat first part of the step above.
4. Centrifugation time is by sperm morphology and total number of motile sperm. The most normal sperm come down first. A 5 min longer centrifugation time is

allowed when sperm recovery is poor. In general, centrifuge gradients at 1000 rpm for 10 min to recover sufficient sperm with a proportion of normal forms higher than in the ejaculate.

5. Insert a sterile 9-in. pipette attached to a red bulb along the edge of the centrifuge tube to the bottom of the conical portion while producing bubbles. Withdraw pellet or approximately a third to a half of the 95% fraction only.
6. Pool 95% fractions for each sample unless sperm density is great, then place 95% fractions in separate centrifuge tubes. Wash once with 2 mL HH-5% HSA (10 min at 500 rpm). Resuspend pellet with 1 mL HH-5% HSA, take a 5 μL sample to count and calculate final resuspension volume. Place resuspended sample into centrifuge for final wash (10 min at 500 rpm). Resuspend final pellet with calculated volume of media.
7. Prepare and read morphology slide on prepared sample.
8. Allow sperm in reconstituted sample to recover from centrifuging (10–20 min at room temperature) before checking count, progression and total number of motile sperm on a Makler or glass slide.
9. Transfer sample to a sterile labeled centrifuge tube, if for intrauterine insemination (IUI) or into a labeled sterile 12 × 75 mm round bottom test tube, capped tightly.
10. Place centrifuge tube with patient's final sample into a heat block (37°C) until time of use or until it is picked up for an IUI at another physician's office. If for use in assisted reproductive technology (ART), the patient's insemination tube is placed in the embryology lab test tube rack at room temperature.

Oocyte retrieval

The patient is prepared for transvaginal aspiration. The gamete laboratory staff supply the heating block unit and flush medium.

The surgical room must be kept at optimal temperature. It is well established that extremes of temperature and light exposure are detrimental to oocytes. Therefore it is necessary to have a temperature-controlled environment under reduced lighting conditions.

Embryology lab

1. Turn off all fluorescent lights and turn on incandescent light.
2. Turn on the laminar flow hood, dissecting microscope and stage warmer, inverted microscope stage warmer and power supply.
3. Wipe down the counter top of the hood and microscope stage with 70% ethyl alcohol.
4. Retrieve tubes of media (prepared the day before) from the water bath rack, dry with sterile towel and place in heating block of the embryology laminar flow hood.

5. Place two round bottom 12 × 100 mm tissue culture tubes in block (holder), each containing a sterile short Pasteur pipette with red bulb.
6. Place appropriate number of culture dishes (Falcon) next to the microscope in the laminar flow hood.
7. Place an organ culture dish (Falcon no. 3037) on the stage warmer, in the hood, and using one of the glass Pasteur pipettes add approximately 1.5 mL HH-5% HSA (egg wash) to the inner well and approximately 0.5 mL in the outer well. The organ culture dish containing the egg wash should be kept on the stage warmer to maintain optimum temperature.
8. Each follicle is aspirated. The circulating nurse will label each tube with the follicle number and the side it was aspirated from (for example: R1 or L1). If the aspirate is a rinse the tube is labeled as such: R 1F. The tubes containing the aspirated follicles are then placed in the heating block in the laminar flow hood of the gamete laboratory.
9. Place the lid or the bottom of a culture dish on the dissecting microscope. Remove the aspirate from the heating block, note the volume of the aspirate, uncap the culture tube and decant entire content into the dish.
10. Scan the dish for the cumulus mass while noting other particulate material (i.e. granulosa cells). The size of the expanded cumulus cell–oocyte complex is easily identifiable even in the presence of red blood cells and granulosa cells. It is often easier to see the cumulus mass in slightly bloody fluid than in clear follicular fluid.
11. Once an oocyte is located, remove the cumulus mass containing the oocyte using the small Pasteur pipette. Make sure there is sufficient medium in the pipette prior to aspirating the oocyte–cumulus mass. This will ensure that the mass does not stick to the inner surface of the pipette and that, should it occur, there is sufficient media to be able to dislodge the oocyte.
12. Place the oocyte–cumulus mass in fresh culture medium (HH-5% HSA) for proper identification and scoring.

NOTE: If a cumulus mass containing an egg is found, the remainder of the sample should not be discarded. More than one follicle may have been aspirated (biovular follicles have been observed).

13. Score oocyte–cumulus mass: Parameters used to determine maturity include size of the follicle, volume of follicular fluid and direct observation of the cumulus–corona complex.

NOTE: Determination of oocyte quality or maturity is important since it dictates the length of time required for *in vitro* maturation prior to sperm/egg mixing. The more immature the oocyte the longer the maturation period required before the addition of sperm (insemination).

- *Immature oocyte*: Characteristically recovered from a follicle in which preovulatory maturation has not been initiated and therefore the oocyte remains arrested in prophase I of meiosis with an intact germinal vesicle (GV). The GV is difficult to visualize directly because of obscuring cell layers but appears as a large, centrally localized nucleus (GV intact oocytes may be

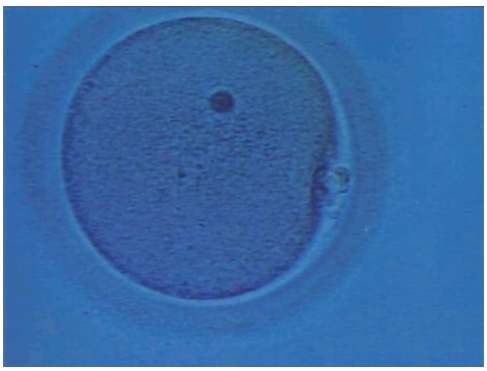

FIGURE 24.1 Mature (MII) oocyte.

difficult to find in normal follicular aspirates since they are not usually surrounded in a large, highly visible cumulus mass). Immature oocytes will be surrounded by several layers of tightly adhering cumulus–corona cells.

- *Mature oocyte*: Usually recovered from follicles of a mean diameter exceeding 15 mm. These follicles contain oocytes (Figure 24.1), which have resumed meiosis from prophase I progressing to metaphase II; the stage normally ovulated in most mammalian species. Mature oocytes will be surrounded by expanded and radiating cumulus–corona cells.

14. Transfer the oocyte to a small tissue culture tube containing 0.5 mL HH-5% HSA. Cap the tube tightly and label with follicle number and side (i.e. R1). Place tube in the heating block and continue scanning other aspirates.
15. Record follicle, volume and oocyte information.
16. Once the retrieval is complete, wash the oocytes and transfer into 30 μL droplets of HH-5% HSA using a short sterile glass Pasteur pipette with a rubber bulb. Aspirate and expel the cumulus–oocytes complex in a sterile organ culture dish containing approximately 1.5 mL HH-10% HSS. It may be necessary to strip the cumulus complex, using two sterile syringes with 30G 1/2″ needles to remove excess cells or blood clots.
17. Transfer the oocyte into an organ culture dish, prepared the day before, containing three 30 μL drops of QB XI-5% HSA under mineral oil (one oocyte per drop).
18. Label each drop with the respective egg (i.e. drop no. 1 – R1, etc.), return the organ culture dish to the incubator and allow eggs to equilibrate.
19. Initial the appropriate space on the patient identification flow sheet found in the patient's chart.

Oocyte insemination

1. Inseminate the oocytes approximately 3–5 h post-retrieval.
2. Each oocyte is inseminated with 5×10^3 motile sperm. The volume of sperm added to each oocyte is dependent on the concentration. For example, if the

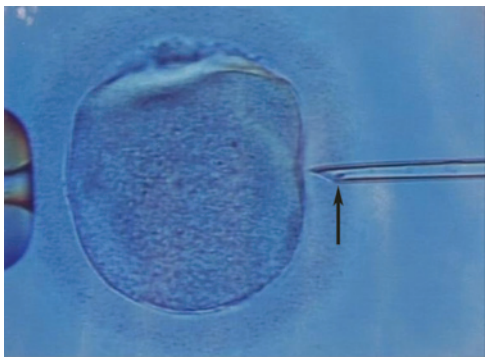

FIGURE 24.2 Oocyte insemination via intracytoplasmic sperm injection.

patient has a concentration of 45×10^6 motile sperm/mL, each drop will be inseminated with approximately 1.1 μL of the prepared sperm sample. In some cases, insemination is actively accomplished by intracytoplasmic sperm injection (ICSI, Figure 24.2). In this case, a single sperm is injected into each oocyte.

3. Retrieve the patient's organ culture dish from the incubator and place on the heated stage of the dissecting microscope in the laminar flow hood.
4. Inseminate each drop/oocyte using a sterile Elkay disposable, individually packaged, yellow pipette tip and P20 Pipetman.
5. Record the time and volume of sperm added to each organ culture dish on the lid of each dish.
6. After each dish has been inseminated, return the organ culture dish to the incubator on the appropriate shelf marked with the patient's name.
7. Initial the appropriate space on the patient identification flow sheet.
8. Record the volume, concentration and time sperm added to each dish.

Day one, post oocyte retrieval

Fertilization check

Fertilization check is done approximately 15–17 h after insemination (sperm–egg interaction). By this time, the majority of the cumulus mass has dispersed from the cumulus–oocyte complex and the presence or absence of motile sperm can be recorded.

1. Retrieve the patient's organ culture dish from the incubator and place on the heated stage of the dissecting microscope in the laminar flow hood.
2. Using an orally controlled, finely drawn sterile Pasteur pipette fitted with a 0.22 μm Millipore filter unit, strip the remainder of the cells from the oocyte. The tightly adherent corona cells can be dispersed so that the presence of pronuclei within the vitellus can be confirmed. The diameter of the Pasteur pipette should not be smaller than the diameter of the oocyte; otherwise

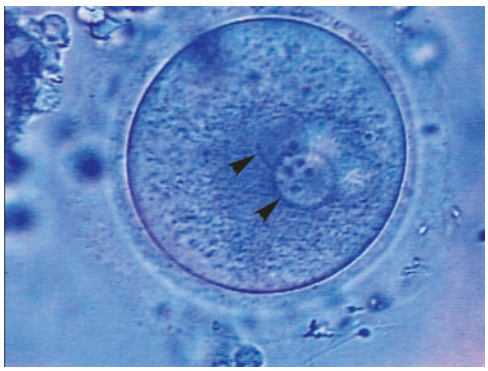

FIGURE 24.3 Normal fertilized egg (2PN).

excessive compression may result in the rupture of the zona pellucida. Conversely, if the bore of the pipette is too large, then insufficient numbers of cells will be removed.

3. After confirming the presence of two pronuclei, wash the eggs through three drops of QB XI-5% HSA and then transfer to a clean 30 μL droplet of QB XI-5% HSA under oil. Place approximately 5–6 eggs in each drop but do not put all the patient's eggs in one dish.
4. Mark polyspermic eggs, more than 2PN, as PSP and do not place them with normal fertilized eggs (Figure 24.3). Vigilance is required to identify these embryos at the pronuclear stage because they often cleave normally and are morphologically indistinguishable from normal fertilized embryos.
5. Leave all eggs that do not fertilize in the original drop and note the presence or absence of a polar body.
6. In the event of failed fertilization several things should be noted: the presence or absence of motile sperm in the culture dish, whether or not a sperm is bound to the zona pellucida and the quality of the eggs, which is more easily ascertainable once the cumulus cells have dispersed (i.e. GV, the presence or absence of a polar body and/or atretic eggs).
7. Place all organ culture dishes containing viable eggs/embryos back in the incubator on the appropriate shelf marked with the patient's name. Proper information should be noted in the patients chart (ART patient summary – data sheet, fertilization check).

Day two, post oocyte retrieval

Embryo check

Embryo check is done approximately 39–42 h after insemination (sperm–egg interaction).

1. Retrieve the patient's organ culture dish from the incubator and place on the heated stage of the dissecting microscope in the laminar flow hood.

2. Observe and note embryo cleavage stage/development of each embryo in the patient's chart (ART patient summary – data sheet, embryo check). The morphologic criteria for each embryo are based on cleavage rate, size and appearance of blastomeres and the presence or absence of cytoplasmic fragments. It may be necessary to move/rotate embryos in culture to better facilitate observation of embryo quality. This may be done by utilizing an orally controlled finely drawn sterile Pasteur pipette fitted with a 0.22 μm Millipore filter unit. The diameter of the Pasteur pipette should not be smaller than the diameter of the embryo, otherwise excessive compression may result in the rupture of the zona pellucida.
3. Place all organ culture dishes containing embryos back in the incubator on the appropriate shelf marked with the patient's name. Proper information should be noted in the patient's chart (ART patient summary – data sheet, embryo check).
4. Initial the appropriate space on the patient identification flow sheet found in the patient's chart.
5. Confirm embryo transfer time with the physicians.
6. Prepare embryo transfer media (HH-37.5% HSA) in a round bottom culture tube, tightly cap and place in incubator to equilibrate overnight. Label the tube with the patient's name, date, and type of media.
7. In the event that there are extra embryos that the patient may want cryopreserved, media should be prepared for extended culturing.
8. Ham's F10-5% HSA.
9. Prepare organ culture dishes and vaginal preparation medium.

Day three, post oocyte retrieval

Embryo check

Embryo check is done approximately 63–66 h after insemination (sperm–egg interaction).

1. Retrieve the patient's organ culture dish from the incubator and place on the heated stage of the dissecting microscope in the laminar flow hood.
2. Observe and note embryo cleavage stage/development of each embryo in the patient's chart (ART patient summary – data sheet, embryo check, see Figure 24.4).

Embryo transfer

The goal of the embryo transfer is the placement of the embryo(s) in the uterus with minimal trauma to both embryo(s) and recipient. The embryo transfer is accomplished in as small a volume of medium as possible.

1. Retrieve the patient's organ culture dish from the incubator and place on the heated stage of the dissecting microscope in the laminar flow hood.

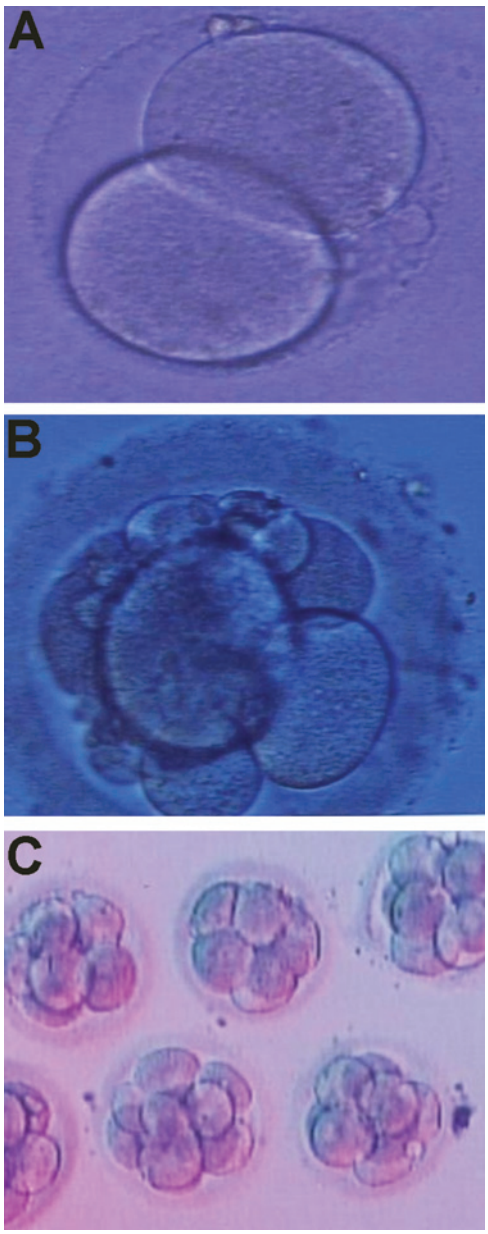

FIGURE 24.4 (A) Day 2 two-cell grade 1 embryo. (B) Day 2 three-cell grade 3–4 embryo. (C) Day 3 grade 1 and grade 1–2 embryos (at various cells stage).

2. Observe and note embryo quality. Examination of embryos before transfer often sheds little light on which embryo will most likely result in a clinical pregnancy. It is not uncommon to examine an embryo and conclude that it is fragmenting or has ceased development only to observe several hours later that extensive reorganization has occurred, resulting in a regular-appearing embryo. Human embryos that are entering cleavage division often look their worst.

3. Using an orally controlled finely drawn sterile Pasteur pipette fitted with a 0.22 μm Millipore filter unit, separate embryos based on quality for transfer.
4. The physician will speak with the patient and discuss the number of embryos to transfer and the end result of the remaining embryos, if there are any (i.e. embryo cryopreservation).
5. Complete the IVF cycle form and return to the technologist.
6. Give the nurse the embryo transfer tray and vaginal preparation medium.
7. Using an orally controlled, finely drawn sterile Pasteur pipette fitted with a 0.22 μm Millipore filter unit, place the embryos to be transferred in the first embryo transfer dish containing HH-37.5% HSA.
8. Retrieve patient's name, uterine depth and any notations from the patient's chart and note them on the outer packing (blue peel pack) of the Tef cat catheter. The embryo transfer catheter is inserted to a depth of 1 cm below the top of the fundus. For example, if the uterus is sounded at 8 cm, the transfer catheter should be marked at 7 cm.

Loading the Tef cat catheter

1. Aspirate HH-5% HSA in a 1 cc Terumo syringe, making sure not to touch the tip of the syringe. Tap out any air bubbles in the syringe and using sterile technique attach the syringe to the Tef cat catheter.
2. Measure the catheter using a ruler (cm) by moving the silicone adjustable positioner ring on the Tef cat catheter.
3. Gently peel back the outer peel pack half way and remove the catheter by handling the inner sleeve of the packing (the Tef cat catheter is in the inner sleeve in a blue peel pack). Do not touch the end of the catheter or the area where the syringe is attached to the catheter.
4. Expel the media from the syringe through the Tef cat catheter to the 0.2 mark on the 1 cc syringe.
5. Place the catheter in the sleeve back in the peel pack.
6. Keep the catheter warm until you are ready to load the embryos.
7. Place the embryos for transfer in the second embryo transfer dish containing HH-37.5% HSA in the same manner as above. With the same pipette aspirate any debris (i.e. plastic) floating in the media so that it is not aspirated into the embryo transfer catheter with the embryos.
8. Retrieve the embryo transfer catheter from the blue peel pack, again handling the catheter by the inner sleeve. Pull back on the plunger of the syringe to create a small air bubble at the tip of the catheter. While viewing the embryos under the dissecting microscope, place the tip of the catheter into the organ culture dish. Aspirate a small amount of the media and then the embryos followed by more media. The total volume of the media, in the Tef cat catheter, containing the embryos should not exceed 40 μL. Again, pull back on the plunger of the syringe to create another small air bubble at the tip of the catheter.

9. Replace the Tef cat catheter in the blue peel pack again and proceed to the patient's room. During the transfer the catheter containing the embryos is maintained at optimum temperature by placing it on a clean blue towel on a heating pad. The temperature of the heating pad is monitored by a thermometer.
10. Remove the catheter from the blue peel pack and the inner sleeve by only touching the syringe and handing it over to the physician. The physician gently inserts the catheter into the cervix/uterus to the measured depth and will notify the embryologist when to transfer the embryos. The embryologist depresses the plunger of the syringe to the 0.2 mark on the syringe emptying the contents of the catheter into the uterus.
11. Once the embryos have been placed into the uterus, wait 60 seconds, the physician will gently remove the catheter and hand it back to the embryologist.
12. Return back to the laboratory and flush the catheter.

After the embryo transfer, it is essential to flush the transfer catheter to rule out the possibility of any retained embryos.

1. Empty the contents of the catheter into a clean culture dish by depressing the plunger.
2. Rinse the catheter by placing the tip in the embryo transfer dish and aspirate and expel media several times.
3. Remove the syringe and allow the contents of the catheter to be expelled into another clean culture dish.
4. View all dishes under the dissecting microscope to ensure that there are no embryos.
5. Notify the physician that "all is clear."

NOTE: In the event that one or more embryos are retained they should be retransferred to the patient. The embryo should be rinsed in culture medium and a new embryo transfer catheter should be used.

6. Record embryo transfer information on the ART patient summary – data sheet, embryo transfer.
7. Initial appropriate space on the patient identification flow sheet found in the patient's chart.

Remaining embryos in culture

If the patient consents to embryo cryopreservation, then the remaining embryos in culture (after the transfer) are placed in Ham's F10-5% HSA and allowed to remain in culture for two more days prior to freezing.

1. Retrieve the patient's organ culture dish containing 30 μL drops of Ham's F10-5% HSA from the incubator and place on the heated stage of the dissecting microscope in the laminar flow hood.

2. Observe and note embryo cleavage stage/development of each embryo in the patient's chart (ART patient summary – data sheet, embryo check).
3. Using an orally controlled, finely drawn sterile Pasteur pipette fitted with a 0.22 μm Millipore filter unit, all embryos are washed through three drops of Ham's F10-5% HSA and then transferred to a clean 30 μL droplet of Ham's F10-5% HSA under oil.
4. Return the patient's organ culture dish containing the embryos to the incubator on the proper shelf labeled with the patient's name.

Day five, post oocyte retrieval

Embryo check

Embryo check is done on day five after oocyte retrieval.

1. Retrieve the patient's organ culture dish from the incubator and place on the heated stage of the dissecting microscope in the laminar flow hood.
2. Observe and note embryo cleavage stage/development of each embryo (Figure 24.5) in the patient chart (ART patient summary – data sheet, embryo check).
3. Initial the appropriate space on the patient identification flow sheet found in the patient's chart.

Embryo freezing

All embryos of good quality at blastocyst stage (early and/or expanded) are cryopreserved.

1. Discard all unfertilized eggs and embryos that are not of freezing quality.
2. Record information in patient's chart.
3. Complete an ART cycle summary sheet and send to physician's office.

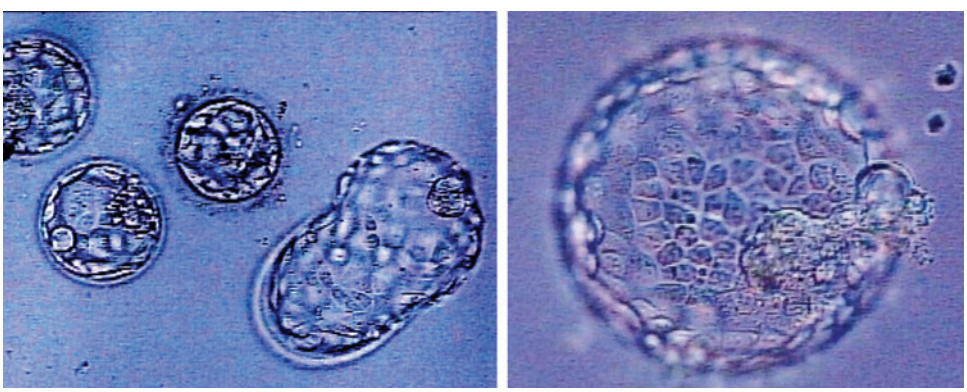

FIGURE 24.5 Day 5 embryos: blastocyst stage.

Pre-implantation genetic diagnosis

The recent application of pre-implantation genetic diagnosis (PGD) by FISH analysis of biopsied blastomeres has shown a surprising discrepancy between gross morphology and genetic normality of the embryos. Even the most "beautiful" embryos may have genetic abnormality; those with less esthetic qualities, including the presence of fragments, may, in fact, have normal implantation potential.

PGD can help the following patients:

- Women of 35 and older.
- Women with a prior history of repeated spontaneous abortions or trisomic conceptions. Regardless of age, these patients could benefit from PGD. In all these patients, higher implantation rates, reduced spontaneous abortion rates, and reduced risk of chromosomally abnormal conceptions are expected after PGD.
- Carriers of single gene disorders. These patients benefit from PGD by reducing the risk of conceiving an affected baby. In addition, through HLA matching, PGD can help select for embryos that are histocompatible with live siblings.
- Carriers of chromosome translocations, or other chromosome abnormalities. Translocation carriers benefit from a reduced risk of miscarriage and chromosomally abnormal offspring.
- Other indications. For patients with repeated IVF failure or extreme male factor, PGD can be useful as a diagnostic tool to determine if high rates of chromosome abnormalities are the cause of their infertility problems. IVF patients over 35 years of age with more than five zygotes and without a history of repeated IVF failure can also benefit from PGD.

After fertilization, embryos are cultured until day 3 of development, also known as cleavage stage, when there are 6–8 cells (Figure 24.6).

A blastomere is a single cell from an embryo. To test the blastomere, an opening is made in the covering of the embryo during its third day of development. A blastomere is removed via aspiration with a pipette (Figure 24.6). The embryo is placed in an incubator while the cell is analyzed. The biopsied cells are analyzed using FISH. Under the fluorescent microscope, the number of chromosome pairs that are of interest in each biopsied cell are counted (Figure 24.7). Also determined are the gender of each embryo and which embryos contain normal chromosome pairs. Abnormal chromosome pairs may lead to spontaneous miscarriage, failed implantation with IVF and, possibly, birth defects. The diagnostic accuracy for PGD is 95% and for gender selection 99%.

Equipment

- Makler Counting Chamber (Sefi Medical Instruments, Israel) supplied locally by Irvine Scientific or Fertility Technologies, Inc.
- P20 Pipetman

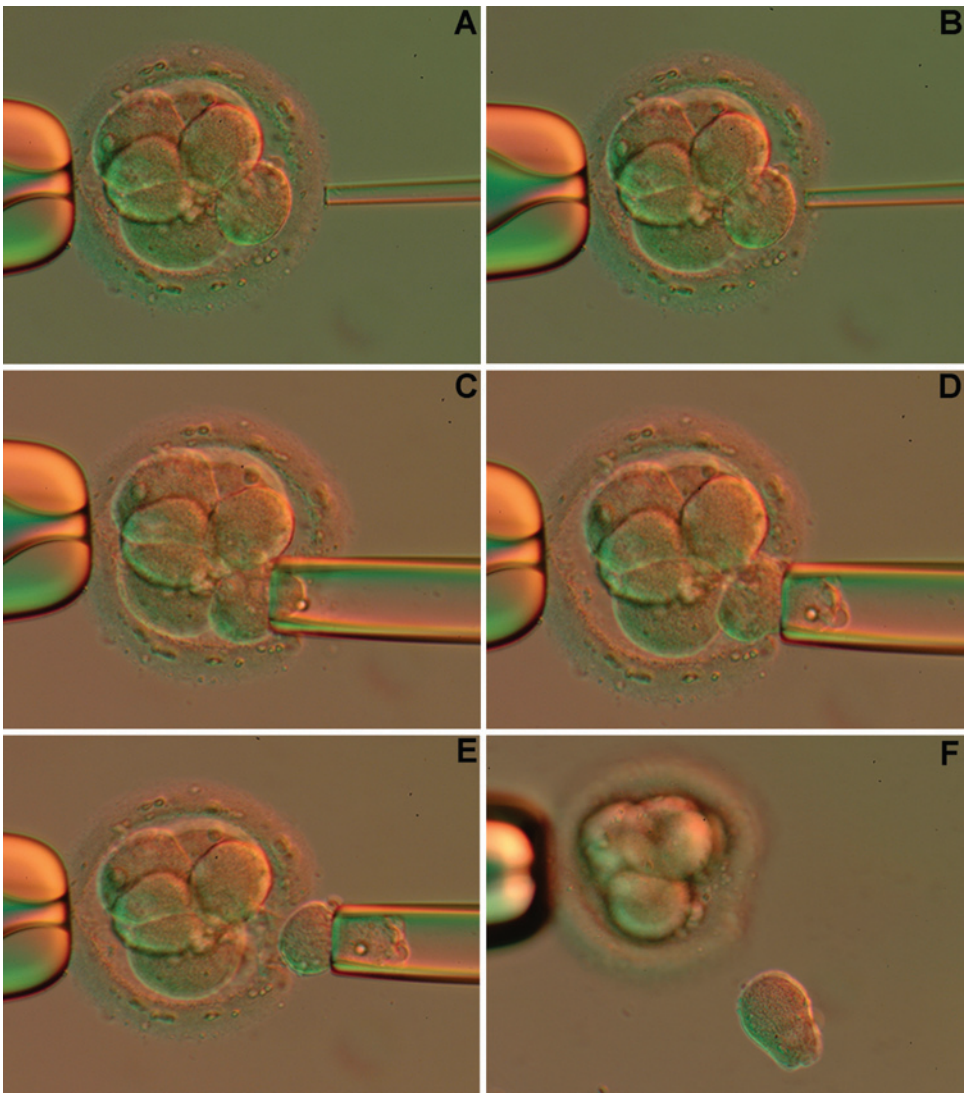

FIGURE 24.6 Extraction of a blastomere for pre-implantation genetic diagnosis (PGD) or hESC derivation. (A) Preparation for perforation of the zona pellucida of a cleavage-stage embryo (shown in Figure 24.4). (B) Perforation of the zona. (C) Insertion of extraction pipette through the zona. (D) Gentle aspiration of a single blastomere. (E) Removal of a single blastomere. (F) Deposition of removed blastomere into medium containing the still-viable embryo.

- Nikon Eclipse E400 compound microscope with A10PL, A20PL, SPLAN 40 PL, bright field phase contrast objectives as well as SPLAN APO 100× oil immersion objective
- Olympus dissecting microscope
- Laminar flow clean hood
- Automatic pipetting device. (Drummond Pipet-Aid)

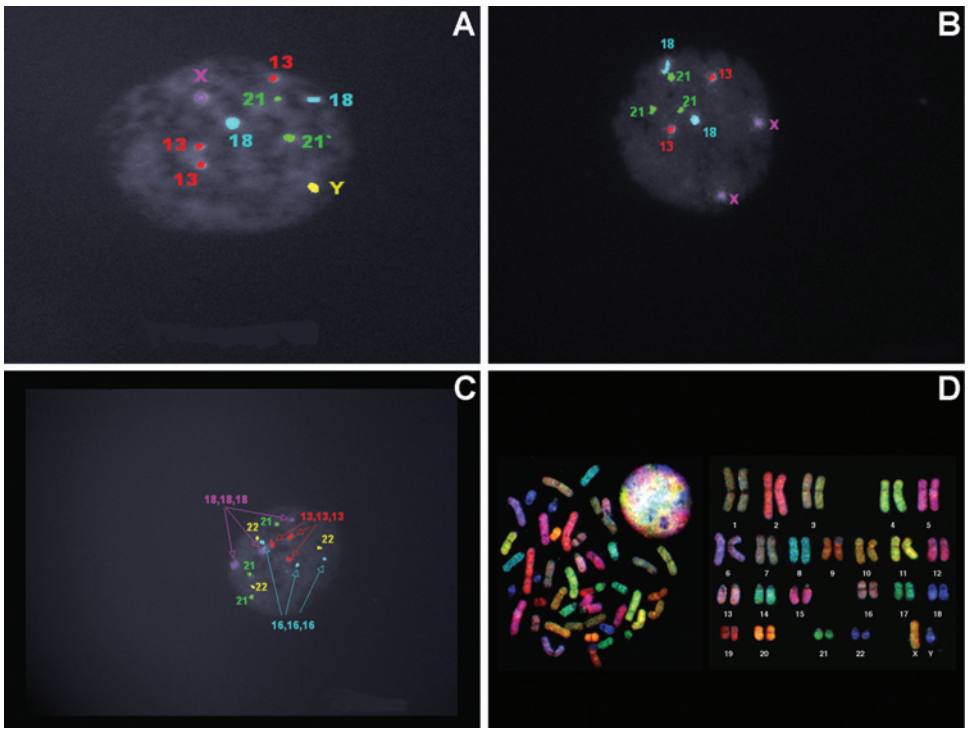

FIGURE 24.7 Results of typical pre-implantation genetic diagnosis with abnormalities reported. (A) FISH showing trisomy 13 (red). (B) FISH showing trisomy 21 (green). (C) FISH showing triploidy (yellow, green, red, purple, aqua). (D) Typical normal spectral karyotyping (SKY) results.

- Biohazard shield or chemical resistant face shield
- Heating block maintained at 37°C (×2)
- Centrifuge: IEC HN-SII
- Water-jacketed CO_2 incubator (Forma, model no. 3110)
- Refrigerator 4–5°C
- Freezer −17 to −20°C
- Heating pad
- Ultrasound machine
- Vacuum pump.

SUPPLIES

- Falcon 2 mL, 5 mL, and 10 mL disposable serological pipettes, sterile, individually packaged
- Falcon 50 mL sterile tissue culture flasks (Falcon catalog no. 2095)

- 15 mL sterile polystyrene, conical centrifuge tubes with screw cap (Corning catalog no. 430790)
- Elkay sterile, disposable, individually packaged or bio-pur rack, yellow pipette tips
- Boehringer-Mannheim Testsimplet prestained slides
- 20 × 30 mm coverslips for prestained slides
- Immersion oil
- Lens paper
- Powder-free disposable latex gloves
- 60–15 mm tissue culture dishes (Falcon catalog no. 3001)
- 60–15 mm organ tissue culture dishes (Falcon catalog no. 3037)
- Terumo tuberculin 20 cc syringe, disposable (catalog no. SS-20S)
- BD tuberculin 3 cc syringe, disposable
- Terumo tuberculin 1 cc syringe, disposable (catalog no. SS01T)
- B-D 30G1/2 disposable needles
- Tissue culture tubes, sterile, individually wrapped, 17 × 100 mm (Falcon catalog no. T-1342-1)
- Tissue culture tubes, sterile, individually wrapped, 12 × 75 mm (Falcon catalog no. T-13431-1)
- Pasteur pipettes, glass, 9 and 5¾ inch, washed and sterilized for tissue culture (Sigma catalog nos P5215-2 and P5215-1)
- Tissue culture tubes, sterile, 25/package, 17 × 100 mm (Falcon catalog no. 4-2057-1)
- Extension tubing, sterile, individually wrapped, 5.0 mL and 89 cm in length (Baxter catalog no. 2C5627)
- 3½ French Tom Cat Catheter (Sherwood catalog no. 8890-703021)
- Sterile ultrasound transmission gel, 25 × 15 mL (Swemed Lab International)
- Aspiration needle, disposable, 16GA 25 cm or 30 cm, Echotip Pivet-Cook Double Lumen (Cook OB/GYN, catalog no. J-OPSD-162501)
- Test tube rack, plastic
- Test tube rack, metal, sterile, individually packed
- Speculum, Graves (large), sterile, individually packed
- Ultrasound needle guide, sterile, individually packed
- Green twist-tie, sterile, individually packed
- Rubber bulbs, sterile, 2 per package
- Frydman catheter, sterile, indivually wrapped (Fertility Tech catalog no. 1307050)

- Frydman catheter for difficult transfer, sterile, individually wrapped (Fertility Tech catalog no. 1307100)
- Tef cat, 4 French catheter with a silicone adjustable positioner, sterile, individually packed (Cook Ob/Gyn catalog no. J-TFCT-40200)
- Tubing for mouth pipettes
- Millex-GS 0.22 μm filter unit, sterile (Millipore catalog no. SLGS0250S)
- Nunc four-well culture dish (VWR catalog no. 267061).

Reagents

Item	Supplier	Catalog no.
HTFM-HEPES (HH) medium	Irvine Scientific	90126
Human serum albumin (HSA)	Irvine Scientific	9988
QB XI	Concept Tech	ED1-100
P-1	Irvine Scientific	99242
Isolate stock	Irvine Scientific	99275
Synthetic serum substitute (SSS)	Irvine Scientific	99193
ET medium (HH-50% SSS)		
Sterile water		
Phosphate buffered saline (flush/vaginal preparation medium)	Irvine Scientific	9236
Sperm wash medium (HH-5% HSA)		

Recipes

QBXI-5% SSS

QBXI-5% SSS is prepared the day before the oocyte retrieval. For example, for 10 mL QBXI-5% SSS (= 0.5 mL SSS in 9.5 mL QBXI):

1. Add SSS to QBXI in a labeled orange-capped centrifuge tube.
2. Mix with a sterile serological pipette.
3. Store in a refrigerator until it is time to prepare culture dishes.

Isolate gradients (95%, 70%, 50%)

Prepare gradients as needed based on patient volume. For example:

- 95% Isolate: 9.5 mL isolate stock + 0.5 mL HH-5% HSA.
- 70% Isolate: 7 mL isolate stock + 3 mL HH-5% HSA.
- 50% Isolate: 5 mL isolate stock + 5 mL HH-5% HSA.

Seal labeled (media, expiry date, lot no., date made) tubes with parafilm.

HTFM-HEPES-50% SSS

HH-50% SSS is prepared the morning of the transfer(s). For example, for each patient 2.0 mL HH-50% SSS (= 1 mL SSS in 1 mL HH):

1. Add SSS to HH in a sterile centrifuge tube. Mix well with serological pipette and cap tightly. Do not allow foaming to occur.
2. Place in water bath (37°C) to equilibrate until time of transfer.

Egg wash (HH-5% HSA)

Egg wash is prepared the day before the oocyte retrieval. For example, for 10 mL HH-5% HSA (= 0.5 mL HSA in 9.5 mL HH):

1. Add HSA to HH in a sterile centrifuge tube, mix well with serological pipette and cap tightly. Do not allow foaming to occur.
2. Place in water bath (37°C) to equilibrate until time of retrieval.

Mineral oil (mouse embryo tested)

Wash 30 mL of mineral oil with 20 mL of QBXI, invert several times and place into an incubator (37°C, 5% CO_2), with cap loose, 2 days before use. The expiration date of the mineral oil after gassing is one week.

HTFM-HEPES-5% HSA

HH-5% HSA is prepared the day before the oocyte retrieval. For example, for 100 mL HH-5% HSA (= 5 mL HSA in 95 mL HH):

1. Add HSA to HH original bottle and invert gently. Do not allow foaming to occur.
2. Aliquot into labeled (media, expiry date, lot no., date made) sterile centrifuge tubes (12 mL per tube).
3. Place in refrigerator or into water bath to warm on morning of use.

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CHAPTER

25

Functional Characterization of Neurons

Scott McKerchner, Maria Talantova, and Stuart Lipton

INTRODUCTION

Diseases and injuries that affect the CNS, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis (ALS), macular degeneration, and traumatic injury afflict large numbers of people and often result in permanent debilitation, paralysis, and death. There are many juvenile onset diseases, such as the lysosomal storage diseases, that are either fatal in early childhood or contribute to a lifetime of suffering. The prevalence of such pathologies of the CNS and the nearly total absence of curative treatments for them has contributed to great expectations for the use of stem cell therapies to cure the disease, correct the neuronal damage or at least control the symptoms.

Routine clinical use of stem cell therapies for the CNS is still years away, but major investigative efforts are underway from basic research to early clinical trials. A variety of delivery methods are being tested, including injection into the brain ventricles or the eye, directly into neural tissues, administration through the bloodstream, implantation within various matrices and other techniques. One issue common to all of these

Human Stem Cell Manual, edited by J. F. Loring, R. L. Wesselschmidt, and P. H. Schwartz.
ISBN: 978-0-1237-0465-8.

experimental investigations in model animal systems is the ability to characterize the transplantation cells. Approval of a cell-based therapy by the US Food and Drug Administration (FDA) will require extensive data demonstrating the nature of the cells to be administered, the cells during the progress of the therapy, and the cells at the endpoint of the therapy. Perhaps the most compelling question for stem cell therapy is, can they repair damage by differentiation into mature, functional cells of the target tissue? This chapter presents a current state of the art set of procedures for neural cell identification and characterization *in vitro* and *in vivo* as it pertains to the use of neural stem/progenitor cells for therapeutic purposes in the CNS.

Overview

The two issues confronting the researcher in experiments involving transplantation of cells targeting the CNS are identification and characterization of the implanted cells.

Identification of the transplanted cells within the host may seem simple, but it is the source of much concern. Do the transplanted cells survive? Do they migrate or proliferate? What is the explanation for a situation in which the transplanted cells fail to survive, but the transplant appears to have improved the neurodegenerative disease? Identification of the cells can be done by several techniques. Many of these are described in more detail in the chapter on neural transplantation (Chapter 22). Of these techniques, only fluorescence-based methods offer the ability to visualize the cells in living tissue. Cells can be made fluorescent by introduction of green fluorescent protein (GFP) or other fluorescent proteins, or by incorporation of fluorescent dyes or particles. Considering the concern of the FDA with genetically engineered cells, we prefer to use direct labeling of cells with inert fluorescent particles, such as quantum dots; this approach presents virtually no risk of genetic alteration or cell toxicity, while allowing for visualization of labeled cells in live tissue or post-mortem identification of transplanted cells.

Characterization of the transplanted cells is important to understand how they might be functioning in a transplanted animal. Are the cells differentiated? Have they become neurons, glia, both, or neither? Techniques for the characterization of neural cells can be divided into those for use with fixed cells or tissue, and those for use with live cells or tissue. For fixed cells and tissue, standard cytochemistry, histochemistry, immunocytochemistry, and immunohistochemistry methods are the most routinely used. However, neurons and their processes can also be labeled by anteriograde and retrograde transport of a variety of molecules that will provide specific identification and morphological characterization. Also, the most powerful technique for characterization of neurons or astroglia, the two most numerous cells in the CNS, is electrophysiology.

Co-localization of the identification signal and the characterization signal is the essential requirement for concluding that the nature of the identified cell has been determined. Standard staining techniques combined with confocal or deconvolution microscopy can establish co-localization of identification and characterization markers in fixed tissues. In live acute brain slices, biocytin (a fluorescent molecule) can be added to the intracellular electrode solution during electrophysiological recording in

order to stain the recorded cell. This provides unequivocal identification of the cell from which electrophysiological data is obtained. Live pictures can be taken to show the quantum dot or GFP fluorescence. After subsequent fixation of the tissue slice, use of a strepavidin conjugated fluorescent molecule and additional staining (e.g. for neuronal or glial markers) can be done to show co-localization of the fluorescent dyes by microscopic analysis.

This chapter presents a set of procedures for labeling cells with quantum dots *in vitro*, injecting them into the brain, characterizing them by electrophysiological criteria, and then confirming cell identity by co-localization of fluorescent signals.

PROCEDURES

Labeling of cells

This procedure can be used with any cells isolated from primary tissue or cultured cell lines.

Qtracker® fluorescent quantum dots allow inert labeling of any cell type. The quantum dots are coated with a peptide that enables pinocytosis of the quantum dots by virtually any cell. Dots cannot then be taken up by another cell (except by phagocytosis of a dead cell). Cells can be labeled just prior to injection into the mouse or rat brain or the day before.

1. Human fetal brain-derived neurospheres are grown in neurobasal medium, B27 supplement, plus glutamine, pen-strep, heparin (5 μg/mL), and FGF2 (20 ng/mL). (Gentamicin and Fungizone are added to initial culture through first split.)
2. For suspension cells (including neurospheres), prepare single-cell suspension just prior to labeling by digestion with Accumax (a proprietary formulation of enzymes with proteolytic, collagenolytic, and DNase activities).
3. Label 1×10^6 cells with the following procedure.
4. Add 1.0 μL each of Qtracker reagents A and B to a sterile 1.6 mL microfuge tube and incubate at room temperature for 5 min.
5. Add 0.2 mL of preferred cell complete growth medium (including serum) and vortex for 30 s.
6. Use medium with Qtracker to resuspend up to 1×10^6 cells and leave in microfuge tube or place in well of 96-well plate; or use to replace medium on attached cells. Place in cell culture incubator for 45–60 min (expand to larger volume for overnight incubation).
7. Wash cells twice with complete growth medium and resuspend in injection buffer; or trypsinize attached cells and resuspend at approximately 1×10^5 cells/μL or as per experimental protocol in injection buffer.
8. Place a small number of cells into a well of the 96-well plate (or other tissue culture dish, with or without glass coverslip for future staining) and assess labeling by fluorescence microscopy (Figure 25.1).

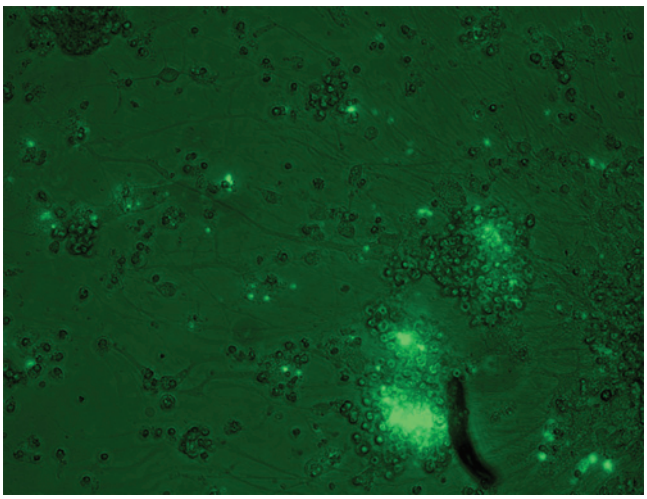

FIGURE 25.1 Human fetal neurosphere cells labeled with Qtracker 525 after 15 days in culture. Neurospheres derived from an 18-week human fetus were digested with Accutase and labeled with quantum dots during 1 h of incubation. Cells were then plated in neural basal medium containing B27 supplement, but without FGF2, on polyornithine/laminin-coated glass coverslips. Quantum dots are found nearly exclusively in the cell body as opposed to the cell processes.

9. To check to see whether cells become uniformly labeled, plate cells in their preferred medium in eight-well Lab-Tek chambered coverglass system (or equivalent) at 2×10^4 cells/well. Incubate and fix overnight for two hours.

NOTE: Invitrogen recommends a final labeling concentration of quantum dots (reagent A) of between 2 nM and 15 nM. Reagent A stock is 2 μM, so the above procedure results in a 10 nM final concentration. Optimization may be required for specific cell types.

Cell transplantation

The cells should be transplanted according to the desired protocol. In general, these methods involve a mouse/rat of the experimental model system.

The techniques vary in detail, but in broad outline, the animal is anesthetized with isoflurane or equivalent, an incision is made in the skin of the skull, and a hole is bored in the desired location through the skull bone with a dentist's drill. The mouse/rat is placed in a stereotactic injection apparatus and the coordinates of the injection calculated; cells are taken up into a 26G to 33G needle or glass pipette and the syringe placed into the micromanipulator; the needle is lowered into the brain to the desired depth and 1 μL of the cell suspension is injected at a rate of about 1 μL/min; the needle is allowed to remain in place for an additional 3 min before withdrawing slowly; the hole in the skull is filled with bone wax and the skin is sutured. The mouse/rat is allowed to recover and from a few days to several weeks are allowed for cell migration and/or differentiation to occur, depending on the experimental model.

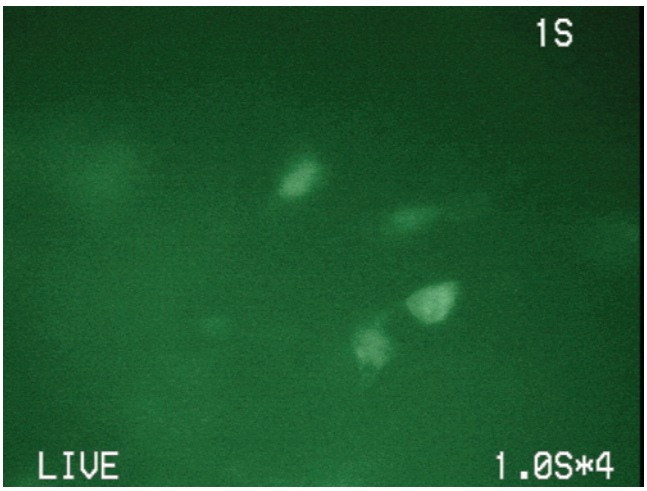

FIGURE 25.2 Live neurons in neonate rat brain acute slice labeled with Qtracker 525. Quantum dots were prepared as if for cell labeling *in vitro,* but were injected directly into the hippocampus of a normal 5-day-old rat pup. Two days later acute brain slices were prepared for electrophysiology, the slice was illuminated with a mercury lamp and cells were visualized through a GFP filter with a 40× water objective.

Electrophysiological techniques to assess transplanted cells for neuronal characteristics

It is important to test for neuronal properties *in situ*, where physiological connections have formed, and brain slice cultures are a good way to approximate *in vivo* conditions. A variety of techniques are used to analyze neurons of different types in different areas of the brain.

We generally wait three to six weeks after transplantation of neural progenitor or stem cells to assess the developing neuronal characteristics of the transplanted cells. Since the object of analyzing transplanted neurons by electrophysiology is to determine what type of neuron it is and how functional and how integrated it is, several variations are described for this analysis. The following procedures outline the approach we use for functional analysis of transplanted cells in the hippocampus.

Ligand- and voltage-dependent currents

N-Methyl-D-aspartic acid (NMDA) and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor-mediated excitatory post-synaptic currents (EPSCs) in acute slices with hippocampus; voltage-gated sodium channels.

1. Transverse brain slices (250–400 μM thick) from rats or mice are obtained using standard methods.
2. The animal is anesthetized, decapitated, and the brain is quickly removed in gassed (95% O_2/5% CO_2), and ice-cold artificial cerebrospinal fluid (aCSF).
3. Slices are prepared with a vibratome and immediately transferred to an incubation chamber where a continuous flow of warm (30°C) and gassed aCSF is supplied for 30 min of recovery.

4. Slices are then incubated in aCSF at room temperature for an additional 60 min to stabilize prior to electrophysiological recording.
5. Slices are transferred to a holding chamber mounted on an upright infrared-DIC-video-microscope with a 40× water-immersion objective, where they are continuously superfused with aCSF equilibrated with 95% O_2 and 5% CO_2 at room temperature and checked for the presence of fluorescent cells (see Figure 25.2).
6. For whole-cell recordings, CA1 pyramidal cells or other neurons of interest are visualized using Nomarski-type differential interference contrast imaging with infrared illumination. The presence of fluorescent quantum dots indicates that the cells have been transplanted.
7. Neurons are identified morphologically and electrophysiologically (firing trains upon depolarization, resting membrane potential).
8. Patch pipettes are pulled just prior to experiment from borosilicate glass tubing (1.5 mm outer diameter, 0.86 mm inner diameter). By filling with internal potassium gluconate solution, pipettes will have a resistance of 3–6 MΩ.
9. Identified neurons are approached with patch pipettes under visual control with positive pressure.
10. GABAergic and AMPAR-mediated responses are blocked by addition of 100 μM picrotoxin (or 200 μM bicuculine) and 10–20 μM CNQX to the bath solution. Sodium channels activated by depolarizing voltage pulses are blocked by 1.1–1 μM textrodotoxin (TTX).

In *cortical* slices, the EPSCs are evoked by stimulation of pre-synaptic fibers in neocortical layers II/III.

- A bipolar tungsten electrode is connected to a stimulus-isolator unit (WPI; stimulus frequency 0.1 Hz, current stimulus amplitude ranging from 150 μA to 1.5 mA).

In *brain stem* slices, stimulation electrodes are placed in the vicinity of the motor neuron from which EPSCs are recorded.

- The stimulation frequency is the same as above.
- In order to relieve the block by extracellular Mg^{2+}, EPSCs are recorded at +40 mV, digitized and evaluated for rise time, amplitude, deactivation, and desensitization, with pCLAMP 9 or pCLAMP10 and MiniAnalysis software.
- Input–output curves are generated: the "input" represents stimulus intensity and the "output", charge transfer of NMDA receptor EPSCs at a holding potential of −40 mV. In order to eliminate the effect of cell size, current amplitude is normalized to cell capacitance (Cm).

Whole-cell patch clamp

The whole-cell configuration of the patch clamp technique is used for recording macroscopic currents at room temperature (22 ± 1°C).

- The recording chamber is mounted on the stage of a Zeiss Axiovert inverted microscope.
- Signals are amplified using an Axopatch200B (or equivalent) amplifier, filtered below 2 kHz via a Bessel low-pass filter.
- Data are sampled and analyzed using pCLAMP9 and pCLAMP10 software in conjunction with a DigiData interface.
- The patch pipettes are pulled from standard wall 1.5 mm OD glass tubing with a final tip resistance of 3–6 MΩ. The access resistance (R_a) is measured via a membrane test prior to recording and monitored during and after recording. Recordings from cells with R_a values over 25 MΩ are not analyzed.
- For the recording of voltage-gated currents we use intracellular solution no. 1. For the initiation of voltage-gated currents we use voltage steps from −60 to +30 mV, with Δ10 mV; the duration of each step is 100 ms. Each step is initiated after hyperpolarization to −90 mV for 300 ms.
- The solutions of the agonists and antagonists of the different receptors are prepared in bath solution, and applied by an array of tubes placed 50–75μm from the cells.
- Solution changes are achieved rapidly, within 50–100 ms, by moving the array of constantly flowing pipette tips relative to the cell with the micromanipulator driver. A control pipette containing bath solution is used to wash away applied drugs rapidly.
- To study endogenous ligand-gated receptors and also the formation of new synapses, we use intracellular solution no. 2.
- EPSCs are evoked by stimulating the afferent terminals with fine bipolar stainless-steel electrodes.
- NMDA receptor-mediated EPSCs are isolated by adding 5 μM glycine and holding the recorded cell at −40 mV (in low Mg^{2+} solution) in the presence of CNQX (to block AMPA receptors), bicuculline or picrotoxin (to block GABA receptors).
- The AMPA component of EPSCs is isolated by APV to block NMDA receptor-mediated currents.

Single-channel activity in response to various neurotransmitter ligands

- Outside-out patches can be obtained from both soma and dendrites under visual guidance with Nomarski-type differential interference contrast imaging with infrared illumination.
- Outside-out patches are positioned in front of a two-barreled application pipette connected to a piezoelectric element.
- A continuous flow of control solution is provided from one barrel and test solution (e.g. containing NMDA, glutamate, glycine or other agonists) from the other.

- The piezo-controlled two-barreled pipette is moved in a defined time window, and receptors in the outside-out patch are exposed to the test solution.

Extracellular recording of LTP in hippocampal slices

- Transverse hippocampal slices (250–400 μM) are prepared with a tissue chopper, then treated as above. Extracellular field excitatory post-synaptic potentials (EPSPs) in the stratum radiatum of area CA1 are recorded in response to stimulation of CA3 Schaffer collaterals.
- The field EPSP is evoked, and an input-output curve performed to determine the stimulus intensity required to produce a half-maximal response. The input is the peak amplitude of the fiber volley, and the output is the initial slope of the field EPSP.
- All subsequent stimuli are delivered at that intensity at 0.1 Hz.
- Paired pulse stimuli are delivered at interstimulus intervals ranging from 40 to 200 ms and the degree of paired pulse inhibition or facilitation calculated for each interval using the initial EPSP slope values.
- After obtaining a stable baseline for 10–20 min (tested every 10 s), LTP (long-term potentiation) is induced by four tetanic stimuli delivered to the slice (100 Hz, 1 s, 20 s apart).
- LTD (long-term depression) is induced by low-frequency stimulation at 1–3 Hz for 900 stimuli.
- Stimuli are then applied every 10 s for 60–90 min. Initial field EPSP slope (% change) after tetanus is compared with pre-tetanus values.

Whole-cell recordings of LTP and LTD in hippocampal slices with patch electrodes

As described above, whole-cell recordings are made from visualized CA1 pyramidal or other cells that are labeled with quantum dots to ensure that they represent the transplanted cells. These recordings can be compared with endogenous neurons that are not labeled with quantum dots.

- To evoke synaptic current, stimuli are delivered through fine bipolar stainless steel electrodes placed in the stratum radiatum.
- For both LTP and LTD experiments, two independent pathways are stimulated (0.05 Hz), using bipolar stainless-steel stimulating electrodes placed in the stratum radiatum equidistant from the pyramidal layer on each side of the recording site.
- A pairing protocol is used to induce LTP, which consists of depolarization of the cell to 0 mV paired with 100 stimuli at 2 Hz in one pathway under voltage clamp conditions.
- To induce LTD, 200 stimuli at 0.5–1 Hz are paired with depolarization of the cell to −40 mV. The second pathway is used as a control.
- The magnitude of LTP or LTD transplanted cells can be compared with that of control host cells in the same slice.

Histological analysis of recorded slices

Optimally, the fluorescent labeling agent biocytin will be loaded into the patched transplanted cells. This will unequivocally identify the specific neurons from which recordings were taken by subsequent co-localization of the biocytin (red) with the quantum dots (green) (or the reverse combination of fluors) in histological examination of the tissue. Blue and far red fluorescence remains available for additional staining of the cells with DAPI and/or cell type-specific antibodies.

1. Fix sections in fresh 4% paraformaldehyde in PBS, cryoprotect in 30% sucrose in PBS, embed in OCT and quick freeze on a block of dry ice or in an isobutane/dry ice bath.
2. Cut 10–20 μm thick sections on a cryostat and stain with standard immunohistochemical techniques.
3. Use confocal or deconvolution reconstructions to obtain absolute correlation of the electrophysiological behavior of the recorded cells with the results of subsequent histological processing.

Useful markers for transplanted cell characterization are: nestin (neural precursor); βIII-tubulin, MAP2, and NeuN (neuronal); GFAP and S100β (astroglial); O4, myelin basic protein (MBP), and myelin proteolipid protein (PLP) (oligodendrocyte). See the list of antibodies in this chapter and others in this book. Note that GFAP can also label neural precursor cells.

Pitfalls and Advice

- To label cells from neurospheres, or other clumped cells, with quantum dots, it is very important to have the cells in a single-cell suspension (they can be allowed to clump again following labeling, if desired).
- Molecular Probes now recommends using Cytoseal 60 from Richard Allan Scientific (catalog# 8310-4) with all Qdots. Other mounting media, including ProLong Gold sold by Molecular Probes, actually quench the fluorescence of the Qdots.
- Pull pipettes in small numbers daily, and store them in a dust-proof container; avoid bubbles during the filling.

Equipment

- Cell culture incubator
- Cell culture hood
- Centrifuge for cell culture
- 95% O_2/5% CO_2 compressed gas cylinder
- pH meter
- Confocal or deconvolution microscope.

Supplies and Reagents

Supplies

Item	Supplier	Catalog no.	Alternative
Small animal stereotaxic instrument	David Kopf Instruments	Model 900	
Anesthesia machine	Matrx Medical Inc.	VMC	
Drill	Dremmel	Model 732	
Syringe, 5 μL	Hammilton, Model 65 26G blunt end	7633–01	
Vibratome	Leica	VT1000S	
Microscope	Zeiss	Axioskop2	
40× water-immersible objective	Zeiss		Hamamatsu
CCD camera and controller	Hamamatsu	C5985	
Amplifier	Axon Instruments (Molecular Devices)	Multiclamp 700B	Axopatch200B
Digital Interface	Axon Instruments (Molecular Devices)	DigiData 1440a	
Software	Axon Instruments (Molecular Devices)	PCLAMP 9	
Software	Synaptosoft-Molecular Devices	MiniAnalysis	
Electrode, tungsten	WPI		
Glass tubing	Warner Instruments	G150F-3 G150F-4	
MicroManipulator	Luigs & Neumann		
Cryostat	Leica	CM3050	

Antibodies for immunocytochemistry

Item	Supplier	Catalog no.	Alternative
Nestin (Ms IgG1)	Becton-Dickenson	N17220-050	Chemicon AB5922 (Rb)
βIII-tubulin (Rb polyclonal)	Covance Research Products, Inc.	PRB-435P	Cymbus CBL412 (Ms IgG1)
MAP2 (Ms IgG1)	Sigma	M4403	Chemicon AB5622 (Rb)
NeuN (Ms IgG1, clone A60)	Chemicon	MAB377	Abcam ab13938 (Ms IgG1, clone 4G2)
Neurofilament 200 (Ms IgG1)	Sigma	N-0142	Chemicon AB1981 (Rb)
GFAP (Ms IgG1)	Sigma	G-3893	Santa Cruz sc-7170 (Goat)
S-100β (Ms IgG1)	Sigma	S 2532	Swant 37 (Rb)
Myelin proteolipid protein (Ms IgG2a)	Chemicon	MAB388	Calbiochem NB38 (Ms IgG2a, clone plpc 1)

(*Continued*)

Item	Supplier	Catalog no.	Alternative
Myelin basic protein (Rt IgG2a)	Chemicon	MAB386	Chemicon AB5864 (Rb)
O4 (Ms IgM, clone 81)	Chemicon	MAB345	Sigma O7139 (Ms IgM, clone O4)
PDGFR-α (Rb IgG)	Santa Cruz	sc-338	Abcam ab20274 (Ms, clone SPM473)
FITC-donkey α-mouse	Jackson Laboratories	715-095-150	Molecular Probes A-21042 (goat α-Ms, Alexa 488)
Cy3-donkey α-mouse	Jackson Laboratories	715-165-150	Molecular Probes A-21426 (goat α-Ms, Alexa 555)
Cy5-donkey α-mouse	Jackson Laboratories	715-175-150	Molecular Probes A-21238 (goat α-Ms, Alexa 647)
FITC-donkey α-rabbit	Jackson Laboratories	711-095-152	Molecular Probes A-21206 (donkey α-Rb, Alexa 488)
Cy3-donkey α-rabbit	Jackson Laboratories	711-165-152	Molecular Probes A-31572 (donkey α-Rb, Alexa 555)
Cy5-donkey α-rabbit	Jackson Laboratories	711-175-152	Molecular Probes A-31573 (donkey α-Rb, Alexa 647)
FITC-donkey α-rat	Jackson Laboratories	712-095-153	Molecular Probes A-21042 (goat α-Rt, Alexa 488)
Cy3-donkey α-rat	Jackson Laboratories	712-165-153	Molecular Probes A-21426 (goat α-Rt, Alexa 555)
Cy5-donkey α-rat	Jackson Laboratories	712-175-153	Molecular Probes A-21238 (goat α-Rt, Alexa 647)
Normal donkey serum	Jackson Laboratories	017-000-121	Normal goat serum (005-000-121)

Reagents

Item	Supplier	Catalog no.	Alternative
Neurobasal medium	Gibco	21103-049	D-MEM/F12 medium (Gibco)
B27 supplement	Gibco	17504-044	N2 supplement (Gibco 17502-048)
Penicillin/streptamycin	Gibco	15140-122	Omega PS-20
Gentamicin	Sigma-Aldrich	G-1397	Gibco 15710-064
Fungizone	Omega	FG-70	
FGF2	Invitrogen	13256-029	Sigma F-0291
Hoechst 33342	Invitrogen	H1399	DAPI (Invitrogen D1306)

(*Continued*)

Item	Supplier	Catalog no.	Alternative
Qtracker 525 cell labeling kit	Invitrogen	Q25041MP	Other kits have Qdots with E_m of 565–800 nm
Accutase or Accumax	Innovative Cell Technologies, Inc.	AT-104 AM-105	Trypsin 0.05% EDTA (Gibco 25300-062)
D-AP5	Tocris Bioscience	0106	Calbiochem 165304
IsoFlo (isoflurane)	Abbott	05260-05	Avertin or pentobarbitol
(+)-Bicuculline	Tocris Bioscience	0130	Sigma B-9130
Biocytin	Sigma-Aldrich	B-4261	
Calcium chloride	Sigma-Aldrich	C-5080	Calbiochem 208291
Cesium chloride	Sigma-Aldrich	C-3032	Gibco BRL 15507-015
Cesium hydroxide	Sigma-Aldrich	C-8518	
CNQX, disodium salt	Tocris Bioscience	1045	AG Scientific C1053
γ-Amino-*n*-butyric acid (GABA)	Tocris Bioscience	0344	Sigma A-5835
Glucose	Sigma-Aldrich	25,307	Calbiochem 346351
L-Glutamic acid	Tocris Bioscience	0218	Calbiochem 3510
Glycine	Tocris Bioscience	0219	Calbiochem 3570
HEPES	Sigma-Aldrich	H-3375	Calbiochem 391340
Magnesium ATP	Sigma-Aldrich	A-9187	
Magnesium chloride	Sigma-Aldrich	M-4880	Calbiochem 442611
NMDA	Tocris Bioscience	0114	Calbiochem 454575
Phalloidin	Calbiochem	516640	
Picrotoxin	Tocris Bioscience	1128	Calbiochem 528105
Potassium chloride	Sigma-Aldrich	P-3911	Calbiochem
Potassium gluconate	Sigma-Aldrich	P-1847	Calbiochem 346125
Potassium phosphate (monobasic)	Sigma-Aldrich	P-0662	Calbiochem 529568
Sodium bicarbonate	Sigma-Aldrich	S-6297	
Sodium chloride	Sigma-Aldrich	20,443-9	Calbiochem 56744
Sodium hydroxide	Sigma-Aldrich	S-8045	Calbiochem 567530
Sodium phosphate (dibasic)	Sigma-Aldrich	S-0876	Calbiochem 567547
Sucrose	Sigma-Aldrich	S-7903	Calbiochem 5737
Tetraethylamonium chloride (TEA-Cl)	Sigma-Aldrich	T-2265	Calbiochem 584128
Tetrodotoxin (TTX)	Tocris Bioscience	1078	Calbiochem 554412
Water (HPLC grade)	Sigma-Aldrich	27,073-3	Fisher W5-4
Bone wax	Fine Science Tools	19009-00	Harvard Apparatus (no. 599864)

Recipes

- Prepare electrophysiology bath solutions without $CaCl_2$ and store at 4°C for up to two weeks; add $CaCl_2$ to warmed solution prior to use.
- aCSF must be prepared fresh the day of use.
- Intracellular solutions: prepare 50–100 mL without Mg-ATP or $CaCl_2$ using HPLC-grade water, sterilize with 0.2 μm filters and store at 4°C. These solutions can be used up to one month or frozen in aliquots of 1–2 mL and used for up to six months. Add Mg-ATP and $CaCl_2$ after warming prior to use.
- Prepare a 100 mM solution of Mg-ATP by adding 1.97 mL of HPLC water to a vial containing 100 mg of Mg-ATP; carefully adjust to pH 8 with KOH. Aliquot this solution at 20 μL into 1 or 1.5 mL Eppendorf tubes and immediately freeze it. Just prior to the experiment, add 980 μL of potassium gluconate solution and keep it on ice between pipette fillings; discard the remaining solution after 6–8 h.

Stock solutions

Component	Amount	Stock concentration
Magnesium ATP	100 mg in 1.97 mL	100 mM

Working solution: aCSF, pH 7.4 (1.0 L)

Component	Amount (g)	Final concentration (mM)
NaCl	6.779	116.0
Na_2HPO_4	0.145	1.02
$NaHCO_3$	2.200	26.19
KCl	0.400	5.37
$MgSO_4$	0.096	0.8
$CaCl_2$	0.470	3.2
D-Glucose	1.802	10.0

Working solution: intracellular solution no. 1 (voltage-gated currents, low EGTA) (100 mL)

Component	Amount (g)	Final concentration (mM)
Potassium gluconate	3.279	140.0
NaCl	0.029	5.0
$MgCl_2$	0.0095	1.0
EGTA		0.25
HEPES	0.477	10.0
KOH		to pH 7.25
Sucrose		to 290 mOsm

Working solution: intracellular solution no. 2 (ligand-gated receptors and synapses, high EGTA) (100 mL)

Component	Amount (g)	Final concentration (mM)
CsCl	2.021	120.0
TEA-Cl	0.331	20.0
HEPES	0.477	10.0
$MgCl_2$	0.0095	1.0
EGTA	0.086	2.25
CsOH		to pH 7.4
Sucrose		to 300 mOsm
Mg-ATP	20 μL of 100 mM stock added to 980 μL of this solution	2.0

Quality Control Methods

- Check all intracellular solutions prior to experiment for their ability to maintain live cells while recording.
- Check R_a before, during and after recording. R_a values should be between double the initial resistance and 10–25 MΩ. For example, for a pipette with an initial resistance 5 MΩ, the R_a after whole-cell formation should be between 10 MΩ and 20 MΩ, ideally around 12–17 10 MΩ.
- For the study of NMDA-mediated whole-cell currents or $EPSC_{NMDA}$, it is desirable to add phalloidine into the intracellular solution from 1 mM stock solution prepared in DMSO to a final concentration of 1 μM to prevent rundown of currents.

Reading List

Chen H-SV, Wang YF, Raydu P, Edgecomb P, Neill JC, Segal MM, Lipton SA, Jensen FE (1998). Neuroprotective concentrations of the NMDA open-channel blocker memantine are effective without cytoplasmic vacuolization following post-ischemic administration and do not block maze learning or LTP. *Neuroscience* 86: 1121–1132.

Goetz T, Kraushaar U, Geiger J, Lubke J, Berger T, Jonas P (1997). Functional properties of AMPA and NMDA receptors expressed in identified types of basal ganglia neurons. *J Neurosci* 17: 204–215.

Stuart GJ, Dodt HU, Sakmann B (1993). Patch-clamp recordings from the soma and dendrites of neurons in brain slices using infrared video microscopy. *Pflugers Arch* 423: 511–518.

Vogt K, Mellor J, Tong G, Nicoll RA (2000). The actions of synaptically released zinc at hippocampal mossy fiber synapses. *Neuron* 26: 187–196.

PART

VI

Setting up a Laboratory

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CHAPTER

26

Setting up a Facility for Human Embryonic Stem Cell Research

Ian Lyons, David Tan, Philip H. Schwartz, and Mahendra Rao

Introduction

Here we have distilled our collective experience of setting up over ten stem cell laboratories on three continents, with the intention of lessening the pain of others who are setting up such laboratories for the first time. We are sure of two things: first, the effort extended in setting up systems and processes for a laboratory will be repaid many fold in future efficiencies, and, second, the effort to set systems in place from the very beginning is much less than retro-fitting them. So if you are fortunate to be setting up a laboratory from scratch, our advice is to try to get it right, right from the beginning.

Overview

The recommendations here are for a small-to-medium scale academic or biotech research laboratory; those setting up manufacturing or therapeutic laboratories will have a different set of regulatory and logistic constraints. Most of the design features for a human embryonic stem cell (hESC) laboratory are the same as for any other cell

Human Stem Cell Manual, edited by J. F. Loring, R. L. Wesselschmidt, and P. H. Schwartz.
ISBN: 978-0-1237-0465-8.

culture laboratory. The physical laboratory space needs to be set up in a way that efficiently reflects the expected workflows, taking into account the numbers of workers who will be sharing the space and the equipment and the expected throughput. Like any well-run cell culture facility, materials and supplies need to be monitored to ensure that quality and amounts match the demands, and waste is disposed of appropriately. Similarly, the ancillary activities for molecular analysis of cell phenotype are not unique to hESCs. However, given the need for a regular supply of cells with defined characteristics and uncompromised differentiation potential, a careful system of cell banking is even more critical for hESC than for other cell types.

The critical issue in setting up the laboratory is planning for long-term success. This will rest on the functional design and utilization of the space and the appropriate equipment. Most important is the implementation of quality control systems that allow a continuous supply of validated cells and reagents. This book provides most of the protocols and methods for these systems, and other resources are also available; however, wherever possible, we recommend that key personnel receive specific training in hESC manipulation, and that the laboratory develop a network of communication and collaboration with other hESC groups.

Laboratory

General considerations

- hESC culture laboratories are similar to other cell culture research facilities. Space is required for tissue culture, standard biochemistry and molecular biology, and cell imaging. Incoming material and waste disposal logistics need to be accommodated.
- When setting up a laboratory, we typically define two distinct work areas: a cell culture area with rooms that can be dedicated for different types of work (Figure 26.1) and a molecular biology area with laboratory benches and appropriate equipment organized for common work flows (Figure 26.2). If possible, construction should consist of a modular framework that allows the laboratory to be reconfigured easily, if needed. A centralized CO_2 gas system with all CO_2 gas cylinders located outside the laboratories saves valuable laboratory space and simplifies the monitoring of gas levels, while the exchange of cylinders does not compromise cleanliness of the culture space. Drop lines carry CO_2 gas to equipment in each tissue culture room and in the fluorescence activated cell sorting (FACS) and microscopy rooms, if required for live cell imaging.
- Chemical fume cabinets are located within the molecular biology laboratory and are vented to the external environment as appropriate under local regulations. While not a high demand facility, access is needed for tissue perfusion, for fixation procedures, and for organic extraction of nucleic acids.
- High-quality water for the laboratory is supplied through a laboratory-specific Milli-Q or similar system and regular testing procedures for the quality of the water is needed, as water quality is critical to all cell culture.
- In order to facilitate cleaning and maintenance, vinyl flooring, gloss paint (low in volatile organic compounds), and bench tops of impermeable materials

FIGURE 26.1 Compartmentalized cell culture facility showing adjacent but separate rooms that are used for culturing different types of stem cells (adult versus embryonic, human versus animal). Arrow shows second biological safety cabinet in adjacent room.

FIGURE 26.2 Arrow shows the molecular biology area that is adjacent to but outside of the cell culture facility, allowing easy access but maintaining the integrity of the cell culture area.

FIGURE 26.3 The air handling system of the cell culture rooms are HEPA-filtered (arrow) and maintain positive pressure with respect to adjacent non-cell culture areas.

should be used as appropriate. Ideally, the rooms should be lit with fluorescent light and ambient temperature should be constant (typically 18–22°C), while circulating air should be passed through 0.22 μm HEPA filters (Figure 26.3). Cell culture laboratories should be under positive pressure, while molecular biology areas or those used for viral work should be under negative pressure.

- Standard safety requirements for laboratories, conforming to safety standards in the local jurisdiction, need to be implemented, but should include eye-washes, safety showers, first aid kits, fire extinguishers, and chemical spill kits at appropriate sites along the corridors. Sinks are typically located at the end of each workbench and, ideally, the taps will be foot activated (Figure 26.4).
- Common work areas for procedures such as media preparation will be conveniently located in the middle of the laboratory. Computers, internet connection points, and telephones are provided at the end of designated workbenches for communal use.
- When the layout of the laboratory has been determined and equipment locations decided, electrical wiring needs can be planned. Depending on local supply voltage and supply type, special circuits will probably be needed to supply higher voltage or three-phase power to some of the large equipment. In

FIGURE 26.4 All sinks in the cell culture areas are foot-pedal controlled (arrow), allowing hand-washing without contamination of or by fixtures.

addition, certain equipment, such as ice flake machines and autoclaves require easy access to floor drains and water inlet points.

- We recommend that all storage freezers and incubators be connected to an emergency back-up supply and that a centralized alarm system, linked to key equipment such as freezers and incubators, should be considered.

Cell culture area

- Depending on available space, we recommend that cell culture areas be functionally separated into dedicated rooms for hESCs (Figure 26.5), for non-human cells (e.g. mouse feeder cells), and for viral work. Ideally, each would have automatic doors such as elbow-activated glass sliding doors for easy access, and cell culture rooms should be under positive air pressure, while the room for viral work should be under negative air pressure.
- We have found it useful to equip each tissue culture room with independent workstations that consist of a biological safety cabinet (BSC), a CO_2 incubator, vacuum aspiration system, a complete set of personal equipment such as pipetting aids, micropipettes (2–1000 μL), and a drawer unit containing a selection of serological pipettes, filters, syringes, needles, culture vessels (flasks, plates, dishes), and cryopreservation supplies. From these workstations, there is easy access to shared equipment such as sinks, water baths, vortex mixers, centrifuges, and inverted microscopes for bright field/phase contrast visualization of cells in culture. Microscopes should be physically isolated from centrifuges and other vibrating equipment.

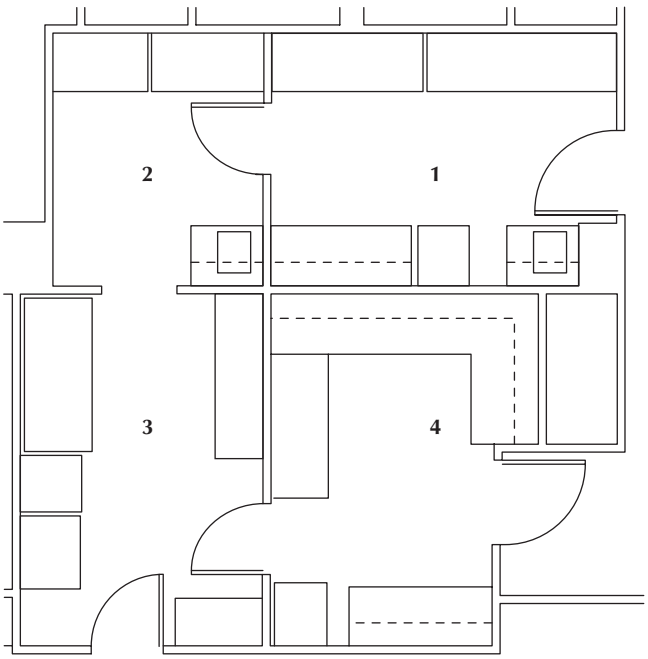

FIGURE 26.5 Example of adjacent cell culture rooms (1, 2, 3, 4) that allow efficient use of space and allow safe culture of disparate cell types in the same facility.

- For hESC culture, we have each BSC equipped with a heated base and a stereomicroscope to facilitate colony picking or other manipulations, and also the more usual germicidal ultraviolet lamps (under timer control), outlets for CO_2 and vacuum, 2 L collection flasks, and power supply for equipment such as pipetting aids.
- Fitting a zoom dissecting microscope to a class II BSC is not trivial. hESC colony picking requires a relatively large working distance and a heated stage is useful for the extended time the cultures are manipulated. A low microscope base with sloping sides allows easier transfer of the cell culture dish from the worktop to the stereomicroscope with minimal movement, and reduces chances of spillage or contamination.
- We have found the Gelman BH Class II Series (type A/B3) BSCs coupled with the Leica MZ6 to be a good combination. The microscope can be fitted with extended eyepiece tubes, and the BSC is modified to allow the extendable eyepieces to protrude from the sash toward the user (Figure 26.6). A pneumatic window sash is available and swings forward and back, allowing access to the BSC interior for equipment transfer and cleaning without having to move the stereomicroscope.
- An alternative and very cost effective approach, which is not recommended by the BSC manufacturer but has been used successfully by one of this book's editors, is the following: raise the BSC sash to its fully raised position, block all of the rear air vents with tissue paper (i.e. Kimwipes), and place a standard inverted microscope into the BSC. Most of the air flow then is into the front

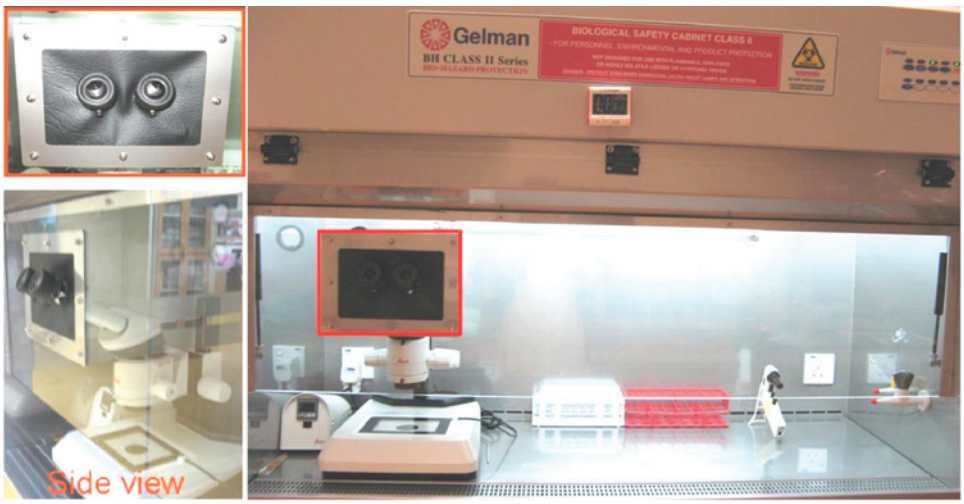

FIGURE 26.6 Biological safety cabinet with fitted dissecting microscope.

vents, creating a sterile curtain behind which culture manipulation on the microscope stage can take place (Figure 26.7) and in front of which the operator can view the process through the microscope's objectives. It should be noted that, in this arrangement, the user may not be adequately protected from the sample; thus, only samples that have been proven negative for potential human pathogens should be used. Dedicated laminar flow hoods, which are relatively inexpensive, can also be used for this purpose.

- The CO_2 incubators are more conveniently situated beside the BSCs instead of behind the user, minimizing movement of culture dishes from BSC to incubator and reducing the chance of accidental spills. Electric outlets in some of the incubators allow temporary installation of equipment inside the incubator (for example an orbital shaker for embryoid body formation).
- There are many CO_2 incubator options available. Important criteria include: readily cleaned internal surfaces without cracks or seams, monitored CO_2 level and temperature, with tight tolerances, and alarms. We like the Binder CB 150 with a data recording system which allows CO_2 levels and changes in temperature levels to be recorded for up to 14 days. Installation of tri-gas control incubators should be considered if the effects of manipulation of oxygen tension are likely to be investigated at some stage. The extra cost is incremental, since these incubators can also be used conventionally at ambient oxygen.

Molecular biology area

The organization of the molecular biology area will be determined by both the space available and the numbers of workers employed. For greatest efficiency in high-density laboratory areas, the space can be organized into functional areas for specific work-flows. In this system, all the equipment and reagents are readily at hand for specific processes and workers can come to a dedicated and shared area and find all that is needed for their task. Examples of this would be an area for immunostaining, with all

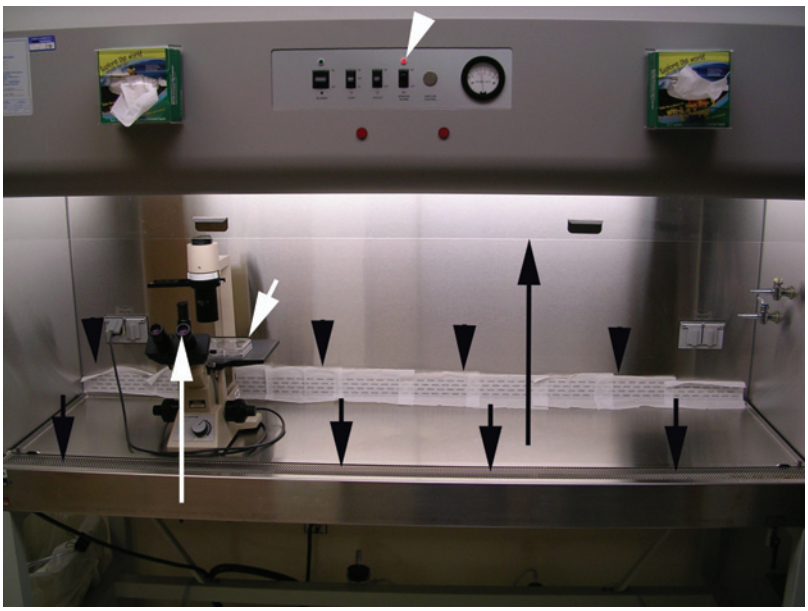

FIGURE 26.7 Standard biological safety cabinet modified to permit manual dissection of hESC colonies on an inverted phase-contrast microscope (Olympus CK-2 in this case). Alternatively, a dissecting microscope can be used. The sash is raised to full height (long black arrow) resulting in the sash alarm being activated (white arrowhead), the rear vents are "blocked" with lab tissue paper (Kimwipes, black arrowheads), and the microscope is placed inside the cabinet. Most of the HEPA-filtered airflow, therefore, is directed toward the front vents (black arrows), creating a sterile air barrier near the front of the hood. After a 1–2 min equilibration on the microscope stage, dishes or plates (white arrow) can be uncovered and manual dissection can take place under direct microscopic observation (long white arrow).

equipment and antibodies easily at hand. Another example is a separate bench (preferably a laminar flow cabinet) for RNA preparation and for polymerase chain reaction (PCR) set-up which is physically remote from the area for PCR product manipulation and analysis. The latter has the advantage of separating the PCR set-up from analysis, reducing the chances of reagent contamination with PCR products.

If space is less limited, a separate bench or area can be assigned for each worker. Each person will have access to a basic set of tools including micropipettes (2–1000 μL), a PCR machine, centrifuge, vortex mixer and an under-the-counter refrigerator and freezer.

In addition to the functional areas described above, we have found it convenient to have "communal areas" with more generally used equipment such as analytical balances, centrifuges, spectrophotometers, gel imaging equipment, pH meters, plate readers, ultracentrifuges, and power supplies for electrophoresis.

Additional considerations

In addition to laboratory areas dedicated to cell culture and to molecular cell analysis, there are a number of other activities that might require dedicated areas. These may

be part of the hESC culture facility itself or may be part of core institute facilities; but if access is needed, it should be taken into account during the planning phase.

Dedicated rooms for microscopy, image analysis and flow cytometry equipment will reduce dust and traffic flow. Depending on the equipment used, purpose-built benches with vibration isolation are a good investment.

Sufficient storage space should be available to the laboratory. In particular, disposable plastic cell culture supplies are bulky and having a separate storage room will help avoid bringing potentially dusty cartons into the laboratory. Storage for flammable liquids should be available in fireproof cabinets. Fridges, freezers, and ultra-low freezer units should be located carefully. Apart from under bench fridges and freezers, we recommend that these units be housed separately wherever possible, since they are a major source of ambient noise in laboratories.

Long-term cell storage has traditionally been done in liquid nitrogen freezers. If these are to be used, they should be housed separately from the laboratory in a well-ventilated space with low oxygen alarms to reduce dangers of asphyxiation. This could be in the same area used to store consumables. We also recommend all critical derivations and lines be stored in two different freezers, preferably with one at another site. Ultra-low (−152°C) freezers are an alternative to liquid nitrogen storage; they are reported to have become increasingly reliable and do not need a constant supply of liquid nitrogen, the regular replenishment of which brings dirt into the laboratory and is also expensive.

Hazardous biological waste needs to be disposed of according to local regulations. In most regions this will require access to an autoclave, and this should be taken into account when the laboratory is being planned.

Record Keeping

General procedures

Good book keeping is essential; paperwork ranging from quotations, purchase orders, delivery orders, invoices, certificates of analysis (C of A), material safety data sheets (MSDS), material transfer agreements (MTAs), research agreements, and licenses should be filed and organized in an easily understood system. This is particularly important in the case of hESCs, where regulations can be stringent in some countries. Careful documentation from the start will save considerable time and stress in the future.

Reagent and consumable tracking

A reliable supply of validated reagents is critical to hESC research quality. In general, reagents from major suppliers can be used with confidence; however, some are known to be subject to lot-to-lot variation. There are a number of reasons for this, including the unfortunate fact that some reagents are quality tested against cell types less fastidious than hESCs. An example of this is KnockOut™ serum replacement (KSR; Invitrogen catalog no. 10828-028), a product that is reliable for the cells it was designed for, mouse embryonic stem cells (mESCs), but for which lot-to-lot variation

has been described for hESCs. To maintain a continuity of supply of reliable reagents, it is important to have a system that:

- ensures that the laboratory does not exhaust any reagent, and
- provides a system that allows timely testing of incoming reagents to ensure comparability with those being replaced.

For hESC culture, some of the critical reagents that should be tested well in advance of being brought into general use include fetal bovine serum (FBS), KSR, and mouse embryonic fibroblasts (MEFs). Approaches to testing are described below. Other reagents are less variable and are usually used untested.

Stock and reagent inventory

The levels of stocks of reagents that need to be held to ensure continuity of supply will depend on the rate of usage by the laboratory, on the delay between ordering and delivery, the probability of an exceptional delay, and the risk aversion of the laboratory director. For a guideline, we recommend that stocks of all reagents be maintained at a minimum of three times the delivery lead time. So for reagents that typically can be delivered one week after ordering, we recommend that a minimum of three weeks normal usage should be maintained. If these reagents are being tested to validate lots, then the holding time should be increased to account for the time it takes to test. Maximum holding times will depend on the storage capacity of the facility and the shelf life of the reagents.

For all reagents, it is important to keep track of the manufacturers' stock keeping units (SKUs) and lot numbers. These should be maintained as part of the laboratory record keeping and inventory records, and each worker should record those details in their laboratory notebook for each experiment or process undertaken. It is relatively rare to have a lot fail, especially if rigorous lot matching and testing is undertaken systematically; however, rare, sporadic lot failure will occur, and recovery from a cell culture "crash" or unexplained failure will be more efficient if all reagents can be traced. The savings in time and money will justify the upfront effort to establish such a system.

Lab index system

In addition to labeling the outsides of drawers and cabinets with their contents, we have found it useful to number each of the laboratory drawers and cabinets and to keep a database of their contents. This is particularly useful in a laboratory that is likely to have frequent visitors or changes of staff. This also facilitates stocktaking and auditing. The laboratory manager should ensure that these lists are updated regularly so that finding irregularly used items becomes quick and easy.

Freezer inventory systems

Keeping track of the stored cell stocks is critical for the smooth operation of the facility on a day-to-day basis, and also allows for the longer term planning. Whether cell stocks are stored in an ultra-low temperature mechanical freezer or in liquid- or vapor-phase nitrogen, a system of cell labeling and record keeping is essential to allow retrieval of cells, or to keep track of the rates of cell stock usage. The cell freezer will typically come

with a system of racks to store the ampoules and allow them to be quickly located. This must be backed up by an inventory system that keeps track of each ampoule. This can be done electronically, using a purpose-designed system or a more generic database software, or it can be done manually using a dedicated laboratory notebook or ring binder. In either case, the data need to be regularly backed up. Each freezing vial must be unambiguously labeled either using printable cryoresistant printer labels, or with a cryoresistant marker pen. Details should include cell type and passage number, date, technician, and laboratory notebook reference. Like any system, this will fail if there is not compliance, and every vial added or removed should be noted immediately. Proper adherence by the users is essential to the smooth functioning of the system, so creating an environment where users have ownership over their work is important.

Examples of cryostorage record systems can be found at http://www.cryotrack.com/, http://www.cclims.com/, http://www.nalgenelabware.com/techdata/technical/cryo.pdf.

Equipment Maintenance

Equipment maintenance is essential for the smooth operation of the laboratory and for the generation of reproducible data. We recommend providing all equipment and accessories with maintenance schedules and a local and alternate contact person for sales and service. All major equipment must be tested and commissioned before use, and maintained on a regular basis by the vendor or reputable service organization. It is important to keep track of dates and records of these procedures so as to prolong the lifespan of each piece of equipment and ensure their proper use. Usage of common large equipment such as centrifuges and spectrophotometers should be tracked using a logbook. For regular maintenance of equipment, there should be clear ownership and visibility.

- *Incubators* should be checked on a weekly basis using a digital thermometer and a Fyrite device. The readings obtained can be recorded on the door of the incubator so that all users are aware of any fluctuations.
- *Water baths* are a common source of contamination. To minimize this, the water should be changed at least weekly. Locating the water baths adjacent to the sinks simplifies the changing of water and cleaning.
- *Microscopes* are often used while not optimally aligned. They should be adjusted regularly, and all scientists and staff using them should be trained in their correct use.
- *BSCs* should have their interior wiped down after every use with 70% ethanol. The germicidal ultraviolet lamps should be switched on at the end of each day. For this reason, it is important that the equipment kept inside the BSC is able to withstand exposure to UV light; remove all vulnerable equipment and supplies before operating the UV light.

Safety

Staff should be trained in the safe handling of all equipment, reagents, and biological materials and in the use of first aid and safety equipment. Laboratory gowns and

safety glasses should be supplied and be worn at all times. Personal protective equipment (PPE) should be available for all pertinent laboratory work. In particular, full-face shields should be worn when removing and thawing ampoules from cryostorage.

Biological hazards

Human and other primate cells should be handled using Biosafety Level 2 practices and containment appropriate for the institute and local authorities. All work should be performed in a BSC with appropriate PPE (gloves, glasses, laboratory coats), and all waste material should be decontaminated by autoclaving or disinfection before discarding. For a discussion of recommendations and requirements refer to "Biosafety in Microbiological and Biomedical Laboratories" at http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm.

Quality Control

The aim of the laboratory set-up is to have systems in place that lead to reliable and reproducible experimental outcomes (Figure 26.8). This is dependent on four critical factors:

- *Reliable techniques* that yield a minimum of variability between processes. Use of methods developed by others and shown to be reliable is a good way to establish a laboratory, and to have the results of that laboratory comparable to the work of others. Refer to other chapters of this book for these methods.
- *Validated reagents*. As this is critical, we describe below a stock keeping and validation system for laboratory reagents.
- The *quality of the hESCs*. We also describe a method for generating a structured stock of hESCs that have been validated molecularly and functionally, and which has sufficient size to cover the demands of the laboratory.

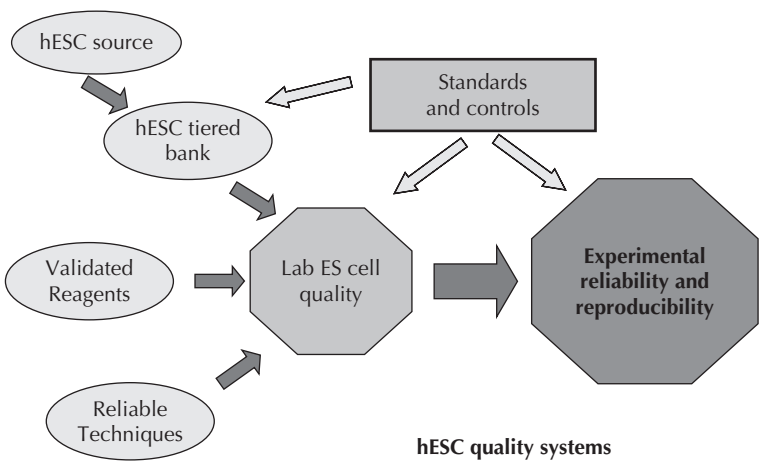

Figure 26.8 hESC quality systems.

- A *system of testing* and standards that confirms comparability of hESCs being used. They need to be free of contamination, genetically stable, to express defined markers, and capable of differentiation into representatives of the three germ lineages. Much of this is described in other chapters of this book, but below we describe techniques for monitoring of and recovery from contamination.

Reagent testing

Because lot-to-lot variation has been described for FBS, KSR, and MEFs, we recommend that a regimen be introduced to test lots before they are brought into general use. Other reagents are less variable and are usually used untested.

Lot testing FBS for MEFs

Almost all FBS lots are able to support MEFs, but they still need to be tested in advance, because the cost may be significant. We confirm FBS lot suitability on the basis of cell expansion over two passages, but do not monitor biological activity. A positive control in this assay is either an FBS sample that has been shown previously to support MEFs, or if unavailable, a commercially available FBS validated for support of mESCs (e.g. Invitrogen catalog no. 10439-016). mESC-qualified FBS could be used routinely for MEF propagation, but is significantly more expensive than unvalidated FBS.

Using the protocols described in the chapter on preparing MEFs:

1. Make medium with each of the test FBS samples, and with the control.
2. Seed MEFs at usual density in triplicate 10 cm dishes in medium.
3. Harvest cells and count after 5 days (but before the control reaches confluence).
4. Count cells and replate a second passage in each medium.
5. Again harvest cells and count after 5 days (but before the control reaches confluence).

Suitable batches of FBS will have cell yields in excess of 80% of that of the control lot.

Testing FBS and KSR for hESCs

When testing lots of serum or KSR for use with hESCs, we passage cells four times before confirming morphology and marker expression patterns, since many medium components do not have an obvious effect until at least three passages. Ideally, one would use a positive control serum or KSR batch, but these are not commercially available. Typically most laboratories would use a batch that has been previously used successfully. A new laboratory that is being set up will not have that option, though may be able to obtain samples from an established laboratory.

Using the protocols described in Chapter 1 on hESC culture:

1. Make medium with the test serum or KSR batches and with a positive control, if available.
2. Passage cells in the usual manner for three passages in the test media.

3. Passage into appropriate culture systems for immunocytochemistry for SSEA-4, POU5F1/OCT4, and alkaline phosphatase staining, and for expansion to extract RNA for microarray or PCR analysis.
4. Analysis: Serum or KSR batches are acceptable if they support cells as well as the control lot that has been used successfully and/or have these minimal performance characteristics:
   - Immunocytochemistry: >85% of cells express SSEA-4 and POU5F1/OCT4.
   - RT-PCR: We use conventional and quantitative PCR to assess expression of genes that are markers of undifferentiated cells and to confirm absence of markers of differentiation for each of the three germ lineages (see below).
   - Morphology and alkaline phosphatase: more than 95% of colonies express alkaline phosphatase and are morphologically undifferentiated.

Lot testing MEFs

During preparation, MEFs should be tested for microbiological contamination; testing biological function of each new lot should not be necessary given that the appropriate strain and embryological stage of fetus were used and that methods are reproducibly followed. Testing new batches of MEFs before they are brought into general use, however, is a wise precaution, at least until several lots have been generated successfully. The testing procedures and readouts are essentially the same as those described above for serum and KSR. Cells are maintained on test and preferably control MEFs for four passages and then marker expression is analyzed as described above in the section on Testing FBS and KSR for hESCs.

Cell Banking

Rationale

A well-structured cell bank is important for successful cell culture in general, but is critical for ESCs, with their inherent karyotypic instability and tendency to differentiate. A tiered or master cell banking system brings validated and reproducible cells to each experiment and procedure. Cells will be:

- At defined range of passage number for all experiments
- Of normal karyotype
- Free of microbiological contamination
- Capable of differentiation to desired lineages
- Of undifferentiated phenotype with expression of appropriate molecular markers.

Establishment of a tiered banking system

A tiered banking system typically consists of three levels of cryopreserved cells: the earliest "mother stock," the critical "master cell bank," and the "working cell bank" (Figure 26.9). The number of vials of each depends on the cell type and projected

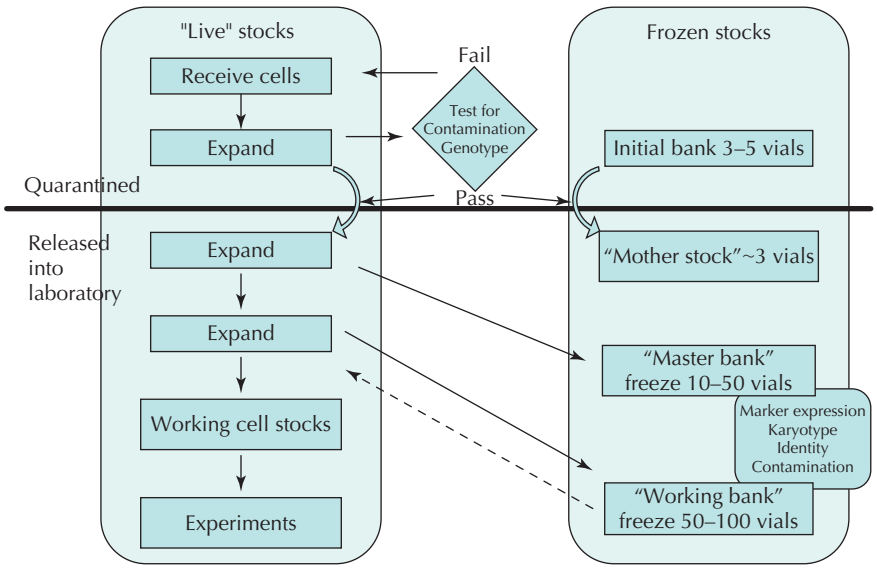

FIGURE 26.9 hESC workflow and tiered cell bank.

usage, and on the extent of the testing required for that cell type. For hESCs, the testing recommended is extensive and relatively expensive, and so we suggest that large banks of cells be frozen.

At each level of banking, we recommend the cells be tested for:

- Karyotype
- Genotype
- Differentiation capacity
- Marker expression (both presence of markers of undifferentiated cells and absence of markers of differentiation).

Other aspects of cell status can also be tested since the cells have been shown to drift with extended culture (e.g. imprinting status, etc.); however, that drift has not been shown to be associated with systematic loss of pluripotency and so may be seen as less critical.

Incoming cells

When any cells first arrive, it is prudent to treat them as if they were microbiologically contaminated. Preferably, they should be handled in a separate quarantine facility, though in usual practice this is not always possible and they are handled carefully in the same laboratory, with care to avoid aerosols that would be capable of distributing contaminant microorganisms. Biosafety cabinets are diligently swabbed after handling new cells, and if a separate incubator is not available, the culture vessels are placed in an uncovered, or vented, plastic box to contain any inadvertent spills.

Thawing and plating cells

If the cells arrive on dry ice (or more preferably in $N_{2(l)}$), transfer to $N_{2(l)}$ storage immediately. Some distributors recommend immediate thawing of samples shipped on dry ice rather than transfer to $N_{2(1)}$. Cells can then be recovered after all reagents have been assembled and validated as appropriate.

Initial cell recovery and maintenance should be done using the instructions of the cell providers, since troubleshooting by the providers is difficult if they are not familiar with your methods. In general, cells should be recovered as gently as possible, and patience must be exercised. Cryopreserved vials typically contain a few to a few tens of pieces of viable hESC colonies and these can easily be lost. Further, these colony pieces are slow to recover, and often do not appear for up to two weeks (or even after a passage is required due to the exhaustion of the MEFs).

Recovery of cells shipped "live"

Although cells are typically shipped frozen, and preferably in liquid nitrogen in a "dry shipper," it is also possible to ship them as living cultures in a flask at ambient temperatures. Cells sent this way will usually arrive in a "T25" flask completely filled with equilibrated medium and closed tightly give the flask greater compressive strength and to reduce swirling that might dislodge cells. Cells in a flask, however, are much more difficult to passage mechanically.

Swab the flask with 70% ethanol, transfer to the BSC, remove all but 6–8 mL of medium, and inspect the cultures microscopically. If they are not ready to be passaged (see Chapter 1), incubate and feed, per usual, until they are ready to passage. When the cells are ready to be passaged, carefully use a heated knife or scalpel blade to cut an aperture in the top of the flask and passage the cells using the microdissection protocol described in Chapter 1. Alternatively, all of the colonies in the flask may be dislodged with a cell scraper and the colonies may be broken up with trituration prior to re-plating. This technique, however, will generally lead to the plating of colonies that are larger than desired and may lead to greater than normal levels of differentiation.

Initial expansion: "mother stock"

The aim of this phase is to establish the earliest bank of frozen cells as soon as possible after receipt, and to confirm that they are not contaminated with microorganisms.

Expansion and cryopreservation

About three vials should be frozen within two or three passages of the cells' arrival. For this phase, the cells should be maintained using the protocols provided by the supplier of the cells. Cells will be maintained in culture and further expanded to generate the next level of stocks, while simultaneously some cells will be tested for contamination and genotyped to confirm the identity of the cells.

Cells can be cryopreserved using variations on commonly used methods; however, we recommend that, for the first freeze, the methods recommended by the supplier of the cells be followed. Again, this will allow more efficient troubleshooting, if necessary. Alternative methods are described in Chapter 4. One way to confirm cryopreservation viability of another cryopreservation method is to thaw one of the vials

from the first freezing to confirm recovery before the freezing of additional cells for the master cell bank.

Testing

Microbial contamination
We routinely test for mycoplasma, bacteria, and fungus, using protocols described in Chapter 1. The most frequent contaminants of hESC cultures are mycoplasma, which are small prokaryotes without cell walls. Since most antibiotics do not kill mycoplasma, it often persists at low levels without developing the turbidity that indicates bacterial contamination, and may induce a variety of deleterious effects, ranging from changing cell metabolism to chromosomal changes. If the cells are contaminated at this early stage, abandon the expansion and get fresh cells.

Genotype
Cells should be genotyped for positive identification because, although rare, confusion can occur. There are a number of methods available, including single nucleotide polymorphism (SNP), human leukocyte antigen (HLA), and short tandem repeat (STR). These can be carried out in house or by a contract organization such as a paternity testing lab. A protocol for SNP analysis is described in Chapter 1.

Secondary and tertiary expansion: "master cell bank" and "working cell bank"

The master and working cell banks are the two critical levels of the tiered cell bank and utmost care should be taken during the expansion of the cells for them. Diligently change medium as recommended, passage using best practise manual dissection, and dissect away any regions of differentiation as described in the supplier's protocols and in Part 1 of this book. Limits are set to the amount of time that cells are serially passaged during normal laboratory activities, and cells must, therefore, be recovered from the working cell bank. As the working cell bank is depleted, it is replaced by expansion of cells from the master cell bank. At each banking step, the cells are extensively validated as described below.

The number of dishes of cells required for this freeze will depend on the size of the cell bank planned, but as a rule of thumb we aim for 20–40 colony pieces per freezing vial, and we usually get four vials per 60 mm dish or 12 vials per six-well plate. So, for a bank of 20 vials, five 60 mm dishes at moderate density are required.

These cells can be cryopreserved by a variety of methods. We recommend thawing a vial of cells previously frozen by your laboratory to ensure that appropriate recovery rates can be achieved, and expanding these cells to undertake the battery of quality control (QC) tests, including microbial contamination, genotype (genetic identity and karyotype), and phenotype (molecular marker expression and differentiation capacity). These tests are described below in the section on QC standards and in protocols found elsewhere in this book.

QC standards: routine monitoring of cell status

Although hESC lines are highly similar to each other in their expression of cell surface antigens and markers characteristic of the ESC state and are relatively stable over time

in continuous culture, like all other cell types ESCs can undergo changes in culture. Most dramatic are changes in the chromosomal composition of the cells, but other forms of genetic and epigenetic drift have also been detected in hESCs after extended culture. These changes include accumulation of DNA damage, oxidative damage, erosion of telomeres, acquisition of mitochondrial mutations, and loss of imprinting.

The potential for these changes makes it important to monitor cells regularly to ensure maintenance of baseline hESC characteristics. There are many aspects of cell status that could be meaningfully tested; however, to carry out all of these tests while freezing regularly through extended passage would become prohibitively expensive and time consuming. The choice of tests should be most complete at the time of laying down each layer of the tiered frozen cell bank, and less extensive at the time of regular tests through the period of expansion of the cells in culture. We recommend that these regular tests be carried out at least every ten passages and ideally at every fifth. The cost of the tests is currently about US$2000 when performed extensively for a frozen bank and about US$500–800 at every tenth passage. While expensive, it is well worth the savings in time and money that are involved in rescuing a line or retesting interesting results (or, as a worst case scenario, starting all over). Using this strategy, we have been able to maintain five different lines over several years in culture without losing more than an occasional vial to karyotypic or other abnormalities.

Basic set of tests for monitoring passaged cells

Karyotype

We recommend using SNP analysis or G-banding (both are described in detail elsewhere in this book). Although fluorescence in situ hybridization (FISH), spectral karyotyping (SKY), and comparative genome hybridization (CGH) are also possibilities, these require longer time periods, larger cell numbers or cost much more, so are not appropriate for routine monitoring. They nevertheless remain important second stage tests for verification or more precise definition of an abnormality detected by SNP or G-banding. (See section II part 1 for protocols).

Marker expression: RT-PCR, immunocytochemical analysis, and telomerase

We recommend a combination of PCR and quantitative PCR (qPCR) to assess cell populations. PCR is used to confirm expression of markers of undifferentiated cells and to demonstrate the absence of markers of differentiation and the lack of contaminating feeder populations. (This technique is described in detail in Chapter 10) If these are detected, we then use a qPCR protocol to assess the degree of contamination by feeders and the extent of differentiation.

While PCR or qPCR give an impression of the total cell population, immunocytochemistry allows assessment of the status of individual cells. Usually we use a double labeling technique to look at, first, POU5F1/OCT4 or SOX2 and, second, a cell surface epitope such as SSEA-3 or, instead, alkaline phosphatase fluorescent histochemistry. We recommend the *in situ* staining of cells in culture to layer the staining information onto the morphology and appearance of the colonies (see Chapter 9).

While flow cytometry (Chapter 8) may be a more quantitative way to assess the proportion of cells carrying different cell surface epitopes, we have found that obtaining

accurate numbers is difficult as undifferentiated hESCs attach to each other by tight junctions and so obtaining a representative single cell suspension is problematic. Moreover, the total amount of cell sample required is much higher than that required for other tests with equivalent or greater sensitivity.

Telomerase activity is a defining characteristic of hESCs and levels should be assessed regularly.

Additional tests for frozen cell banks

To the tests described above for routine assessment of cells in culture, we add the following to confirm the identity and characteristics of the cells frozen at each level of the tiered cell bank.

Identity

At each major freezing step or before a critical series of experiments, the identity of the cells should be confirmed by STR or HLA typing. This is particularly important when more than one line is being maintained in the laboratory. Mix-ups are inevitable and are most often attributable to human error. However, such mistakes can be readily determined if a genomic fingerprint of the cells is on file for comparison. STR testing can be readily done using off-the-shelf kits, as can HLA typing. Both are also offered as services by forensic and pathology labs. The cost is easily justified by the alternative greater cost of the consequences of working with misidentified cells.

Demonstration of differentiation potential

In addition to the cells being relatively uncontaminated by differentiated cells, it is critical that they retain the ability to differentiate to representative cells of the three germ lineages (endoderm, ectoderm, and mesoderm) and to trophoblast lineages. This can be ascertained most readily by *in vitro* differentiation to embryoid bodies (Chapter 4), followed by molecular analysis (RT-PCR; Chapter 10, or immunocytochemical; Chapter 9). Alternatively differentiation capacity can be demonstrated by the *in vivo* induction of teratomas in immunocompromised mice followed by histological analysis (Chapters 12 and 13).

Other tests

Whenever a new line is derived, it should be characterized even more stringently. In addition to the tests described above, mitochondrial sequences, miRNA expression, global methylation profile, and methylation-specific PCR to assess key developmental and imprinted genes should be considered. Our data suggest that although these aspects of cell biology might be important when cell lines are developed for clinical use, they do not appear to affect most cell culture differentiation protocols, and so these may not need to be included in the routine battery of tests.

Standards

The ideal standard to compare gene expression patterns of hESC cultures may be a perfectly defined culture of hESCs with no differentiation at all, but this is difficult to generate in sufficient quantities to allow the use of the same standard sample across

time. Although there is no perfect cellular control for the validation of hESC cultures, there are cells that may be used for this purpose because they approximate the expression patterns of hESCs, but are more stable in culture. We recommend two cell lines available from American Type Culture Collection (ATCC): the germ cell tumor-derived cell line NTERA-2 cl.D1, and the aneuploid hESC line BG01v. RNA from either of these cell lines can be used as a standard for the comparison of gene expression profiles by qPCR or by microarray. The BG01v cells are useful for immunohistochemistry, since they express the key surface epitopes of undifferentiated euploid hESCs and also are capable of differentiation to the three germ cell lineages. In addition to acting as standards within a laboratory, these cells are very useful in the development of many of the assays and protocols described in this manual and may also be useful for the initial development of new protocols.

Microbial contamination

Mycoplasma testing

Mycoplasma is a common contaminant in mammalian cell culture, and can be very insidious. Cultures can be contaminated with mycoplasma from the reagents or from the technician, and once contaminated, low levels may persist undetected.

Fungal and bacterial sterility testing

This is usually done by classical culture techniques that use fluid thioglycollate medium (Sigma catalog no. 90404) for detection of aerobic and anaerobic bacteria and soya bean/casein broth (Sigma catalog no. S1674) for detection of aerobes, facultative anaerobes and fungi. Samples are inoculated into the broths in duplicate, with one being cultured at 22°C and the other at 32°C for 14 days, after which they are examined for turbidity.

Recovery of normal populations

While it is possible to recover cells that have become contaminated with microorganisms, the treatments are often harsh, and we strongly recommend going back to an uncontaminated stock if possible. If there has been a significant amount of work invested in the generation of a clone or subline, for example, then the following methods might be attempted; however, once the cells are free of contamination, they should be QC tested to ensure that the relevant properties have been retained.

When using multiwell plates, contamination is generally restricted to only one or a few wells, unless the contamination results from a severe compromise of the medium used to feed all the wells. One option, therefore, is simply to aspirate the contaminated well(s), fill the now-empty well(s) with full-strength bleach, aspirate the bleach, wash the well(s) with 70% ethanol, and aspirate the well(s) to dryness. The remaining uncontaminated well(s) are then likely to remain so. This consideration, by the way, highlights one main advantage of using multiple wells of multiwell plates rather than equivalent surface area in a single dish – the entire culture may not be lost in the former situation while it is much more likely to be lost in the latter.

It is also important to remember that, unlike bleach and ethanol, antibiotics do not kill microorganisms, they merely retard or stop their proliferation. In the intact animal this allows the immune system time to do its job of actually eradicating the invading microorganisms. In cell culture there is no immune system, so complete eradication of

contamination is very difficult and sometimes, at best, all one can hope for is to save the culture with continuous antibiotic supplementation, keeping the contamination at a very low level – it may not ever be possible to go back to an antibiotic-free medium. While this may be acceptable for research purposes, it is clearly unacceptable for clinical purposes, so in this latter case complete clearing of the contamination must be proven by an extended period of continuous antibiotic – free culture.

Mycoplasma eradication

Mycoplasma infection is a serious threat in hESC laboratories; if a mycoplasma infection is discovered, the best course is to destroy the cultures, then test all the other cultures in the lab. Mycoplasma can be eradicated by treatment with antibiotic cocktails such as BM-cyclin (Roche), MRA (ICN), or Plasmocin (Invivogen), which are all reported to be effective, although with some cytotoxic side effects; however, their successful use with hESCs has not been reported. This is an action of last resort. If possible, go back to frozen mycoplasma-free stocks because the influence of these antibiotics (much less the mycoplasma) on cell karyotype or genotype is not known.

Recovery from bacterial contamination

Antibiotics that target bacteria are routinely used in many cell culture situations, and some laboratories use them routinely in hESC culture. Bacterial contamination is usually first recognized by cloudiness in the medium. Contaminated cultures should be immediately isolated from other cultures, remembering that bacteria cannot jump from culture to culture, but rather are spread by spills and aerosols. It is recommended that the cells be handled in separate incubators, and kept in a disposable outer container such as a plastic box. Media and reagents for these cells should be entirely separate from those used with other cells; whenever the contaminated cells are handled, the BSC should be swabbed thoroughly with 70% ethanol before other cells are handled there. We recommend a regimen in which these cells are manipulated last thing at night and, after swabbing the work surfaces with 70% ethanol, the UV lights are left on overnight in the BSC.

1. Working as carefully as possible to prevent aerosols, wash away as much of the bacteria as possible.
2. Aspirate the medium, and replace with (Ca/Mg-free) D-PBS. Aspirate and replace the Ca/Mg-free D-PBS six times. This must be done gently as the Ca/Mg-free buffer loosens the attachment of the hESC colonies to the substrate.
3. Aspirate and replace with hESC medium containing penicillin (100 U/mL) and streptomycin (100 μg/mL). Aspirate and replace with hESC medium containing penicillin (100 U/mL) and streptomycin (100 μg/mL) a second time.
4. If possible, passage the colonies by manual dissection to a fresh dish in order to reduce the burden of bacteria further.
5. Put cultures into the incubator and change the medium twice daily for 2 days and then daily until ready to passage.
6. Keep the cells in antibiotic-containing medium for at least two passages before attempting to wean them from the antibiotics, then monitor carefully by microscope, and, if bacteria reappear, repeat this entire process. It may be prudent to use sterile-filtered MEF-conditioned medium for these latter feeding steps.

Eradication of yeast and other fungi

Option 1

There are a number of antimycotics available, including amphotericin B ("Fungizone") and mycostatin ("Nystatin") (Invitrogen) but these drugs should only be used as a last resort. We are not aware of any groups having successfully recovered fully functional hESC cultures after yeast or fungus contamination and most laboratories simply destroy yeast- or fungus-contaminated cultures.

Contamination is usually recognized microscopically as chains of yeast or filaments of fungus in the culture. Contaminated cultures should be immediately isolated from other cultures, remembering that pre-sporulating fungi and yeast are spread by spills and aerosols. It is recommended that the cells be handled in separate incubators, and kept in a disposable outer container such as a plastic box. Media and reagents for these cells should be entirely separate from those used with other cells, and whenever they are handled, the BSC should be swabbed thoroughly with 70% before other cells are handled there. We, again, recommend a regimen in which these cells are manipulated only at the end of the day and, after swabbing the work surfaces with 70% ethanol, the UV lights are left on in the BSC overnight.

1. In the case of fungal contamination, first carefully aspirate the visible fungal colonies with a Pasteur pipette.
2. Then, working as carefully as possible to prevent aerosols, wash away as much of the fungus as possible. Aspirate the medium, and replace with (Ca/Mg-free) D-PBS. Aspirate and replace the Ca/Mg-free D-PBS six times. This must be done gently as the Ca/Mg-free buffer loosens the attachment of the hESC colonies to the substrate.
3. Aspirate and replace with hESC medium containing the commercial antimycotic. Aspirate and replace with hESC medium containing the antimycotic a second time. If possible, passage the colonies by manual dissection to a fresh dish in order to further reduce the burden of fungi.
4. It is possible to use 2–3 times the usual concentrations of antimycotics during this process but antibiotics must not be used simultaneously with the higher antimycotic concentrations as the toxicity of the antimycotics to the cultured cells is greatly increased in their presence.
5. Put cultures into the incubator and change the medium twice daily for 2 days and then daily until passage.
6. Keep the cells in antimycotic-containing medium for at least two passages before attempting to wean them from the antimycotics, then monitor carefully by microscope, and, if yeast or fungi reappear, repeat this entire process. It may be prudent to use sterile-filtered MEF-conditioned medium for these latter feeding steps.

Option 2

It is also possible, but very difficult, to recover cultures contaminated with fungus without the use of antimycotics. Visible fungal colonies are removed and the cultures are washed repeatedly as described above, first with PBS and then with medium to

remove the bulk of the contamination. The colonies are then passaged by microdissection under a high-powered dissecting microscope, taking care to remove pieces of colony without bringing along fungal cells. Those skilled with a pipette can even "clean off" fungal cells from pieces of colony. Individual pieces of colony are transferred to small culture wells (such as Nunc 4 well; SKU 167063). As mentioned above, splitting the colonies up into multiple wells allows for the possibility that separate uncontaminated and contaminated cultures may be generated.

Cultures are monitored daily for contamination. Using this method we have been able to recover about a quarter of the transferred colony pieces. A proportion will carry the fungi, however, and can be discarded, or treated with the antimycotic agent as described above in option 1.

Recovery of karyotypically normal cells

hESC populations are prone to accumulation of karyotypic abnormalities over time. This does not happen spontaneously for all the cells of a given culture, but, presumably, by the appearance of cells with abnormal karyotype which have a growth advantage. The appearance of karyotypic abnormalities has been hypothesized to be associated with the method of passaging; passage by microdissection appears to be less prone to abnormalities than enzymatic passage. The appearance of karyotypic abnormalities is not sudden, but rather there is accumulation of abnormal cells and, by the time the abnormality is recognized, abnormal cells will represent a significant proportion of the culture. A carefully constructed tiered cell bank, as described above, can reduce the impact of aneuploid cells by allowing the researcher, with relatively little cost, to go back to a validated cell population with normal karyotype and with known differentiation capacity.

Sometimes, however, the amount of effort that has been invested in the generation and characterization of a population of cells makes it cost effective to clone out normal cells from the population.

This process is not very efficient, however, and with normal culture conditions a plating efficiency of less than 1% is expected. Cloning is generally done by FACS sorting directly into 96-well trays and it may be made more efficient using medium supplemented with neurotrophins, which are reported to allow cloning of cells with 15% efficiency. In this way, it is quite feasible to clone and expand 10–50 clones and to identify those with normal karyotype.

Reading List

General

European Collection of Cell Cultures (2001). *The Fundamentals of Cell Culture – A Laboratory Handbook*. http://www.ecacc.org.uk/default.asp?itemid=29673

Overview of general cell culture methods

Freshney R Ian (1999). *Freshney's Culture of Animal Cells: A Multimedia Guide*. New York: John Wiley & Sons.

General cell culture methods and laboratory set-up

Centers for Disease Control and Prevention and National Institutes of Health (1999). *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 4th edn. http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm

Safe handling of human cells and tissues

Protocols and manuals published on the internet:
ATCC stem cell methods:
http://www.atcc.org/common/catalog/cellBiology/stemcells/technicalInfo/protocols.cfm
BresaGen hESC methods:
http://stemcells.nih.gov/research/registry/
ESI manual and other Singapore protocols:
http://www.stemcell.edu.sg/resources/methodsProtocols.php
Geron hESC methods:
http://www.geron.com/showpage.asp?code=prodstprot
Melton Laboratory hESC methods:
http://mcb.harvard.edu/melton/HuES/
WiCell hESC protocols:
http://www.wicell.org/forresearchers/index.jsp?catid=12&subcatid=20

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PART

VII

Stem Cells and Society

CHAPTER

27

Intellectual Property: Owning the Stem Cell

Cathryn Campbell and Jeanne F. Loring

Introduction

On August 9, 2001, US President George W Bush, citing ethical and moral issues, announced that Federal funding for human embryonic stem cell (hESC) research would be limited to the small number of hESC lines then in existence (NIH Human Embryonic Stem Cell Registry). President Bush confirmed this position by vetoing the "Stem Cell Research Enhancement Act of 2005," (HR 810) which would have expanded Federal research funding to hESC lines regardless of when they were derived. This restriction on government funding forced individual states and private foundations to support hESC research on the much larger number of hESC lines derived after the President's announcement. However, while these measures compensate for the lack of Federal funding for research, a more daunting barrier to innovations exists within the stem cell field: the intellectual property rights for hESCs and their uses. The foundational concepts for deriving, maintaining, and using stem cells, including the stem cells *per se*, are the subject of a handful of key patents, giving the "owners of the stem cell" significant control over innovations in this field.

The US Patent System

Abraham Lincoln declared that "[t]he patent system added the fuel of interest to the fire of genius." Himself a patent holder, Lincoln called the introduction of patent

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laws one of the three most important developments in the world's history, along with the discovery of America and the perfection of printing. A patent is, in effect, a limited property right that the government offers to inventors in exchange for their agreement to share the details of their inventions with the public. Like any other property right, it may be sold, licensed, mortgaged, assigned, or simply given away. Although many object to anyone having a monopoly on an idea or invention, such monopoly rights have always been a fundamental part of the patent system. The importance of granting monopolies for new inventions has been recognized in the United States since the adoption of the US Constitution, which states: "Congress shall have power . . . to promote the progress of science and useful arts, by securing for limited times to authors and inventors the exclusive right to their respective writings and discoveries" (US Constitution, Article I, section 8, clause 8).

Congress used this Constitutional power to enact the Patent Act (35 US Code), which established the United States Patent and Trademark Office (USPTO). To obtain protection under US law, the applicant must submit a patent application to the USPTO, where it will be reviewed by an examiner to determine if the invention is patentable. There are three types of patents issued by the USPTO. The most common type of patent is a utility patent, which has a duration of 20 years from the date of filing but is not enforceable until the day of issuance. The other types of patents include design patents, which protect ornamental designs, and plant patents, which protect new varieties of asexually reproducing plants.

The patent system fuels interest by incentivizing innovation while concomitantly encouraging the exchange of ideas. The protection of intellectual property provides incentives for costly research and development (R&D), since corporations would be much more conservative with R&D investments if third parties were free to exploit any developments. However, these incentives come at a cost. The *quid pro quo* for receiving a government-sanctioned and government-enforced monopoly is a duty to put the public in possession of the invention. If inventors did not have the legal protection of patents, they may choose to keep their inventions secret, whereas awarding patents makes the details of new technology publicly available for further improvement by other inventors during the life of the patent or for exploitation after patent protection ends.

What Can be Patented?

According to the patent statute, any person who "invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvements thereof, may obtain a patent," subject to the conditions and requirements of the law (35 USC § 101). Thus, laws and products of nature are not patentable unless they are in a form not found in nature. For example, a nucleic acid that exists naturally in a cell is not patentable; however an "isolated" nucleic acid can be patented.

The patentability of a product of nature, such as a living organism, that is in a form not normally found in nature was explored in the United States Supreme Court case Diamond v. Chakrabarty (*Diamond* 1980) which was dealing with whether genetically

modified microorganisms can be patented. Ananda Mohan Chakrabarty, while working for General Electric, had developed a bacterium derived from the *Pseudomonas* genus that was capable of breaking down crude oil, which he proposed could be used in treating oil spills. He requested a patent for the bacterium in the United States but was turned down by a patent examiner who believed that living things were not patentable. The Patent Office Board of Appeals agreed with the original decision; however, the Court of Customs and Patent Appeals (CCPA) overturned the case in Chakrabarty's favor, writing that "the fact that micro-organisms are alive is without legal significance for purposes of the patent law." Sidney A Diamond, Commissioner of Patents and Trademarks, appealed to the Supreme Court. In a 5–4 ruling, the court ruled in favor of Chakrabarty, and upheld the patent, concluding that Congress intended statutory subject matter to "include anything under the sun that is made by man."

Thus, in terms of biotechnology, patentable subject matter generally includes (1) compositions, e.g. drugs, proteins, antibodies, medical devices, and cell lines; (2) methods of making the compositions, e.g. synthesis, isolation, screening, and purifying a composition; and (3) methods of using the compositions, e.g. diagnostic, therapeutic, and prognostic uses. Thus, for stem cells, patents can be directed to methods of making stem cells, methods of using stem cells, and, most significantly, to the stem cell *per se*. Examples of such patents and the impact they are having on the field are discussed below.

What are the Requirements for a Patent?

Any process, machine, manufactured article, composition of matter, or improvement of the same may meet the legal definition for "invention" and qualify for patenting if it is (a) new, (b) useful, and (c) non-obvious. The novelty requirement states that an invention cannot be patented if certain public disclosures of the invention have been made. The statute that explains when a public disclosure has been made (35 USC § 102) is complicated and often requires a detailed analysis of the facts and the law. The most important rule, however, is that an invention will not normally be patentable if the invention was known to the public before it was "invented" by the individual seeking patent protection; the invention was described in a publication more than one year prior to the filing date; or the invention was used publicly, or offered for sale to the public more than one year prior to the filing date. Although the United States grants the one year grace period described in the last two rules above, most other countries do not grant such a period. Therefore, it is almost always preferable to file a patent application before any public disclosure of the invention.

The specification must also contain an assertion of a specific, substantial and credible utility for the invention (*Federal Register* Vol 66, 1092–1099). The patent need not be limited to this asserted use, but at least one use that is credible must be provided. This requirement excludes "throw-away," "insubstantial," or "non-specific" utilities, such as the use of a complex invention as landfill. This also excludes incredible assertions such as for cold fusion or a perpetual motion machine. If an invention is not exactly the same as prior products or processes (which are referred to as the "prior art"), then it is considered novel. However, in order for an invention to be

patentable, it must also be a non-obvious improvement over the prior art according "to one of ordinary skill in the art." As can be imagined, the determination of whether a particular change or improvement is "obvious" is one of the most difficult determinations in patent law. In order to make such a determination, an examiner in the patent office will normally review the prior art to find those publications that are closest to the invention in which a patent is sought. If no single publication contains all of the features, the examiner will attempt to find all of the features in a combination of two or more prior publications. If there is a motivation to combine these references in order to arrive at the claimed invention and an indication in the prior art that the invention would have had a reasonable likelihood of success, then the invention would be obvious and unpatentable.

Other important legal requirements for patenting, all of which are part of the *quid pro quo* that serves to protect the public's interest in understanding and applying the knowledge embedded in the patent, are that the patent must (a) be explicit and detailed enough to enable the person with "ordinary skill in the art" to reproduce the invention without undue experimentation (35 USC § 112, paragraph 1); (b) present the best configuration and use of the technology known to the inventors (35 USC § 112, paragraph 3); and (c) conclude with claims that are precise, clear, correct, and unambiguous (35 USC § 112, paragraph 2). Patent claims define the metes and bounds of the patentees property right, much like a deed to a piece of land, but in a specific legal style that sets out the essential features of the invention in a manner to clearly define what will infringe the patent.

What If There is Already a Patent?

The purpose of a patent is, after all, to exclude others from making, using, or selling the invention. However, the rights given to the patentee do not include the right to make, use, or sell the invention themselves. It is necessary to determine the "freedom to operate" for a new invention, because the patentee may have to obtain a license from another patent holder and/or comply with other laws and regulations to make use of the claimed invention. A perfect example of this necessity exists for stem cells. A researcher can patent a novel therapeutic use for hESCs; however the stem cells themselves are the subject of several patents (see below), which could also be true for the other reagents and materials used in the method. In addition, there may be other method patents with generic claims that dominate those of the researcher. It would therefore be necessary to get some form of rights to the invention from the patent holder in order to make, use, or sell the invention. These rights usually come in the form of either assignment (i.e. sale or transfer) or license of the patent.

A patent license can take many forms but the most common types are exclusive and non-exclusive licenses. A non-exclusive license means that multiple parties may concurrently license the technology, even if they are competing with each other in the same industry and territory. As a general rule, a non-exclusive licensee cannot sue a non-licensed entity for patent infringement. The non-exclusive licensee must demand that the patent owner take steps to enforce the patent rights. In contrast, an exclusive licensee can sue for patent infringement, because the license affords sole rights to the invention within the confines of the license. However, an exclusive license can be

conditional or limited, such as to a particular industry or geographic territory. It is therefore common for a company to obtain exclusive rights to a particular use of an invention, but not to others. As an example, Geron Corp. received a license from the Wisconsin Alumni Research Foundation (WARF) that grants Geron exclusive commercialization rights to three hESC derivatives (cardiomyocytes, neural cells, and pancreatic islet cells) and non-exclusive rights to three other cell types (hematopoietic cells, osteoblasts, and chondrocytes) for therapeutic and diagnostic products. Thus, while they are allowed to produce cardiomyocytes, neural cells, pancreatic islet cells, hematopoietic cells, osteoblasts, and chondrocytes from hESCs, they are only allowed to sue others for patent infringement based on cardiomyocytes, neural cells, and pancreatic islet cells.

What is the Status of Stem Cell Patents for ESCs?

On December 1, 1998, the US Patent and Trademark Office (PTO) issued a broad patent claiming primate (including human) ESCs, entitled "Primate Embryonic Stem Cells" (Patent 5,843,780). On March 13, 2001, a second patent (6,200,806), with the same title but focused on hESCs, issued from a "divisional application." Finally, on April 18, 2006, the PTO issued a third "continuation" patent application to Thomson under the same title (7,029,913). These three patents have considerable consequence for hESC research in the United States, because they have claims to the cells themselves, not just a method of deriving them. The claims give the patent owner, WARF, the legal right to exclude everyone else in the United States from making, using, selling, offering for sale, or importing any hESCs covered by the claims until 2015. The broadest claim of both the 1998 and 2001 patents is claim 1, which claims the "composition of matter" of primate ESCs. The 1998 patent reads as follows:

> We claim: 1. A purified preparation of primate embryonic stem cells which (i) is capable of proliferation in an *in vitro* culture for over one year, (ii) maintains a karyotype in which all the chromosomes characteristic of the primate species are present and not noticeably altered through prolonged culture, (iii) maintains the potential to differentiate into derivatives of endoderm, mesoderm and ectoderm tissues throughout the culture, and (iv) will not differentiate when cultured on a fibroblast feeder layer.

Composition of matter claims are generally more powerful than method claims because they cover the matter itself, regardless of how it is made or used. As the characteristics listed in claim 1 in the 1998 patent describe the essence of primate ESCs the patent effectively covers all primate ESC lines regardless of who makes them or how they are generated (see Loring and Campbell, 2006, for a review of how hESCs became patented). While many new stem cell creation methods, such as those not involving the isolation of the inner cell mass from a human blastocyst (e.g. using blastomere biopsy or cellular fusion) may not be covered by the WARF process claims, the composition claims are much more difficult to avoid. This is partly because the scope of the composition claims is somewhat ambiguous, because the Thomson patents do not define the term "embryonic stem cell." In the absence of a clear definition in the specification, this term should be given its ordinary and customary meaning

according to one of ordinary skill in the art at the time the application was filed (Phillips, 2005). A definition that is consistent with the prosecution history of the Thomson patents and expounded by the National Institutes of Health (NIH Report) is that an embryonic stem cell is defined by its origin, i.e. is derived from the blastocyst stage of the embryo. Thomson filed comments disputing a request for interference in Application No. 09/982,637 partially on the basis that the contestant's cells were not derived from the inner cell mass of blastocysts. While the Thomson patents were the first to successfully claim a hESC, i.e. derived from a human blastocyst, there are alternative sources of pluripotent stem cells that have therapeutic potential. One such alternative, the embryonic germ cell, is discussed below.

What is the Status of Stem Cell Patents for Embryonic Germ Cells?

Embryonic germ cells (EGCs) are pluripotent stem cells derived from primordial germ cells. As they are not derived from the inner cell mass of a blastocyst, they are not covered by the Thomson patents. Instead, the USPTO issued claims to human EGCs and their uses to John Gearhart (US Patent Nos. 6,090,622, 6,245,566, 6,331,406, and 6,562,619). The broadest claim is claim 1 of the '622 patent, which claims the "composition of matter" of human EGCs. The '622 patent reads as follows:

> We claim: 1. Human pluripotent embryonic germ cells, wherein the cells exhibit the following culture characteristics during maintenance:
> (a) dependence on a ligand which binds to a receptor which can heterodimerize with glycoprotein 130 (gp 130); and
> (b) dependence on a growth factor.

As Dr Gearhart's research was supported by Geron, these patents, like those for the Thomson hESCs (at least for three cell types), were exclusively licensed to Geron. Companies looking to pursue alternatives to hESCs would still have had to acquire a license from Geron. However, there is an earlier patent on embryonic germ cells. Brigid Hogan was the first to determine that an ESC line could be generated from primordial germ cells of an embryo using fibroblast growth factor (FGF), leukemia inhibitory factor (LIF), and steel factor (Labosky *et al.*, 1994). In 1997 the USPTO issued a patent application directed to an "isolated human PGC-derived pluripotential stem cell," i.e. a human EGC. Dr Hogan's patents have been licensed exclusively to Amphioxus Cell Technologies, Inc., a wholly owned subsidiary of Stem Cell Innovations (SCI). Thus, SCI presents an alternative source of EGCs. As interest in EGCs grows, this potential conflict may have important consequences for stem cell researchers, funding agencies, and companies.

What is the Status of Stem Cell Patents for ESCs in Europe?

While the USPTO has, to date, granted at least 46 patents that claim hESCs or their uses, the European Patent Office (EPO) has not granted a single patent on hESCs.

This is because rule 23d(c) of the European Patent Convention (EPC), which has its origins in the European Commission's directive of Article 6.2(c) against the use of human embryos for industrial or commercial use, prohibits the patenting of the "human embryo" on moral grounds. While the EPO is issuing patents on human adult stem cells and to non-human ESCs, since neither of these involves the destruction of a human embryo, there are many applicants that have been awaiting examination, hoping that the EPO's stance on hESCs changes. This stance is the basis for appeal for two cases involving hESC rulings, the University of Edinburgh (European Patent No. EP 0695351) and WARF (European Patent Application No. 96903521.1), wherein the EPO interpreted Rule 23d(c) EPC to exclude not only patents that destroy human embryos (i.e. as part of the process of extracting stem cells from a human blastocyst), but also patents relying on already established hESC lines as their starting point (Laurie, 2004).

While this stance has not been adopted by all countries, no other country has allowed hESCs to be patented as broadly as in the US. Therefore, it remains to be seen what impact this will have on stem cell research and commercialization. This is in part because, even though the US patent rights can only be enforced within the United States, hESCs made in another country become subject to US patent law if they are imported into the United States.

What Effect are Stem Cell Patents Having on Innovation?

Although many patent holders choose to license others to practice the patented invention in exchange for royalties, in the United States, licensing is not compulsory; patent holders can choose to license on their own terms or not to license at all. Because WARF controls the rights to hESCs, researchers who wish to use these cells must be aware of their obligations to the patent owners under US law. The NIH took steps to engage WARF's cooperation, signing a memorandum of understanding (MOU) with WARF. The NIH retains rights to the 1998 ('780) patent, because the work was supported by Federal grants (NIH NCRR Grant No. RR00167). This MOU gave researchers employed by the NIH, the Food and Drug Administration, and the Centers for Disease Control and Prevention a license to use hESCs for research. Also, WARF agreed that it would not impose more restrictive terms for any other not-for-profit institutions.

In early 2002, the NIH made similar MOU agreements with other groups that had made lines that were eligible for funding, including the University of California at San Francisco, Mizmedi (Korea), BresaGen (Australia), Technion (Israel), Cellartis (Sweden), and ES Cell International (Singapore). These institutions received Infrastructure grants from the NIH of about $200 000–500 000 a year to facilitate the distribution of their own hESC lines under a license from WARF, and their prices were limited by the MOU.

As of publication, WARF requires a license agreement for distribution of any hESC lines in the United States, whether or not they are on the NIH registry. The Harvard hESC material transfer agreement (MTA), for example, requires that the recipient of

their cell lines acknowledges WARF's patent rights. Only the institutions that have MOUs with the NIH have price regulations; other suppliers of hESCs can charge as much or as little as they wish for the cells. For example, Harvard charges nothing for its lines. However, because the WARF patents are only valid in the United States, non-US-based hESC researchers do not need a license unless they import the cells into the United States.

As a result of an NIH contract to serve as the main distribution center for hESCs in the United States, WARF reduced the price of cells to $500 for academic investigators. Although the academic price is now less onerous, the situation for commercial biotechnology and pharmaceutical companies remains difficult. Because Geron holds an exclusive license for broad therapeutic use in the United States of hESC-derived cardiac, nervous system, and pancreatic cells, if a company wishes to develop therapies in these areas, they must negotiate with Geron for fees and royalties.

But what if a company simply wants to use the ESCs for basic research? Even if the company's research is non-commercial, WARF still requires a commercial license, which costs an upfront fee (typically $125 000), with $40 000 annual maintenance fees to retain the license. This fee gives commercial entities the same research freedom as academic researchers, and, with negotiated royalty payments, they may commercialize reagents for research.

The research license cost has complicated the situation for start-up biotechnology companies that want to obtain NIH funding for hESC research. Small companies may find themselves in what we call the "SBIR paradox." The NIH is willing to fund hESC research through its Small Business Innovative Research (SBIR) program, but the company is not allowed to use NIH money, usually $100 000 for a phase 1 SBIR (R43), to pay WARF for a commercial research license. Therefore, the company must come up with separate funding of perhaps $125 000 for a license to do the NIH-funded research with the cells. As a result of discussions with the NIH, WARF has offered to take equity instead of cash for a license for some biotechnology companies.

Thus, debates rage on about the ethics of using US tax dollars to fund stem cell research while states and private foundations are attempting to compensate for this lost funding. However, companies on the verge of innovations are going to be less concerned with whether the funding comes from the government or not than they will be about who "owns the stem cell" when they market a therapy or a new diagnostic test.

Reading List

Loring JF, Campbell C (2006). Science and law. Intellectual property and human embryonic stem cell research. *Science* 311: 1716–1717.
This Policy Forum article is written for scientists to explain the process by which hESCs became patented.

US Patent Office website: www.uspto.gov
Searches of issued patents and patent applications: www.uspto.gov/patft/index.html (use keywords "embryonic stem cells" and "human").

Official correspondence and documents (called File Wrappers): http://portal.uspto.gov/external/portal/pair

Public Patent Foundation: www.pubpat.org
This is a non-profit organization that investigates the effects of issued patents on the public and files requests for re-examination for targeted patents.

Stem Cell Community: www.stemcellcommunity.org
This is a non-profit website that posts information about hESCs, including patents and links to other stem cell sites.

Other references cited in the text

Diamond v. Chakrabarty, 447 U.S. 303, 1980.

Federal Register Vol 66 1092–1099 January 5, 2001. The training materials can be found on the Internet at http://www.uspto.gov/web/menu/utility.pdf

Harvard hESC material transfer agreement (MTA): www.mcb.harvard.edu/melton/hues/HUES_request.html

HR 810: The Stem Cell Research Enhancement Act of 2005. The NIH has a tracking site for information about health-related legislation: http://olpa.od.nih.gov/tracking/109/house_bills/

Labosky PA, Barlow DP, Hogan BLM (1994). Mouse embryonic germ (EG) cell lines: their transmission through the germline and differences in the methylation imprint of insulin-like growth factor 2 receptor (Igf2r) gene compared with embryonic stem (ES) cells. *Development* 120: 3197–3204.

Laurie G (2004). *Eur Intellectual Property Rev* 26: 59–66.

NIH Grant Records: http://grants1.nih.gov/grants/award/state/state.htm

NIH Human Embryonic Stem Cell Registry: http://stemcells.nih.gov/research/registry

NIH NCRR Grant No. RR00167; the patent states: "This invention was made with United States government support awarded by NIH NCRR Grant No. RR00167. The United States government has certain rights in this invention." The principal investigator on this grant was John P Hearn, the Director of the NIH Regional Primate Research Center.

NIH Report: Stem cells: scientific progress and future research directions. http://stemcells.nih.gov/info/scireport/chapter2.asp

Phillips v. AWH Corp., 415 F.3d 1303, 75 U.S.P.Q. 2d 1321 Fed. Cir. 2005.

US Code: www.gpoaccess.gov/uscode/
The United States Code is the codification by subject matter of the general and permanent laws of the United States.

CHAPTER

28

Ethical Concerns for Stem Cell Research

Philip H. Schwartz and
Michael W. Kalichman

INTRODUCTION

In this chapter we attempt to categorize ethical concerns for various areas of stem cell research. The research areas are each assigned to one of three categories of ethical concern: low, moderate, and high.

These categories are not intended to encompass all possible ethical issues, but instead to focus on those new issues that arise from the derivation or experimental use of stem cells in research. The lowest level of concern is reserved for those research projects that should normally require little or no ethical review beyond that already required by institutional boards responsible for oversight of animal and human research. While these projects should still be monitored, it is expected that review could be expedited by Embryonic Stem Cell Research Oversight (ESCRO) Committees. The intermediate, or moderate, level of concern is reserved for those research projects that raise significant ethical concerns and will normally require review beyond that already required by other review committees. These projects will require the closest attention of ESCRO Committees. The highest level of concern is reserved for those research projects that should not be approvable now or in the foreseeable future. These projects will normally be prohibited by ESCRO Committees.

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Although the subject of this discussion is ethics, it is not the intent of this chapter to present various viewpoints of the ethical concerns involved, which have been discussed in many other forums. Differences in starting assumptions will result in different views of what is and is not ethical. Ethical choices are rarely black and white, but instead depend on how we choose to define our terms and on our choices of ethical principles. For example, for those who view the fertilized egg as equivalent to a human life, it would be unethical (or immoral) to destroy human embryos for the purpose of research. Conversely, for those who view the fertilized egg as a necessary, but insufficient, criterion for a new human life, it would be unethical (or immoral) to prohibit research that has tremendous promise to improve the lives of those who have been born. Rather than attempt to resolve or weigh the many possible, and often competing, ethical criteria, the purpose of this review is only to identify those types of research that are sufficiently worrisome as to warrant greater consideration by ESCRO Committees.

Overview

For each of the following levels of ethical concern, two types of experiments are discussed. The first is those that involve the derivation or source of stem cells and the second is the proposed experimental uses of those cells. Animal research has also been included in this discussion to parallel the human studies and because several of the categories include the mixing of human and animal cells. Over and above that consideration, however, is the mandate to treat experimental animals humanely. To illustrate the different levels of concern, examples of research activity in the scientific literature have been included when available.

Levels of Concern About Stem Cell Research

Low level of concern

This first category includes stem cell research with little or no ethical concerns beyond basic protections of human subjects (covered by Institutional Review Board (IRB) review) or animal subjects (covered by Institutional Animal Care and Use Committee (IACUC) review).

Derivation of stem cells

Stem cells harvested from animals
This area covers the production of animal stem cells by the researcher, operating under an IACUC-approved protocol. It includes the harvest of animal eggs and sperm, the production of fertilized animal eggs and blastocysts, and the harvest of stem cells from animal blastocysts or fetuses and post-natal animals, from any tissue of the animals' body.

Stem cells harvested from living human donors
This area refers to the harvest of stem cells from human donors and includes bone marrow, mobilized peripheral blood, umbilical cord blood, and adipose tissue. The donor is required to consent to the donation.

Stem cells harvested from human cadavers
In this area the next-of-kin consents to the donation of tissues for stem cell harvest, which may be from any organ system, including brain. Literature examples include the harvest of neural stem cells from human cadaveric brain or hematopoietic stem cells from human cadaveric bone marrow, among others.

Uses of stem cells

Use of existing animal stem cells
This area covers the *in vitro* use of animal stem cells that have been created by others under an IACUC-approved protocol.

Use of existing non-ESC/fetal human stem cells
This area covers the *in vitro* use of human stem cells, other than embryonic or fetal stem cells, that have been created by others under an IRB-approved protocol. Examples include mesenchymal stem cells, neural stem cells, and multipotent adult progenitor cells.

Production of animal stem cell-into-animal chimeras
This refers to the implantation of animal stem cells into any animal developmental stage, blastocyst through adult, such that an animal is produced that is comprised of its own cells plus those derived from the implanted stem cells. The resulting animal is referred to (loosely) as a chimera because it is comprised of the cells of more than one animal. In Greek mythology, a chimera was a creature with a lion's head, the tail of a serpent or dragon, and the body of a goat.

This technique, wherein genetically modified embryonic stem cells (ESCs) are injected into a blastocyst, comprises the initial stage for creating a new strain of animal that possesses the genetic modification introduced into the stem cell. A requirement for the success of this procedure is that one or more of the genetically modified stem cells becomes one or more germ cells ("goes germline") in the resulting animal.

Breeding of animal stem cell-into-animal chimeras
In this area is included the concept of breeding the chimeras that were created in the technique described immediately above. This represents one of the final stages in the creating of a new genetically modified strain of animal. That is, the modified stem cell, becoming a germ cell, can now develop into an animal in which all of the animal's cells possess the genetic modification. Additional cross-breeding is necessary to produce a homozygote of the modification.

Somatic cell nuclear transfer (SCNT) for the production of an animal
This area includes the production of an animal by the technique of somatic cell nuclear transfer or parthenogenesis. Although the purpose of these procedures is not to derive or use stem cell lines *per se*, the initial steps are identical and therefore warrant inclusion on a list of possible uses. The resulting animals may be used primarily for agricultural or biomedical purposes although recent applications include the reproduction of pets. This area also includes the production of an animal fetus, or post-natal animal, by the technique of somatic cell nuclear transfer or parthenogenesis, specifically for the harvest of organs.

Moderate level of ethical concern

The uses of stem cells listed under this area comprise areas that have ethically diverse concerns. Some may engender concern over issues of social justice, while others may involve the use of tissue taken from human abortuses or the destruction of human embryos. Although both of the latter are legal in this country, they both are highly controversial and divisive areas and, thus, they are put into the present category. In any event, all the research areas included in the present category have given rise to vigorous ethical debate. And since there are staunch proponents and opponents of each area they cannot easily be recategorized into a higher or lower ethical stratum.

Derivation of stem cells

Creation of human embryonic stem cells (hESCs) without embryo destruction
In this technique, which has only been shown to be successful with animal embryos, a similar technique used to extract blastomeres for preimplantation genetic diagnosis (PGD) is used, except that the removed blastomeres are used to create a hESC line. As with PGD, the embryo is not destroyed. This technique has not yet been devised for use with human embryos so an additional ethical concern is the use/destruction of human embryos while perfecting the technique.

Stem cells harvested from human abortuses
Although harvest of human fetal tissue for therapeutic purposes has been practiced in many countries for over a decade, in the US a substantial minority are opposed to abortion, much less the harvest of tissues from abortuses.

Production of hESCs from **in vitro** *fertilization (IVF) supernumerary blastocysts*
Currently, the only hESC lines that researchers can work with using Federal dollars are those that have been created from supernumerary blastocysts, blastocysts that were in excess of those needed for the reproductive purposes for which they were created and were donated for hESC production. When these lines are created, by necessity it requires the destruction of the embryo. As a minimum, stem cell lines are considered "ethically derived" only if the embryo donation meets the following criteria: (1) the embryos were supernumerary; (2) the written consents of the male and female gamete donors were obtained; and (3) no financial inducements were offered for the donation. The original lines created by the Thomson, Stice, and other laboratories and the more recent lines created by Melton were all made from supernumerary blastocysts obtained in this way.

Interestingly, the Stice group used embryos that had been graded as non-viable, a consideration recently elaborated by Schwartz and Rae, suggesting that different ethical considerations may pertain to embryos of different qualities. Alikani and Willadsen and Landry and Zucker have suggested that it may be possible to identify embryos that are not viable or "dead," that is, they are not viable for normal, further development, and that to use these embryos lowered the ethical concern associated with stem cell harvest.

Blastocysts created for research
hESCs could be derived from blastocysts created for research in at least three ways. These include blastocysts created through IVF or SCNT, altered blastocysts created by alternative nuclear transfer (ANT), and parthogenetic blastocysts.

- *IVF or SCNT*. In this area are included embryos that were not created for reproductive purposes but, instead, were created specifically for research or therapeutic purposes. In the case of IVF, both male and female gametes would have to be donated specifically for this purpose (the egg donations are considered below). In the case of SCNT, the requirement is for egg donation and a source of somatic cells, whether it is from the proposed stem cell recipient in the case of therapeutic cloning or for the production of an hESC line with particular genetic characteristics (e.g. a genetic defect). In the latter case, the lines would be used for the study of the genetic defect, while the former case would presumably provide an immune match for a patient to be treated. In both cases, the created embryo would have to be destroyed to harvest the hESCs.
- *"Altered" blastocysts*. In this area are included hESCs produced from embryos that have been genetically modified to preclude their full human developmental potential. As originally proposed by Hurlbut, an altered blastocyst is created by SCNT, but only after the genetic material from the somatic cell donor has been altered such that stem cells could be derived, but the blastocyst would not have the genetic and/or epigenetic features necessary for a trajectory of continued embryonic development. It is not yet known whether the genetic defects would preclude the use of the stem cells for the purposes for which they were intended; however, this approach has been successful in a mouse model.
- *Parthenogenetic blastocysts*. These embryos, exceedingly rare in mammalian species, arise from duplication of the single haploid set of chromosomes present in the unfertilized egg. Although a technique for doing this has not yet been published for human cells, it has for animal cells. hESCs produced in this manner would be immunologically competent with the egg donor.

Donation of human eggs for research

Although egg donation is only a first step for several different approaches to deriving hESCs, it is included here because it is a necessary and challenging first step. Since egg donation is a clinical/surgical procedure that involves the administration of powerful hormones, it is not without risk to the donor. Moreover, since, at present, the success rate for the production of an hESC line from a blastocysts is less (and sometimes much less) than 100%, more eggs are required than will produce a given number of hESC lines. The same is not only likely to be true for SCNT but the success rate is likely to be much lower than for IVF, requiring even more eggs. Issues arise, therefore, over how egg donors will be recruited, whether or not they will be paid, and even whether or not enough egg donors can be recruited. It should be noted that recruitment and payment of egg donors for reproductive purposes has been going on for several years. This has usually taken the form of recruitment of young, physically attractive, intelligent women on college campuses to be egg donors for anonymous recipients. Payments have been in excess of $10 000 per cycle.

Uses of stem cells

Production of human stem cell-into-animal chimeras

In this case human stem cells are injected into an animal at the fetal stage or older. This method is used primarily for proof-of-principle for establishing certain properties of the human stem cells (i.e. differentiation potential, engraftment potential, or their potential therapeutic efficacy for specific diseases or injuries). There are many

examples in the literature of the production of this type of chimera for experimental purposes. These include the production of a human hematopoietic system after bone marrow transplantation in the fetal sheep and the investigation of the neurogenic potential of human neural stem cells transplanted into the fetal mouse brain. A concern that has been raised about the production of this type of chimera is one regarding the possible induction of "human qualities" in the recipient animal. The two primary concerns are higher level neurological function and the appearance of recognizable human features. For most, the more worrisome of these two possibilities is the development of human-like intelligence, consciousness, or emotion. Fortunately, this is highly unlikely. Many of the critical developmental signals for the formation of the human brain will have already occurred or signaling necessary for a developing human nervous system will be absent in the developing animal nervous system. Introduction of human stem cells at the blastocyst or very early embryonic stage, however, may give entirely different results; therefore, this type of transplantation is considered of high ethical concern and is dealt with below.

Use of existing ethically derived hESCs
Use of hESCs that are ethically derived, as described above, is currently permissible, even with Federal funds, if the cells were derived before August 9, 2001.

Production of animal stem cell-into-human chimeras
This area includes the xenobiotic transplantation of animal stem cells into humans. Although this has not yet been done, or even proposed to our knowledge, xenobiotic organ transplantation has. It is not unreasonable to imagine that a clinically useful stem cell population might more easily be differentiated from animal rather than human source material.

Clinical uses of stem cells
The primary long-term goal for stem cell research is to develop strategies for preventions, treatments, and cures that can be used in humans. Both the necessary clinical trials and the resulting clinical applications raise issues that are distinct from the basic research now underway. The possible experimental approaches are extensive, but it is noteworthy that a variety of clinical applications are already widely, even if not universally, accepted: (1) transplantation of tissue-specific (e.g. bone marrow, cord blood, peripheral blood) stem cells to treat hematologic or metabolic diseases; (2) IVF for the purpose of reproduction; (3) donation of eggs for reproduction; and (4) PGD for reproductive screening.

High level of ethical concern

This area includes areas that are generally agreed by most, but not all, to have severe ethical challenges because of the nature of the source material or because the resulting "product" may have detrimental clinical consequences. It is noteworthy that at this point, this level of concern is generally applicable to uses of stem cells, not to their derivation.

Uses of stem cells

Culture of human embryos past 14 days
One of the overriding concerns for experimentation with human embryos, whether it has been for the purpose of examining different culture conditions or the effects of

various cytokines or deriving stem cells, has been the capacity of the embryo to "feel." At 14 days, the primitive streak, a thickening of the epiblast and precursor of the nervous system, appears. This has been taken as the earliest representation of the nervous system. It is generally agreed that embryos should not be cultured beyond this stage.

Breeding of human stem cell-into-animal chimeras
Although it is unlikely that a stem cell transplant performed after the embryonic (or more particularly, blastocyst) stage would result in germline transmission, the consequences of such an event would be so severe that a chance of it happening should be avoided altogether. That is, if a human cell in an animal gives rise to germline cells and the animal is allowed to breed, the possibility exists of producing a true human–animal hybrid.

SCNT for the production of a human fetus for organ harvest
In this area we include the possibility, alluded to above, of producing an embryo by SCNT, implanting that embryo, and then subsequently aborting the fetus for organ harvest for transplantation.

SCNT for the production of a living child
It is almost universally agreed that SCNT for the purpose of producing a living child should be prohibited because of the high probability of adverse consequences. This risk has been seen, repeatedly, in animals.

Production of human stem cells-into-human chimeras with human blastocysts
As the plasticity of stem cells is highest in the embryonic stage and as the developmental signaling is its most potent at that stage as well, implantation of human stem cells into a human blastocyst includes the very real possibility of germline transmission as well as of producing a human–human hybrid. The consequences of producing such a hybrid are completely unknown; thus, the risk of untoward effects on the resulting human makes this approach, at the present time, of high ethical concern.

Production of human–animal chimeras
An area with potentially more severe consequences is the possibility of producing, directly, a true human–animal hybrid by introducing human stem cells into the blastocyst of a non-human animal or by introducting animal stem cells into a human blastocyst. Such a mixing of species raises concerns in its own right about the definition of what it means to be human, and these concerns are only further compounded by the risk of germline transmission.

Changing Levels of Ethical Concern

The above categories of ethical concern are not intended to be comprehensive or immutable. Even with the present state of technology, there are almost certainly experiments that have not been included above. Also, while the different levels of ethical concern will hopefully serve as a useful starting point for categorizing proposed research projects, it is the details of a particular proposal that may raise or lower the level of ethical concern. The need to view the above categories as provisional is further emphasized by the fact that stem cell research is a new and rapidly

evolving line of inquiry. New technologies, new understandings, and new perspectives are certain to arise and will likely require us to move some of the listed lines of research to higher or lower levels of ethical concern. In the meantime, this framework may help to ensure that the attention of review committees will be directed to those projects that warrant the greatest level of concern.

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CHAPTER

29

Guidelines for Embryonic Stem Cell Research Oversight (ESCRO) Committees

Michael W. Kalichman and
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INTRODUCTION

> The fraudulent stem cell research reported by South Korean scientists suggests that American institutions are incapable of policing themselves to prevent similar scandals in this country, the chairman of a Congressional subcommittee said on Tuesday (Brainard, 2006)

Research involving the use of human zygotes and blastocysts to derive pluripotent stem cells is a new and rapidly evolving field. It is therefore to be expected that the best way to address the ethical dimensions of this research will also have to evolve. While it is premature to worry that "American institutions are incapable of policing themselves," it is important to ask how the ethical dimensions of this research will be best addressed. This is not just an afterthought for the research enterprise, but a necessity for the scientists who rely on the integrity of the research conducted by their colleagues. This chapter offers an initial attempt to articulate the specific questions that should be asked in the ethical review of human embryonic stem cell (hESC) research.

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Overview

Research to derive pluripotent stem cells from human embryos is rapidly evolving and highly controversial. This combination of factors is sufficient to make such research the most highly scrutinized academic endeavor, perhaps in the history of science. For at least the past 5 years, this public scrutiny has helped fuel increasing expectations that hESC research should be reviewed by appropriate ethics committees (Office for Human Research Protections, 2002; California Health and Safety Code, 2003; Proposition 71, 2004; Committee on Guidelines for Human Embryonic Stem Cell Research, 2005). Although nearly everyone accepts the need for such review, it is not as clear that we know the purpose of the required reviews.

The challenge is more difficult than it first appears: We already have review committees with well-established processes to protect the welfare of animal subjects of research and human subjects of research. The presumption that we need a new review process is a recognition that hESC research is different, even though it may include aspects that are properly covered by existing review mechanisms. The question to be answered is: "What interests are we protecting with review of hESC research?" The answer to this question will help us to define what needs to be reviewed, what criteria should be used for that review, and who should be responsible for the review.

The goal of this chapter is to propose a framework for the practical task of ethical review of hESC research. As proposed by the National Academy of Sciences, this responsibility should be assigned to Embryonic Stem Cell Research Oversight (ESCRO) Committees (Committee on Guidelines for Human Embryonic Stem Cell Research, 2005). In California, it has been proposed that this responsibility should instead be given to Stem Cell Research Oversight (SCRO) Committees, so as to explicitly cover both embryonic and non-embryonic sources of stem cells (California Institute for Regenerative Medicine, 2006). The full scope of responsibility for ESCRO or SCRO Committees is still in flux. However, because the former terminology is more widely accepted, this discussion will refer to these committees as ESCRO Committees.

Before beginning, it is important to be clear that this summary is not intended to be a definitive set of guidelines; review of this kind of research is still a new endeavor and this framework is presented only as a starting point for discussion. Also, this summary will not focus on separation of funding sources or apportioning intellectual property rights, which are for the most part going to be a matter of regulatory oversight. Instead, the hope is that this discussion might be a useful guide for thinking about the purpose of our ethical review of hESC research.

Review of hESC Research

Why should we review this research?

hESC research is not forbidden in the United States; although it is forbidden to use Federal funds for any project in which a human embryo would be "destroyed, discarded, or knowingly subjected to risk of injury or death" (Dickey, 1996). The exception to this is that Federal funds may be used for research on stem cell lines derived

prior to August 9, 2001 (Bush, 2001; National Institutes of Health, 2001). Also, if non-Federal funds are available, then this research is still allowable. Individuals, private foundations, and industry are all free to fund research on hESCs; many states have already voted to explicitly fund such research or are proposing to do so. In all of these cases, it is reasonable to assume that reviewers will first address the scientific merits of such research before choosing to award funding. If this research involving hESCs is already subject to scientific review, then it is important to begin by asking whether an ethical review is also needed.

Research with hESCs is potentially subject to a variety of ethical reviews already. Because hESCs, or the embryo from which they are derived, are human cells, the original donation requires oversight by an Institutional Review Board (IRB) (Office for Human Research Protections, 2002). And if the research project will involve animal subjects, then it requires review by an Institutional Animal Care and Use Committee (IACUC) (Office of Laboratory Animal Welfare, 2002).

It appears that there are at least two reasons that might warrant a special review of these cells. The first is that although the cells of a human embryo are clearly human cells, the cells of the human embryo are different from other cells of the body. Given the right circumstances, that embryo could become a fetus and with time a newborn child. For this reason alone, an embryo, including the cells that comprise the inner cell mass of a blastocyst, is special. The decisions to destroy embryos for the purpose of research, or to reserve them for reproduction, are qualitatively different decisions than consenting to give a sample of blood for example. For this reason, it seems reasonable that we would grant the embryo a measure of respect greater than that given to other non-embryonic cells of the body. One of the earliest formulations of the concept of this "special respect" was proposed by John Robertson in 1999:

> Persons holding the latter view – that the embryo itself lacks interests or rights because of its extremely rudimentary development – do not, however, necessarily view embryos as identical to any other human tissue. Indeed, many such persons would say that embryos, though lacking rights or interests in themselves, deserve "special respect" because of the embryo's potential, if placed in a uterus, to become a fetus and eventually to be born. Even embryos that will not be placed in the uterus have some meaning in this regard for they operate as a symbol of human life or constitute an arena for expressing one's commitment to human life (Robertson, 1999).

Although the argument for "special respect" is an acknowledgement only of a potential trajectory for a given human embryo, scientists should insist on ethical review of hESC research for at least three reasons. First, based on the principle of respect for life, it is just common sense that research on a developing embryo should be given closer scrutiny than research on adult cells or even tissue-specific stem cells. Second, because the nature of research is typically to study the unknown, the consequences and implications of that research cannot always be known. Just as the scientific merits of a research project are often enhanced by a multidisciplinary perspective, it is only reasonable to expect that the ethical defensibility of a project will be strengthened by an independent ethical review. Finally, the above arguments may not convince everyone, but it is worth considering a purely practical analysis. This area of research is being watched closely by the public, and especially by those who are morally opposed to any research uses that will result in

destruction or possible injury to the human embryo. Under these circumstances, it is in the interest of hESC researchers and of science that we adopt a proactive and serious role in addressing the ethical dimensions of our work. This will not necessarily change the minds of those opposed to any use of the human embryo, but it is likely to be important to many of those who hold views that are more open to the acceptance of hESC research. The cynic might say this is just good public relations. To some extent, this is true. Scientific inquiry is made possible because of the support of the public. If scientists wish to retain the privilege of that support, then it is essential that they act in ways that will help to reassure the public. In the process, as noted above, well-trained scientists recognize that scientific rigor depends on a willingness to question our assumptions and to expose our work to critical analysis. In short, the "special respect" defined by reviewing the ethical dimensions of our work can be seen as a prerequisite for good science.

What dimensions of the research project are at issue?

If the goal of ESCRO review is to ensure that we are giving special respect to the human embryo, then what factors would distinguish this review from existing human subjects research reviews? There are at least five questions that can help define the purview of ethical review for hESC research:

1. How do we give special respect? This question is a motivation for all of the other questions, but it warrants consideration in its own right.
2. What is being studied? The species that we choose to study (e.g. human or mouse) and the stage of development (e.g. zygote, blastocyst, fetus, or adult) are clearly factors to be considered in judging the ethical merit of a project.
3. How is it being studied? This is an issue both for the methods by which tissue is harvested (e.g. destruction of the blastocyst vs. extraction of a single blastomere) and the experimental protocol (e.g. *in vitro* characterization of a cell line vs. creation of a chimera).
4. Why is it being studied? It is widely appreciated that it is difficult to predict the likely success or value of a given line of research. Nonetheless, it is worth considering whether different research goals (e.g. a cure for diabetes vs. a treatment designed to make someone taller than average) deserve different ethical weights.
5. Is it good science? If the research is not being conducted in a manner that will produce useful results, then it cannot qualify as ethical research. Factors that must be considered at some point include appropriateness of an experimental approach, consideration of previous relevant research, and adequate training in the methods, policy, and ethical dimensions of the appropriate research.

Each of these five questions is discussed in more detail below.

How do we give special respect for human embryos?

On first examination, the concept of "special respect" seems untenable. How could it be that we can both choose to respect the human embryo and choose to use that embryo for the purpose of research? The answer is that this kind of value judgment is, in

fact, one we make often. Rather than making absolute judgments, we are comfortable with shades of gray. For example, it is against the law to exceed the speed limit, but we typically have more tolerance for breaking this law than for armed robbery. In research with human subjects, an important principle is beneficence (National Commission for the Protection of Human Subjects of Biomedical and Behavioral Research, 1979), "to do no harm"; but for testing new chemotherapeutic agents for melanoma, we may ask research subjects to accept the risk of harm in exchange for the possibility of finding a new and more effective treatment for an aggressive cancer. In animal research, our society has accepted the principle that research can be conducted on animal subjects, but this research does not occur without restriction. By Federal regulations, all animal research is subject to review by an Institutional Animal Care and Use Committee (Office of Laboratory Animal Welfare, 2002). A central principle of that review is that a necessary condition for approvable animal research is that it satisfies three principles proposed by Russell and Burch (1959): replacement, reduction, and refinement. A similar goal could be applied in our expectations for hESC research.

The principles of replacement, reduction, and refinement (the three Rs) are an obvious way in which research involving hESCs can proceed, but with special respect. If research can be conducted without the need to use human embryos, then consideration for the possibility of replacement with a comparable or better approach would be one standard for measuring the ethical merits of a proposal. If the research goals can be achieved by improvements in experimental methodology or without the need to destroy a human embryo, then the principle of refinement would be an appropriate ethical standard. If the statistical outcomes of an experiment can be accomplished with fewer human embryos then according to the principle of reduction, the ethical goal would be to choose to do so. It is noteworthy that these ethical goals can be practical as well. Replacement, refinement, and reduction will often mean a less costly study.

The three Rs are clearly a minimal standard by which researchers can demonstrate special respect in their use of human embryos (Table 29.1). More generally, the very fact of having a review process to consider the relative merits of different research strategies is also important. In conjunction with the three Rs, an ethical review should reasonably consider all dimensions of the research including species, stage of development, experimental methods, purpose of the research, and the quality of the science.

TABLE 29.1 Checklist for ethics review of hESC research: Replacement, refinement, and reduction

Category	Questions
Replacement	Can the research goals be met without destroying a human embryo, and with an alternative approach that raises less severe ethical challenges?
Refinement	If the research goals are best met by using human embryos, is it possible to do so without destroying the embryo or it is possible to do so by means that will not impair possible future development of the embryo?
Reduction	Can the research goals be met with the use of fewer human embryos?

TABLE 29.2 Checklist for ethics review of hESC research: What will be studied?

Category	Questions
Species	Will this project involve human tissue? Non-human primates? Mixing of human and non-human species?
Oocytes Zygotes Blastocysts	Who will be the donors? Will the research require that human oocytes, zygotes, or blastocysts be destroyed, discarded, or damaged?
Fetus	Who will be the donors? How did the fetal tissue become available?
Child or adult	Who will be the donors? What tissues will be donated?

What is being studied?

A fundamental ethical consideration for any biological research is the choice of species and the stage of development of that species. In the case of hESC research, it is already determined that the study will include tissues or derivatives that have human origin, but this does not preclude the need to consider other questions related to non-human species. For example, will the study involve some form of mixing of species, or will cumulative DNA changes be made to shift the identity of a human organism to one which is "less human" or non-human, or, conversely, shift the identity of non-human organisms toward human? Any judgment about the acceptability of such research would have to take into account an ethical analysis of what it means to be human, the risks or likelihood of creating disabled humans, and the ability to give informed consent (Table 29.2).

In addition to considering the species to be studied, it is also relevant to consider the stage of development of the organism. For example, if the study requires harvesting and use of only adult somatic cells or male gametes, the research is likely to raise far fewer concerns than if it requires the destruction of a blastocyst or fetus. Depending on the purpose of the research, it is reasonable to expect increasing levels of concern for projects that require obtaining somatic cells from an adult, male gametes, female gametes, fertilized eggs, and cells from the inner cell mass of a blastocyst or fetus.

How is it being studied?

While it is important to be clear about what is being studied, the degree of ethical concern depends primarily on the methods of the study. These concerns can be divided into two broad questions. First, how will tissue be obtained? Second, what will be done with the tissue?

Factors related to the harvesting or collection of biological materials depend intimately on the species or stage of organism development (Table 29.3). For example, one type of harm could be described as "pain or suffering." While this is a potential issue in studies that require interventions with an organism that has a sufficiently developed nervous system, it is not as obviously a factor for a frozen blastocyst.

The harm described as "pain and suffering" is only one of many potential harms associated with the ways in which tissues are obtained for the purpose of research. Does

TABLE 29.3 Checklist for ethics review of hESC research: How will the study be conducted?

Category	Questions
Pain and suffering	Will the study cause or risk injury of an organism that has sufficient neural organization to experience physical pain? Self-awareness? Memory?
Informed and voluntary consent	How will informed consent be obtained? What measures will be taken to ensure that the consent is voluntary, not coerced, and informed?
Surrogate consent	If the proposed donor lacks the capacity to provide informed and voluntary consent, will consent be provided by a surrogate who will adequately consider the interests of the donor?
Alternative uses	Are the cells or tissues generated for the purpose of the research project or were they spontaneously or intentionally generated for some other use?
Characterization	Will the study consist only of genetic, biochemical, or morphological characterizations of cells?
Differentiation	Will the study consist of genetic or chemical manipulations to induce differentiation or commitment to more specific lineages?
De-Differentiation	Will the study consist of genetic or chemical manipulations to induce cells to revert to a less differentiated state?
Somatic cell nuclear transfer	Will nuclear material from diploid cells be inserted into oocytes?
Mixing of species	Will genetic material, cells or tissues of a non-human species be inserted into a human organism? Will genetic material, cells or tissues of human origin be inserted into a non-human species?

the procedure require destruction of the organism, or can cells be obtained without causing significant harm? If a donor is capable of doing so, has she provided truly informed and voluntary consent? If a donor is not capable of giving consent because of age, maturity, mental state, or death, then is it possible to identify an appropriate surrogate and has that surrogate provided truly informed and voluntary consent? Is the source of research material incidental to some other purpose (e.g. spare embryos in IVF clinics) or specifically produced for research (e.g. voluntary donation of oocytes and adult somatic cells for the purpose of somatic cell nuclear transfer)? Is the material being provided to researchers before or after having been frozen? Are cells to be obtained from an entity (such as a blastocyst) that is presumed to be viable for implantation and, if so, is it believed to have a high likelihood of continued development? These questions are only examples of the many issues we can already anticipate. The challenge for review committees will be to use these assessments of graded ethical concerns to make decisions about when and how research can proceed.

After tissue has been harvested for research, a qualitatively different set of ethical issues is defined by what will be done with the tissue. At the most basic, and presumably least problematic, level would be *in vitro* studies calling simply for characterization

of cells or a tissue. Slightly more, but still minimal, concern might be raised if those cells are being manipulated to encourage further differentiation. A still higher level of concern would occur for *in vitro* attempts to de-differentiate cells to increase their pluripotency. However, while these *in vitro* approaches have a low likelihood of raising ethical worries, other approaches that involve a trajectory of organismal development and/or mixing of species have the potential for much more serious considerations. Socially and legally, there are already strong prohibitions against human reproductive cloning. However, what are the appropriate parameters for mixing cells of species *in vitro*, *in utero*, or in the adult? While we may have clarity about the extremes, it is inevitable that our decisions will depend not on absolutes, but on gradations such as percentage of identifiable human DNA, proximity to humaniform proteins, similarity in appearance to human features, or percentage of cells in the body that are identifiable as human, and which organs (e.g. brain or heart) are most human. These dimensions of hESC research further clarify the extent to which ethical judgments will be matters of degree rather than black and white absolutes.

Why is it being studied?

For research with human or animal subjects, it is well accepted that while the benefits of a research study are not sufficient, they are certainly necessary to justify approval of a research project. If only because financial and biological resources are finite, the same standard seems appropriate for hESC research. Clearly, a minimal expectation is that any such study should be justified by having a rationale. However, the challenge for reviewing such research is whether all possible rationales are equivalent (Table 29.4).

If the long-term goals of a research study are relevant to assessing the ethical merits of the proposal, then it is worth considering the range of plausible outcomes for a project. For hESC research, it seems reasonable to assume that most projects will correspond to at least one of five possible long-term goals: (1) better understanding of biology; (2) prevention; (3) cure; (4) treatment; and (5) enhancement. At first glance, the first of these goals, a better understanding of biology, may seem less worthy than research targeted to a specific disease or disorder. On the other hand, a case can be

TABLE 29.4 Checklist for ethics review of hESC research: Why is the study being conducted?

Category	Questions
Basic research	Is the primary purpose of the study a better understanding of biology or disease mechanisms?
Prevention	Is the primary purpose of the study to develop treatments or devices that will prevent the occurrence of illness or disease?
Cure	Is the primary purpose of the study to develop treatments or devices that will cure existing illness or disease?
Treatment	Is the primary purpose of the study to develop treatments or devices that will treat, but not necessarily cure, existing illness or disease?
Enhancement	Is the primary purpose of the study to develop treatments or devices that are designed to enhance the human condition?

made that support for basic research will be rewarded by providing a foundation for the more efficient and successful study of many different clinical targets.

Prevention, cure, and treatment are clearly admirable goals. Ultimately, it would be desirable to have success in any of these domains. However, faced with scarce resources, are some diseases or disorders more justifiable targets than others? And should it be considered that prevention may be preferable to treatment and that cure is normally preferable to symptomatic treatment? How might these value judgments be addressed in ethical reviews of research?

The final research goal to be discussed is new technologies that are clearly intended to produce "enhancements." In other words, the goal is not to repair or prevent a readily identifiable deficit, but instead to make an individual something more than "normal." The distinction between enhancement and treatment is not easily made and is the basis for considerable discussion (e.g. Parens, 1998). However, for the purpose of this overview, it is worth asking: Should a study designed particularly for the development of an enhancing technology (e.g. to promote height or intelligence that is greater than average) be given less ethical weight than one which is designed to treat disease? Again, how if at all should these kinds of judgments be weighed in the ethical review of proposed research projects?

Is it good science?

Guidelines from the National Academy of Sciences (Committee on Guidelines for Human Embryonic Stem Cell Research, 2005) and the proposed California Institute for Regenerative Medicine regulations (2006) make it clear that review of hESC research should take into account both science and ethics. As discussed above, the range of ethical issues is diverse without even beginning to look at the quality of the science. Further, because such research is costly, it is likely that funding will have been made available only after favorable review of the research. By this argument, it is reasonable to ask why an additional review of the science should be included as part of an ethical review (Table 29.5).

A full ethics review depends on an evaluation of the scientific merits of a project. Even if all other ethical considerations are met, it would be unethical for a research

TABLE 29.5 Checklist for ethics review of hESC research: Scientific merit of study

Category	Questions
Clear hypothesis or question	Is the proposed study designed to address a clearly stated hypothesis or question?
Awareness of literature	Is the design of the proposed study consistent with what has previously been published?
Duplication of previous work	Does the proposed study duplicate work that has already been done? If so, then is a rationale provided to adequately justify the need for the duplication?
Qualifications of personnel	Are the personnel who will perform the studies adequately trained in science and methodology? in the ethical, legal, and social implications of this line of research?

project to use human or animal subjects or to waste scarce resources on a project that does not have the potential to yield useful results. While it is true that this question will often have been addressed by a qualified scientific review that approved the project funding, this is not always the case. For example, some projects will be proposed based on the use of unrestricted gift research funds and others may reflect new research directions not anticipated in the original funding approval. As a result, review committees will need to make judgments about which research projects might need additional scientific review.

Assessing scientific merit depends on a few key ethical considerations. First, the motivation for the work should be based on clearly articulated hypotheses or questions. Second, the work should be appropriately placed in the context of what has already been done. This would be demonstrated by an awareness of previous work and an appropriate justification if the proposed study will duplicate research that has already been done. Finally, those who are conducting the research should be qualified to do so. This means evidence of training or experience should be available to assure reviewers that the researchers have the knowledge and skills appropriate not only to the scientific methodology but to the ethical, legal, and social implications of this area of research.

Summary

The review of hESC research is a new and essential endeavor. Such review should not be *pro forma*. Review committees need to develop criteria for approving, modifying, and rejecting proposed research projects. The range of issues summarized above is intended only as a framework for that discussion and should not be considered either comprehensive or fully developed. However, it should be clear that there are multiple respects in which hESC research raises ethical questions. The challenge to review committees will be to decide how to translate these shades of gray into judgments that will amount to rejection or approval of individual research projects. This is not just to assuage those who might oppose this line of research, but it is important to ensure our research colleagues of the integrity of our research process and it is an ethical obligation to the public that supports such research.

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Abbreviations

2-ME	2-mercaptoethanol
2PN	two-pronuclei
aCSF	artificial cerebrospinal fluid
ADB	antibody dilution buffer
aGM	aorta-gonad-mesonephoros
ALS	amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
ANP	atrial natriuretic peptide
ANT	alternative nuclear transfer
A-P	anterior–posterior
APC	allophycocyanin
ART	assisted reproductive technology
ASO	allele-specific oligo
ASPE	allele-specific primer extension
ATCC	American Type Culture Collection
bFGF	basic fibroblast growth factor
b-gal	beta-galactosidase
BDNF	brain-derived neurotrophic factors
BGS	bovine growth serum
BMP	bone morphogenetic protein
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
CD	cell differentiation
CDM	chemically defined media
CFUs	colony-forming units
CGH	comparative genome hybridization
ChAT	choline acetyltransferase
DAPI	4′,6-diamidino-2-phenylindole
Days post coitum	dpc
D-MEM	Dulbecco's modified Eagle's medium
D-MEM/F12	Dulbecco's modified Eagle's medium and Ham's F12 medium
D-PBS	Dulbecco's phosphate-buffered saline

D-V	dorsal–ventral
ECM	extracellular matrix
EGCs	embryonic germ cells
eGFP	enhanced green fluorescent protein
EPC	European Patent Convention
EPO	European Patent Office
EPSCs	excitatory post-synaptic currents
EPSPs	excitatory post-synaptic potentials
ESCRO	Embryonic Stem Cell Research Oversight
EST	expressed sequence tag
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FCS	fetal calf serum
FDR	false discovery rate
FGF2	fibroblast growth factor 2 (also bFGF)
FISH	fluorescence in situ hybridization
FITC	fluorescein isothiocyanate
FNR	false negative rate
FSC	forward scatter
GFP	green fluorescent protein
GRM	glial restrictive medium
hdF	human ESC-derived fibroblasts
HEF	primary human fetal fibroblasts
HH	HTFM-HEPES
HM	holding medium
HRP	horseradish peroxidase
HAS	human serum albumin
HSCs	hematopoietic stem cells
HSV-tk	herpes simplex virus thymidine kinase
IACUC	Institutional Animal Care and Use Committee
ICC	immunocytochemistry
ICM	inner cell mass
ICSI	intracytoplasmic sperm injection
IHC	immunohistochemistry
IMDM	Iscove's modified Dulbecco's medium
IRB	Institutional Review Board
ITS	insulin-transferrin-selenium
IUI	intrauterine insemination
IVF	*in vitro* fertilization
KD-MEM	knockout Dulbecco's modified Eagle's medium
KSR	KnockOut™ serum replacement
LIF	leukemia inhibitory factor
LOH	loss of heterozygosity
LSO	locus-specific oligo
LTC-ICs	long-term culture initiation cells
LTD	long-term depression
LTP	long-term potentiation
MAP	mouse antibody pathogen
MBP	myelin basic protein
MEF-CM	MEF-conditioned medium
MEFs	mouse embryonic fibroblasts

MLC2v	myosin light chain 2v
MOI	multiplicity of infection
MOU	memorandum of understanding
MPSS	massively parallel signature sequencing
MS	multiple sclerosis
MTA	material transfer agreement
NMDA	*N*-methyl-D-aspartic acid
NOD-SCID	non-obese diabetic severe combined immunodeficiency
NSCs	neural stem cells
NT3	neurotrophin 3
NT4	neurotrophin 4
NuMA	nuclear antigen
OPCs	oligodendrocyte progenitor cells
OPS	open pulled straw
PCR	polymerase chain reaction
PE	phycoerythrin
PFA	paraformaldehyde
PGD	pre-implantation genetic diagnosis
PGM	phosphoglucomutase
PI	propidium iodide
PLP	proteolipid protein
PPD	papain, protease, DNase
PPE	personal protective equipment
PTO	Patent and Trademark Office
QC	quality control
qPCR	quantitative PCR
RA	retinoic acid
RCR	replication-competent recombinants
RFP	red fluorescent protein
SAGE	serial analysis of gene expression
SAM	significance analysis for microarrays
SBE	single base extension
SBIR	Small Business Innovative Research
SCI	spinal cord injury
SCI	Stem Cell Innovations
SCID	severe combined immunodeficient
SCNT	somatic cell nuclear transfer
SCR	Stem Cell Resource
SHH	sonic hedgehog
siRNA	small interfering RNA
SKUs	stock keeping units
SNP	single nucleotide polymorphism
SRC	SCID-repopulating cell
SSC	side scatter
SSEA	stage-specific embryonic antigens
TAE	Tris/acetic acid/EDTA
TC	tissue culture
TH	tyrosine hydroxylase
TVA	Transvaginal aspiration
USPTO	United States Patent and Trademark Office
VSV-G	vesicular stomatitis G protein

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