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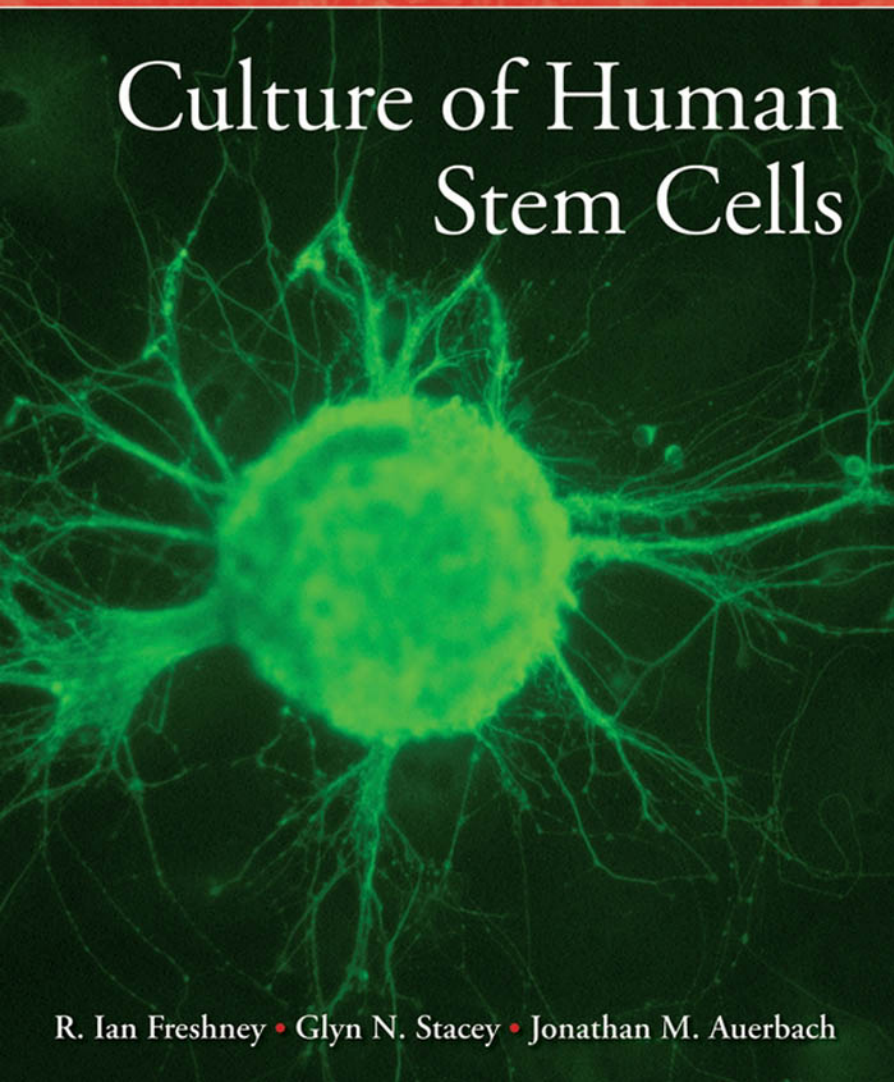
CULTURE OF SPECIALIZED CELLS

Culture of Human Stem Cells



R. Ian Freshney • Glyn N. Stacey • Jonathan M. Auerbach

CULTURE OF HUMAN STEM CELLS

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CULTURE OF HUMAN STEM CELLS

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A JOHN WILEY & SONS, INC., PUBLICATION

Published by John Wiley & Sons, Inc., Hoboken, New Jersey.
Published simultaneously in Canada.

Library of Congress Cataloging-in-Publication Data:

Culture of human stem cells / editors, R. Ian Freshney, Glyn N. Stacey, Jonathan M. Auerbach.
p. ; cm.
Includes bibliographical references and index.
ISBN 978-0-470-05246-4 (cloth)
1. Stem cells. 2. Human cell culture. I. Freshney, R. Ian. II. Stacey, G. (Glyn) III. Auerbach, Jonathan M.
[DNLM: 1. Stem Cells. 2. Biomedical Research. 3. Cell Culture Techniques. 4. Cells, Cultured. 5. Embryo Research. QU 325 C968 2007]
QH588.S83C85 2007
616′.02774—dc22
2007001708

Printed in the United States of America.

10 9 8 7 6 5 4 3 2 1

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PREFACE

The explosion of interest in human stem cells over the past few years has created a discipline able to explore the regulation of cellular differentiation from the most primitive cell to fully functional differentiated cells such as neurons, cardiomyocytes, and hard tissue cells such as bone and cartilage. Not only has this provided models for the investigation of regulatory mechanisms, but it has also created significant opportunities for engineering suitable grafts for tissue repair. Embryonal stem cells, stem cells from umbilical cord and tooth germs, and adult stem cells from bone marrow and other locations all offer potential sources of allograft or autograft tissue. Much has been written about characterization of stem cells, the exploitation of markers to identify them, and the control of changes in marker expression accompanying the expression of the differentiated phenotype in normal stem cells and in embryonal carcinoma. The objective of this book, however, is directed toward the methodology of the culture and characterization of stem cells. Although many of these techniques are still at a developmental stage, there is now a widening repertoire of established techniques that need to be made more generally available to the large influx of workers into the field.

This book follows the tradition of previous books in the *Culture of Specialized Cells* series in that it describes a limited number of representative techniques across a wide spectrum of stem cells from embryonic, newborn, and adult tissue. The emphasis is on practical guidance, and it should be possible to follow all of the protocols without recourse to the primary literature or other publications, other than for background. Hence this book provides a suitable introductory text that will allow incomers to the field, including students and established researchers, both from basic science and a clinical background, to become familiar with some of the techniques in current use, to increase their knowledge of the discipline, or to develop their own research program.

The book progresses from basic quality control issues in the first chapter by Glyn Stacey and Jonathan Auerbach, to deal with the derivation of human embryonal stem (hES) cell lines from the early embryo by Jessica Cooke and Stephen Minger, their differentiation (and that of embryonal carcinoma) into neural cells by Jamie Jackson, Peter Tonge, and Peter Andrews and into cardiomyocytes by Christine Mummery, and then on to primary culture and characterization of primitive germ cells by Lee Turnpenny and Neil Hanley and of embryonal carcinoma by Stefan Przyborski. These six chapters cover the characterization and differentiation and the cryopreservation of these lines. A new and exciting source of stem cells has been found in the newborn, and two chapters describe examples, one from umbilical cord by Young-Jin Kim and another from tooth germ by Wataru Sonoyama, Takayoshi Yamaza, Stan Gronthos, and Songtao Shi. The last five chapters deal with adult mesenchymal stem cells derived from bone marrow stroma by Carl Gregory and Darwin Prockop, cartilage by Charles Archer, Sarah Oldfield, Samantha Redman, Laura Haughton, Gary Dowthwaite, Ilyas Khan, and Jim Ralphs,

cornea by Yiqin Du and James L. Funderburgh, mammary stem cells by Mark Labarge, Ole Petersen, and Mina Bissell, and stem cells from adipose tissue by Kristine Safford and Henry Rice.

Some techniques are described in more than one chapter, for example, the culture of mouse embryo feeder cells in Chapters 2 and 4. As the techniques differ slightly, both have been retained. Other feeder cell systems are discussed in Chapter 2, and protocols using alternative feeder cells are provided in Chapter 5 (STO cells) and Chapter 7 (cord blood mesenchymal stem cells, CB-MSCs). Protocols for freezing cells for storage are provided in Chapters 2, 6, 8, and 9. Most describe variations on the now standard procedure for most cultured cells, namely, slow cooling at 1°C/min, storage in liquid nitrogen, and rapid thawing to recover the cells. However, hES cells have been found to survive better after a rapid vitrification process, and this is described in Chapter 2.

The formatting of each chapter has been standardized, and an attempt has been made to use standardized terms and abbreviations. For example, the abbreviation PBSA is used for Dulbecco's phosphate-buffered saline lacking calcium and magnesium and UPW for ultrapure, tissue culture-grade water, regardless of how it is prepared. Sources of equipment and materials are listed at the end of each chapter, rather than in the text, and a combined list of suppliers' websites is provided at the end of the book. Basic procedures such as trypsinization and cell counting are not always described in detail unless there is a deviation from normal practice, and it is assumed that certain prerequisites will be met in the provision of facilities and equipment, so items such as laminar flow hoods or biological safety cabinets, bench top centrifuges, water baths, pipettes, 70% alcohol, etc., are not listed with every protocol. A description of basic facilities, equipment, and technique is to be found in Freshney (2005), *Culture of Animal Cells*, John Wiley & Sons, and it is assumed that the reader will have some knowledge of basic procedures before embarking on any of these more sophisticated protocols.

We are greatly indebted to our contributing authors for giving up their valuable time to prepare these detailed chapters. We feel we have a good cross section of topics represented but have not attempted to be encyclopedic or totally comprehensive as that would have enlarged the book significantly and increased the cost. We hope the style, format, and coverage of this book will prove of value to those entering the field from a wide spectrum of disciplines as well as those who already have some prior experience. Readers may also wish to consult *Human Embryonic Stem Cells: A Practical Handbook*, edited by Stephen Sullivan, to be published in 2007 by John Wiley & Sons, Chichester.

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ABBREVIATIONS

Abbreviation	*Definition*
2D	two-dimensional
3D	three-dimensional
α-MEM	α-modified minimal essential medium
α-SMA	α-smooth muscle actin
ABCG2	ATP-binding cassette transporter G family member
ALCAM	activated leukocyte cell adhesion molecule
AP	alkaline phosphatase
ASC	adipose-derived stem cell
BCIP	5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt
bFGF	basic fibroblast growth factor (FGF-2)
BHA	butylated hydroxyanisole
BM	bone marrow
BM-MSC	bone marrow mesenchymal stem cell
BMP	bone morphogenetic protein
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
BSC	biological safety cabinet
BSP	bone sialoprotein
CAFC	cobblestone area-forming cell
CBE	cord blood-derived embryonic-like stem cell
Cbfa1	core binding factor 1
CB-MPC	cord blood multipotent progenitor cell
CB-MSC	cord blood mesenchymal stem cell
CDM	chondrocyte differentiation medium
CFU	colony-forming unit
CFU-BL	colony-forming unit-blast
CFU-F	colony forming unit-fibroblast
cGy	centiGray
CMF-Saline G	calcium- and magnesium-free Hanks' Saline G
CNPase	2′, 3′-cyclic nucleotide−3′-phosphodiesterase
CECM	corneal endothelial cell medium
COMP	cartilage oligomatrix protein
DAPI	4′,6-diamidino-2-phenylindole
dH_2O	deionized water
DMEM	Dulbecco's modification of Eagle's medium
DMEM/F-12	Dulbecco's modified Eagle's medium/nutrient mixture F-12, 1:1
DMSO	dimethyl sulfoxide

D-PBS	Dulbecco's phosphate-buffered saline complete with Ca^{2+} and Mg^{2+}
DPSC	dental pulp stem cell
DSPP	dentin sialophosphoprotein
EB	embryoid body
EBM	Eagle's basal medium
EC	embryonal carcinoma
ECC	embryonic carcinoma cell
EDTA	ethylenediaminetetraacetic acid
EGC	embryonic germ cell
EGF	epidermal growth factor
EnC	endothelial cell
ES	embryonic stem (cell)
ESA	epithelial-specific antigen
ESC	embryonic stem cell
ESM	endothelial spheres medium
EtOH	ethanol
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FGF	fibroblast growth factor
GAD	glutamic acid decarboxylase
GASP	gentamicin, amphotericin B, streptomycin, penicillin
gcEB	germ cell embryoid body-like structures
GCT	germ cell tumor
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
GvHD	graft-versus-host disease
HA/TCP	hydroxyapatite/tricalcium phosphate
HAM	human amniotic membrane
HBSS	Hanks' balanced salt solution
HCEC	human corneal endothelial cell
hECC	human embryonal carcinoma cell
hEGC	human embryonic germ cell
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
hES	human embryonal stem (cell)
hESC	human embryonic stem cell
HFEA	Human Fertilisation and Embryology Authority
HG-DMEM	high-glucose Dulbecco's modified Eagle's medium
HLA	human leukocyte antigen
HPP-CFC	high proliferative potential colony-forming cell
HSC	hematopoietic stem cell
HUCPV	human umbilical cord perivascular
ICAM	intercellular adhesion molecule
ICC	immunocytochemistry
ICM	inner cell mass
IMDM	Iscove's modified Dulbecco's medium
ITS	Insulin, transferrin, and selenium
IVF	in vitro fertilization

LG-DMEM	low-glucose Dulbecco's modified Eagle's medium
LMP agarose	low-melting point agarose
LPL	lipoprotein lipase
lrECM	laminin-rich extracellular matrices
LOH	loss of heterozygosity
LSC	limbal stem cell
LTC-IC	long-term culture-initiating cell
mEF	mouse embryo fibroblast
MEF	mouse embryo feeder (cells)
mEGC	mouse embryonic germ cell
mESC	mouse embryonic stem cell
MGS	multipotent germline stem cell
MLPC™	Multi-Lineage Progenitor Cell™
MNC	mononuclear cell
MSC	mesenchymal stem cell
Muc	sialomucin
NBT	nitro-blue tetrazolium chloride
N-CAM	neural cell adhesion molecules
NeuN	neuronal nuclear marker
NFP	neurofilament protein
NOD/SCID	nonobese diabetic/severe combined immunodeficient
NS	neural stem cell
OKT9	anti-transferrin receptor monoclonal antibody
OSC	osteocalcin
P	passage (as in subculture)
PBS	phosphate-buffered saline
PBSA	Dulbecco's phosphate-buffered saline, solution A (no Ca^{2+} or Mg^{2+})
PBSC	Dulbecco's phosphate-buffered saline with 1 mM $CaCl_2$ and 1 mM $MgCl_2$
PCR	polymerase chain reaction
PFA	paraformaldehyde
PGC	primordial germ cell
PGD	preimplantation genetic diagnosis
PI	propidium iodide
PPARγ2	peroxisome proliferator-activated receptor-γ2
RA	retinoic acid
RS	rapidly self-renewing (cell)
RT-PCR	reverse transcription polymerase chain reaction
Runx	Runt-related transcription factor
Sca-1	stem cell antigen-1
SHED	stem cells from human exfoliated deciduous teeth
SIM	Sandoz Inbred Mice
SNP	single nucleotide polymorphism
SR	slowly replicating (cell)
SSEA	stage-specific early antigen
STR	short tandem repeat
TA	transit amplifying
TDLU	terminal ductal lobular unit

TEMED	N,N,N',N'-tetramethylethylenediamine
TGF	transforming growth factor
TH	tyrosine hydroxylase
TMRM	tetramethylrhodamine methyl ester
TPOFLK	thrombopoietin, Flt-3 ligand, and c-kit ligand
UC	umbilical cord
UCB	umbilical cord blood
USSC	unrestricted somatic stem cell
VNTR	variable number tandem repeat
WJ	Wharton jelly
Wpc	weeks postconception
ZO-1	zonula occludens-1

CHAPTER 1

QUALITY CONTROL PROCEDURES FOR STEM CELL LINES

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Culture of Human Stem Cells, Edited by R.I. Freshney, G.N. Stacey and J.M. Auerbach

1.1. INTRODUCTION

The culture of human cells in vitro has provided important insights into cell biology, disease processes, and potential therapies. The advent of the culture of human embryonic stem cells has opened up an exciting new generation of possibilities including their potential for application to human regenerative medicine. However, in vitro cell culture brings a number of challenges; the cells and the cell culture environment are ideal for the growth of numerous microorganisms, and the cells themselves are prone to genetic changes. Furthermore, it is, unfortunately, common for cell cultures to be interchanged, cross-contaminated, or mislabeled during laboratory manipulations. This leads us to define three critical characteristics of cell cultures that are fundamental to the assurance of good-quality cell culture work. These are:

- Purity—The cells are free from microbiological contamination.
- Identity—The cells are what they are claimed to be.
- Stability—The genotype and phenotype remain stable during growth and passage in vitro.

Serial passage exposes cell cultures to the repeated risk of contamination by environmental microorganisms. Such contaminations are generally recognized by a significant change in the pH of the culture medium (as identified by a color shift in the medium) and the sudden appearance of turbidity or colonies of fungal organisms. In these situations the culture is generally not recoverable and should be discarded carefully to prevent contamination of other cultures. However, some microbial contaminants can establish subliminal persistent infections that are not obvious on visual inspection. These are commonly due to mycoplasma but may be caused by other organisms [*see*, e.g. Mowles et al., 1989; Buerhing et al., 1995].

Mycoplasma contamination is known to cause a broad range of permanent and deleterious effects on cells including chromosomal abnormalities [McGarrity et al., 1984] and cell transformation [Zhang et al., 2004, 2006; for a review *see* Rottem and Naot, 1998]. Mycoplasma contamination is not obvious by visual inspection, can spread rapidly to other cultures handled by the same or other operators, and is difficult to eradicate reliably. Accordingly, it is important to perform routine screening for this organism in cell cultures.

The first human cell line HeLa, established in 1952 [Gey et al., 1952], was widely distributed to laboratories also attempting to establish new cell lines. Within a few short years it became apparent that some of the "novel" cell lines being established were in fact cross-contaminated with HeLa cells [Gartler et al., 1967]. The problem was highlighted through the use of karyology and isoenzyme analysis [Nelson-Rees et al., 1981; O'Brien et al., 1977] but was only partially resolved and led to much scientific controversy [Gold 1983]. Since the identification of early cases of cross-contaminated cultures, cases have continued to be identified (Table 1.1). Despite periodic reminders from concerned cell culturists [e.g., Stacey et al., 2000] the problem appears to continue, and recent publications seem to indicate that part of this problem may be due to cross-contamination at the source of the cultures in the laboratories of the originators of the cell lines [MacLeod et al., 1999; Drexler et al., 2003]. It is vital that this situation does not develop with stem cell lines, as this could cause confusion in laboratory experimentation and major

TABLE 1.1 Publications Describing Cell Lines Not Matching Their Purported Origin

Reference	Cell Lines
Gartler [1967]	Breast cancer cell line cross-contamination
Nelson Rees et al. [1977]	Widespread cross-contamination of human breast tumor cell lines and others
Harris et al. [1991]	Putative human Hodgkin disease cell lines cross-contaminated with nonhuman cells
Masters et al. [1988]	Cross-contamination of bladder cancer cell lines
van Helden et al. [1988]	Cross-contamination among esophageal squamous carcinoma cell lines
Chen et al. [1990]	TE671 shown to be a derived from RD cells
Drexler et al. [1993]	Cross-contamination of a leukemia cell line
Reid et al. [1995]	Cross-contamination of U937 cells
MacLeod et al. [1997]	Dami megakaryocytes found to be HEL erythroleukemia cells
Dirks et al. [1999]	ECV304 endothelial cells found to be T24 bladder cancer cells
MacLeod et al. [1999]	18% leukemia cell lines submitted to DSMZ from originators cross-contaminated

problems in potential clinical application and hamper future development and acceptance of the technology.

One of the key issues in the development of hES cell lines has been the consistency and comparability among hES cells isolated at different centers under different conditions. Much work has been published on new culture and differentiation methods; however, each publication generally deals with a very limited number of cell lines. This leads hES cell researchers to ask whether the data produced can be applied more broadly to all hES cell lines. Approaches to technical standardization have been considered [Loring and Rao, 2006], and in addition one attempt to characterize hES cells on an international basis has been initiated [Andrews et al., 2005]. Generally, such attempts at standardization have been based on antibody markers developed for the study of early development in embryonal carcinoma models [Andrews et al., 2002]. Today a growing array of molecular and antibody markers for stem cells is developing that will be of use in the quality control of stem cell lines [Andrews et al., 2005; Loring and Rao, 2006; *see also* succeeding chapters]. In the following sections we explore the various techniques and methods that can be used to test stem cell lines to address issues of purity, identity, and stability and to qualify their use in stem cell research. Although much of this addresses hES cell lines, it is applicable equally to all cell lines whether derived from stem cells or not and whether embryonic, newborn, or adult.

1.2. THE CELL BANKING PRINCIPLE

To limit the chances of contamination and genetic change it is wise to keep the number of passages of cells to a minimum. An important approach to achieve this, called the master/working bank principle, has been adopted in industry for many years. This prescribes the establishment of a *master cell bank* that will provide the reference point for future work with a cell line. This bank should be well characterized and subjected to quality control tests. Ampoules from the master bank are then used to produce larger *working cell banks* that can be made available for experimental purposes or distribution

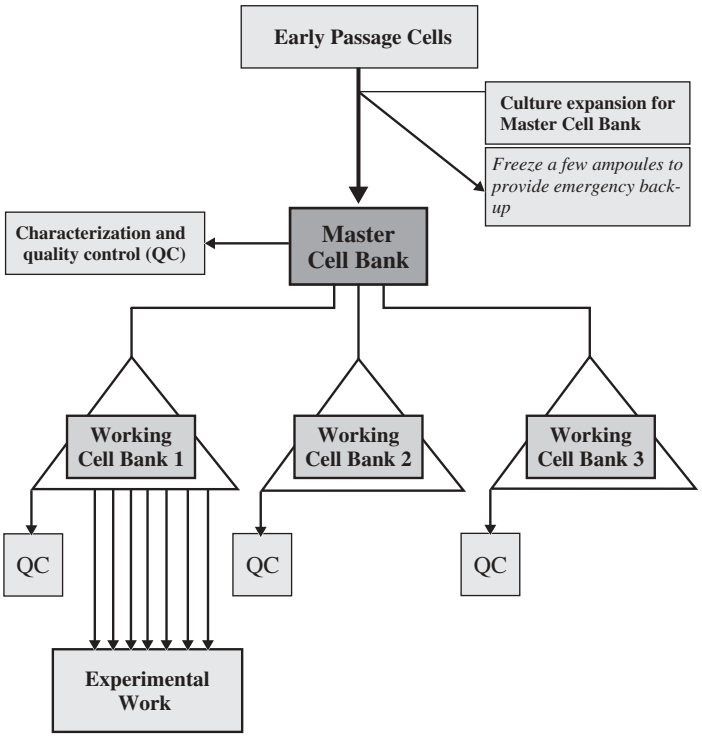

FIGURE 1.1. A scheme for the establishment of master and working cell banks.

to other workers [Hay et al., 2000]. The working cell bank should again be subjected to quality control, although this may be more limited and concerned mainly with identity and absence of contamination. If prepared correctly, this tiered master/working bank system (Fig. 1.1) can provide reproducible and reliable supplies of identical cultures for research work over many decades.

The quality control tests that should performed as a matter of routine for all cell banks include viability (typically Trypan Blue dye exclusion) and testing for absence of bacterial, fungal, and mycoplasmal contamination [Stacey and Stacey, 2000]. These tests should be performed on cultures after a period of at least 5 days, and preferably two passages of antibiotic-free culture, to ensure that any contaminants that may be suppressed by antibiotics do not go undetected. Other tests for authenticity (e.g., karyology, DNA fingerprinting, isoenzyme analysis, surface markers) and assays for the presence of viruses may be performed, but the exact profile of tests will depend on the type of cells involved and the intended use of the cells. For a general reference on cell banking and quality control *see* Stacey and Doyle [2000]. The specific approaches and methods are discussed in the following sections.

Effective cryopreservation protocols are clearly essential for cell banking (*see also* Chapters 2, 6, 8, 9). Standard methodologies for other cell lines and for preservation of mouse embryonic stem cell lines have not been reported as being very successful with hES cells compared to vitrification methods [Reubinoff et al., 2001; Zhou et al., 2004; *see also* Chapter 2]. Most vitrification methods used for hES cells have been adapted from methods established for bovine oocytes and embryos, and the most commonly referenced

modification used for hES cells is that of Reubinoff et al. [2001]. Successful methods reported generally utilize dimethyl sulfoxide and ethylene glycol as cryoprotectants, but details vary between publications. For a review of the methods currently used *see* Hunt and Timmons [2007]. Vitrification, while effective for preservation of hES cells, has a number of drawbacks including the need to carefully maintain storage temperatures close to liquid nitrogen temperatures to avoid devitrification, the costs of shipment under such storage conditions, the small volumes that can be frozen for each vial of cells required to be archived, and the rapid cooling rate required for successful vitrification.

1.3. CELL CHARACTERIZATION

1.3.1. Viability

Cell viability is obviously crucial but is also a characteristic that is all too often poorly addressed in cell culture. Numerous methods are available to determine viability. Each measures a different characteristic of cell biology (e.g., membrane integrity, membrane function, products released by cell damage or death, metabolic functions, enzyme activity, and clonogenic survival), and examples of techniques for measuring "viability" are given in Table 1.2. It is important to remember that the cell characteristics revealed in these tests can be affected differently by particular conditions in culture.

Trypan Blue dye exclusion [Patterson, 1979] is one of the most common methods used, although viability methods based on detection of apoptotic cells are also common [*see*, e.g., Sparrow and Tippet, 2005]. Whatever method is employed, it is important that it is relevant to the cell culture application and that it provides reproducible results. For stem cell cultures it is clear that a viability measurement cannot predict the proportion of stem cells present in a culture after a culture treatment or cryopreservation, but frozen stocks of cells still can and should be checked promptly for viability by recovering a vial of cells into culture immediately after cryopreservation.

1.3.2. Karyology

Visualization of the cell's chromosomes (karyotypic analysis) provides a valuable perspective on the physical structure of the genome. It has been used as a valuable tool for monitoring the genetic stability of a cell culture [*see*, e.g., Rutzky et al., 1980] and for recognizing the appearance of transformed cells, which are often aneuploid (having chromosome loss or duplication, or aberrant chromosomes with translocations, deletions, inversions, etc) and heteroploid (having a wide range of chromosome numbers per cell around or, more often, above the normal number).

The method of visualization of chromosomes most commonly used today employs colchicine or a similar compound to block cell division at metaphase when the individual chromosomes are separate and condensed and thus most readily visualized (*see* Chapter 5). The cells are then harvested, subjected to swelling with hypotonic saline, KCl, or saline citrate, and fixed in acetic methanol before applying them dropwise onto microscope slides to create the characteristic chromosome "spreads." These are then stained with Giemsa to visualize the condensed chromosomes. The ability to identify chromosome pairs and to resolve the nature of fine alterations in chromosome structure was realized through the use of trypsinization before Giemsa staining, which reveals banding patterns characteristic of each chromosome [Wang and Federoff, 1972]. Other

TABLE 1.2 Viability Testing for Animal Cell Cultures

Method	Principle and Comments
Dye exclusion (e.g., Trypan Blue, Naphthalene Black)	Dyes that penetrate cells are excluded by the action of the cell membrane in viable cells; thus cells containing no dye have functional membranes and are probably viable. **Advantages:** Rapid and usually easy to interpret **Disadvantage:** May overestimate viability since apoptotic cells continue to have active membranes and may appear viable.
Neutral red assay	Viable cells accumulate the red dye in lysosomes, and the dye incorporation is measured by spectrophotometric analysis. **Advantages:** Useful for certain toxicology assays **Disadvantages:** Time consuming and incubation conditions need to be optimized for each cell culture.
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay	MTT reduction is measured by the formation of a colored product, and this is indicative of biochemical activity. **Advantages:** Many tests can be performed rapidly in 96-well array in automatic plate readers. **Disadvantages:** Some inhibited cells show a low MTT reduction value that is not necessarily related to cell viability.
Fluorescein diacetate assay	Fluorescein diacetate enters the cell and is degraded by intracellular esterases, releasing fluorescein that cannot escape from cells with intact membranes, and thus the cells fluoresce when observed under UV light. **Advantages:** Rapid setup **Disadvantages:** Requirement for UV microscope or flow cytometer

techniques for studying karyology have been developed, such as Q and R banding and chromosome painting, but the Giemsa banding method described is the most widely applicable and generally useful method that has been used to characterize various stem cell culture systems. A typical method and review of other methods is given in Protocol 16.7 of Freshney [2005].

More recently, studies of hES cell cultures have revealed that they are prone to karyological changes, and a major challenge has emerged in maintaining the cells in the undifferentiated state while preserving a diploid karyotype. No culture system has been able to prevent completely the tendency of hESC lines to accumulate karyotypic abnormalities. It is felt that, owing to the nonideal culture systems for hESC lines, selection pressure is present in favor of chromosome duplications that confer an adaptive benefit [Draper et al., 2004; Andrews et al., 2005]. For hES cells there appear to be common patterns of chromosome alteration representing "adaptation" of these cells to in vitro culture conditions, notably changes involving chromosomes 12 and 17 [Draper et al., 2004]. An example of a karyotype of a normal hES cell line is shown in Figure 1.2.

It is important to verify that frozen stocks of cells retain the diploid karyotype and to check cells in use periodically to ensure that cells used to generate data for publication are

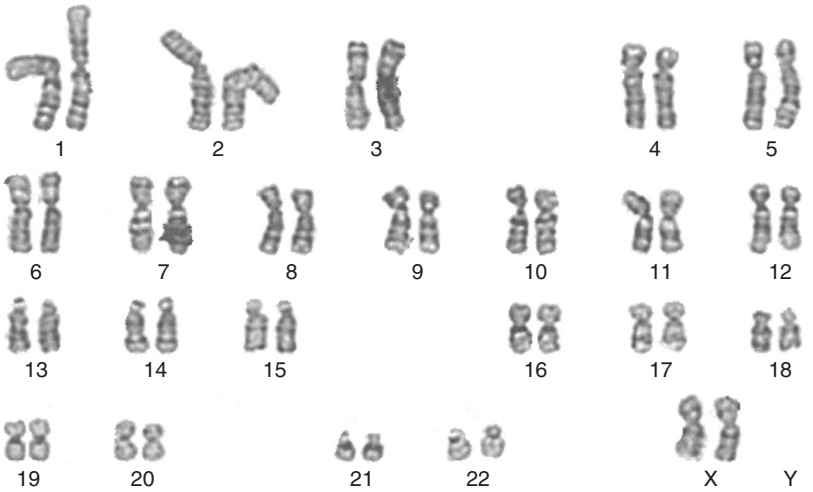

FIGURE 1.2. Diploid human ES cell karyotype.

diploid, unless deliberately studying transformed cultures. Many cytogenetics labs and some contract testing companies will provide a testing service for karyotype analysis. Routine diagnostic cytogenetic tests performed for clinical purposes often give analysis of 10–20 metaphase spreads. While such testing will identify the appearance of a transformed clone that is dominating the culture, it may not detect a low level of transformants occurring at the early stages of culture instability. It is often desirable to count at least 50 metaphase spreads in order to detect (or rule out) lower rates of mosaicism.

There are ongoing efforts to develop a chip-based or molecular assay for the karyotypic stability of hES cells in culture. One of these methods is based on single nucleotide polymorphism (SNP) genotyping. SNP arrays are very useful in mapping markers of genetic diseases and for detecting loss of heterozygosity (LOH) in cancer. Technological advances now enable the use of oligonucleotide SNP arrays to measure chromosomal copy number at high resolution [Zhao et al., 2004; Nannya et al., 2005]. This expands the utility of SNPs to detect nonreciprocal translocations, aneuploidy, and partial amplifications or deletions of chromosomes, and even amplifications or deletions of small chromosomal regions [Maitra et al., 2005]. The SNP array method has some advantages over conventional methods, mostly based on the resolution and size of genomic changes that can be detected. Based on a minimal detectable signal from 10 SNP sequences, currently available arrays of 550,000 SNPs have an effective resolution of about 28 kb, an array of 660,000 SNPs has an effective resolution of 25 kb, and, of course, increased density arrays that are sure to enter the field soon will improve this even further. One limitation that must be kept in mind, however, is the deficiency of molecular methods in analyzing heterogeneous or mosaic cell populations.

1.3.3. Identity Testing

1.3.3.1. Confirmation of Species of Origin. Isoenzyme analysis is based on measuring the charge-to-mass ratio of different isoenzyme activities using an agarose-based gel to separate the various polymorphic enzymes that can be identified for even just one enzyme reactivity. The cells are lysed, and the released enzymes are stabilized

in a buffer. Samples of this preparation are then subjected to agarose electrophoresis before the gel is treated with a specific enzyme substrate and the reaction is visualized by the formation of a purple formazan product. This method has been made more reliable with the advent of a commercial kit (AuthentiKit, Innovative Chemistry), and testing for specific enzymes can be performed within one working day. The enzymes usually used are selected for their ability to identify polymorphism between species while remaining monomorphic within species. A single enzyme test may not identify the species of origin, but certain enzyme substrates provide clear identification of the species of origin using just two or three enzyme substrates [Stacey et al., 1997; O'Brien et al., 1967; Doyle and Stacey, 2000] and may also identify embryonic isoforms. An example of an isoenzyme analysis is given in Figure 1.3.

Such typing enables rapid identification of the species of origin within one working day, and depending on the enzyme substrates used it may also allow the identification of the strain of origin for mouse cell lines. Such levels of differentiation will be valuable in a laboratory using cells from diverse species but may not be so useful in a laboratory that only cultures human cell lines.

Numerous molecular methods are now available for confirming the species of origin based on the amplification by the polymerase chain reaction (PCR) of conserved sequences [*see*, e.g., Stacey et al., 1997] and sequencing of specific genes such as cytochrome oxidase [Folmer et al., 1994; Herbert et al., 2003]. The latter method provides a sequence specific to each species that is supported by a growing database of sequence data maintained by the US National Center for Biotechnology Information [www.ncbi.nih.gov] and may well become a reference method for species identification.

Species identification is a useful part of cell authentication, and which method is used will be a decision based on the types of cell lines used in the laboratory, staff time available to carry out in-house testing, and access to appropriate facilities and equipment.

1.3.3.2. DNA Profiling for Cell-Specific Identification. Variable number tandem repeats (VNTRs) and short tandem repeats (STRs) are interesting sequences in the human genome that are comprised of repeated core units of sequences, some of which, when excised from the human genome with certain restriction enzymes, show polymorphism between individuals in the number of repeat units at a particular genomic locus. It was Alec Jeffreys who discovered that this variation might be used to identify and discriminate between human individuals by means of certain genomic probes and Southern blotting following electrophoresis [Jeffreys et al., 1985]. Other workers identified similar probes based on other VNTR sequences [Vassart et al., 1987].

Application of methods based on the hybridization of various probes to Southern blots of cell line DNA developed rapidly in the 1990s [Gilbert et al., 1990; Hampe et al., 1992; Stacey et al., 1992], and quickly these powerful methods, including PCR-based DNA profiling, revealed numerous cases of cross-contamination [e.g., MacLeod et al., 1999; van Helden et al., 1988].

STR loci consist of short, repetitive sequences, 3–7 base pairs in length. These repeats are well distributed throughout the human genome and are a rich source of highly polymorphic markers, which are routinely detected with PCR. Alleles of STR loci are differentiated by the number of copies of the repeat sequence contained within the amplified region and are distinguished from one another with radioactive, silver stain, or fluorescence detection after electrophoretic separation. Commercial kits that contain labeled primers to detect the number of repeats at 8 or 16 loci are available. These

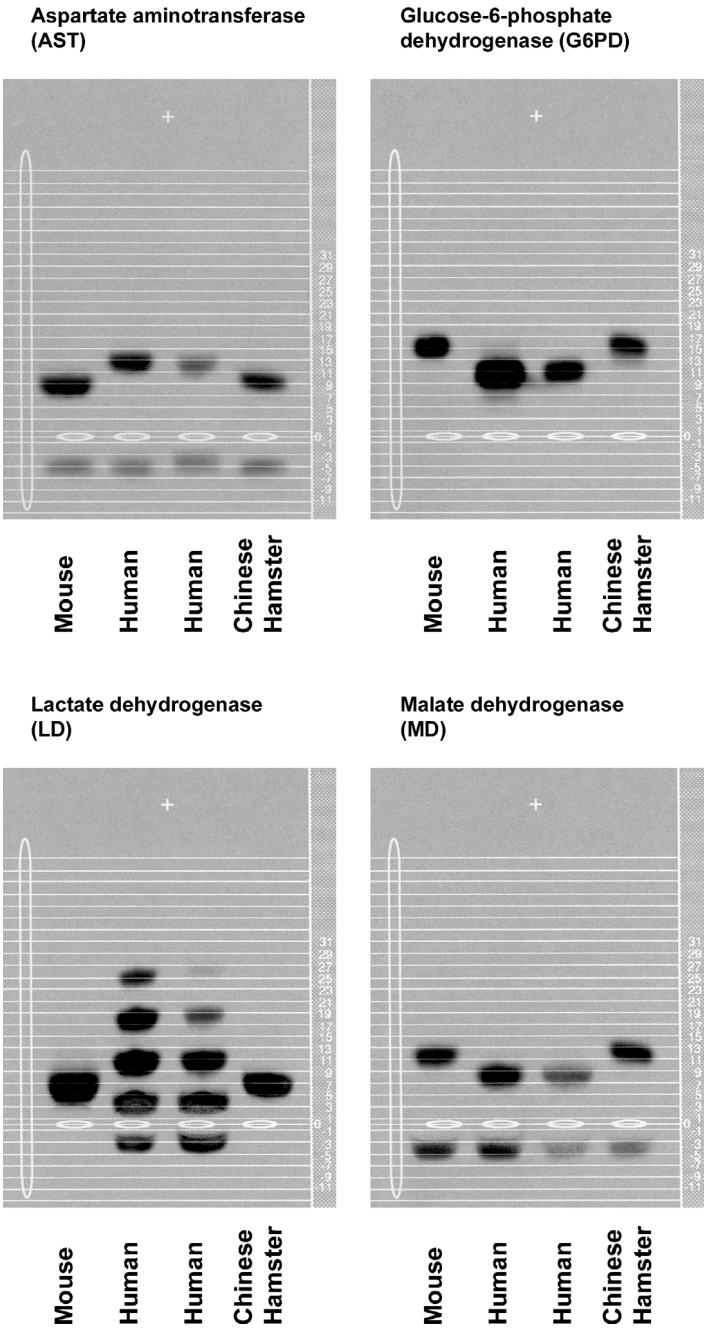

FIGURE 1.3. Isoenzyme profiles for cells from mouse, human, and Chinese hamster cell lines. (Photos and electropherograms courtesy of ATCC; modified from Freshney [2005].)

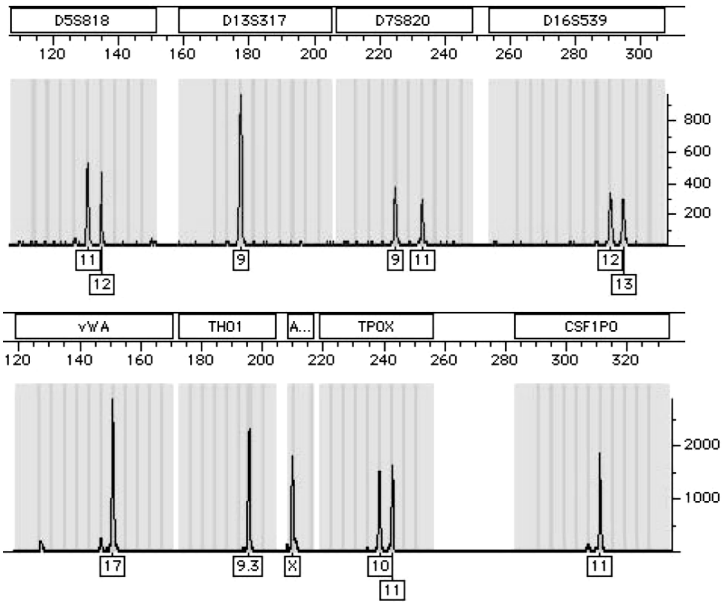

FIGURE 1.4. STR electropherograms.

loci satisfy the needs of several major standardization bodies throughout the world; for example, the US FBI has selected 13 STR core loci from the set of 16 to search or include samples in CODIS (COmbined DNA Index System), the US national database of profiles of convicted offenders. The matching probability of the 8 loci system ranges from 1 in 1.15×10^8 for Caucasian Americans to 1 in 2.77×10^8 for African-Americans, while the 16 loci system ranges from 1 in 1.83×10^{17} for Caucasian Americans to 1 in 1.41×10^{18} for African-Americans. A sample STR electropherogram is shown in Figure 1.4.

1.3.3.3. Antibody Markers. An important characteristic of any cell is its profile of antigen expression. A panel of antibodies has been commonly used to characterize hES cell antigens and show typical patterns of reactivity in such cultures (Table 1.3). Characterization of hES cells by immunophenotyping is best performed qualitatively by using immunohistochemistry together with quantitative analysis using flow cytometry. Several current markers are largely based on a single precursor (lactosylceramide) that undergoes biochemical modification including glycosylation to create the different epitopes representing the stage-specific early antigens (SSEAs) [Gooi et al., 1981; Kannagi et al., 1982, 1983]. Some of these markers have proven useful for tracking the differentiation of hES cells [Draper et al., 2002] and are key identifiers for hES cells, although they are not unique to this cell type. New markers are needed that have a direct functional relationship to stem cell biology.

It is important to take some care when sourcing antibodies for the characterization of stem cell lines. Each antibody used must be of the correct type, specificity, and titer, as described by the supplier. Fundamental approaches to this have been described elsewhere [Hybridoma Bank, USA www.uiowa.edu/~dshbwww/], but at the very least new stocks of antibody should be compared in parallel with existing acceptable stocks and antibody isotype controls should be included in experimental work. It is important

TABLE 1.3 Typical Antigenic Marker Expression of hES Cells [Draper, 2002]

Antigen	Typical Reaction on Undifferentiated hES Cells	Typical Reaction on differentiated hES Cells
SSEA-1	−	+
SSEA-3	+	+*
SSEA-4	+	+*
Oct-4	+	+*
Alkaline phosphatase	+	+*
TRA-181	+	+*
TRA-160	+	+*

*May be downregulated during differentiation [Cai et al., 2006; Draper et al., 2002]

to differentiate between antibodies that might be involved in the detailed characterization of stem cell lines. Table 1.3 identifies a number of antibody markers that might be used. In characterization of master stocks and for publications, comprehensive antigenic characterization may be required. In the case of the UK Stem Cell Bank, the panel of markers such as those highlighted in Table 1.3 are applied to both master and distribution stocks (*see* Fig. 1.1) to ensure that the material released to researchers is of an acceptable quality. Some quality control on cultures used for experimental work is clearly desirable, but it is not practical to carry out detailed profiling in this situation. Observation of hES cell morphology may give an indication of the state of differentiation in routine observation of experimental and stock cultures, but it could be valuable to have flow cytometric data on a marker of differentiation, such as SSEA-1, at critical points in the use of cultures.

1.3.3.4. Gene Expression. The study of gene expression profiles is extremely valuable but is largely just developing at a research level. The application of such tests in routine work has yet to be validated, but there are a number of genes associated with stemness that should be checked (e.g., Nanog, Oct-4) as well as testing examples of genes that are associated with differentiation. Care should be taken to avoid primers that also detect pseudogenes and could cause confusion. At this point in time it is probably not wise to set a specific panel of genes for quality control until better culture methods and knowledge of the nature of stem cells are obtained. However, it will clearly be useful to gather information on gene expression profiles on candidate stem cell genes and markers of different differentiation lineages. Examples of genes being analyzed in parallel in a current international study of over 60 hES cell lines are given at www.stemcellforum.org.uk/.

Recent research comparing the transcriptomes of multiple hES cell lines has identified a set of approximately 100 genes that are highly expressed in undifferentiated hES cells [Bhattacharya et al., 2005]. In a study characterizing 17 different hES cell lines by whole genome gene expression array analysis, almost all of the cell lines show a similar expression pattern for these 100 genes [Josephson et al., 2006]. While global gene expression can be indicative of the state of differentiation of a particular hES cell line, it is most useful when compared side-by-side with a reference standard for the particular differentiation state under investigation. For example, human embryonal carcinoma cell lines and stable, karyotypically abnormal hES cell lines have been proposed as such standards [Plaia et al., 2006]. Microarray expression data are best verified by quantitative real-time reverse transcriptase PCR (RT-PCR), using a marker set for genes commonly associated

with the hES cell differentiation state of interest. For the undifferentiated state, a few accepted markers are listed in Table 1.3.

1.3.3.5. Pluripotency. This is clearly a key measure of stem cell line performance in which the expected outcomes may vary depending on the cell type (hES, mesenchymal stem cells, etc.). There are a number of ways of measuring pluripotency including the following:

- Germ line competence (only acceptable for nonhuman stem cells)
- Teratoma formation in immunocompromized mice (*see* Chapter 6)
- Generation of embryoid bodies with the three germ layers represented (*see* Chapter 3, 4, 6)
- Differentiation in vitro into cell types representing the three germ layers (*see* Chapters 2, 6).

In hES cell research, teratoma formation is generally accepted but is not yet well standardized—molecular assays of gene expression may help in the future. This is clearly a challenging area that requires considerable research effort before reliable QC methods can be selected with confidence. (*See* Chapter 6 for protocols and a discussion of teratoma formation.)

Briefly, undifferentiated hES cells are injected into immunodeficient mice. SCID/Beige is a commonly used strain. The cells are placed either intramuscularly into the hindlimb, in the testis, or under the kidney capsule. There have not been careful comparisons of injection locations, but all three mentioned above appear permissive for pluripotent hESC differentiation. Tumors form in all mice and are excised for analysis after approximately 8–12 weeks, based on tumor size. The first analysis performed is histological, preferably performed by an experienced pathologist. In this analysis, the pathologist attempts to identify tissues representative of the three germ layers (ectoderm, endoderm, and mesoderm). This may be followed by immunohistochemical and gene expression analyses to demonstrate that the teratomas contain specific terminally differentiated lineages. Typical teratomas are generally well demarcated from the host tissue and exhibit organized clusters of cells, which may include cartilage, mineralized bone, villi, smooth muscle, nerve bundles, neural rosettes, liverlike structures, ducts, cystic epithelium-lined spaces, and various types of epithelia.

1.4. STERILITY

Bacterial and fungal contamination generally prevents work with affected cultures as they become turbid with organisms that completely overwhelm and kill the cells. Contamination can arise from a variety of sources in the laboratory environment (e.g., water baths, fridges, sinks, cardboard boxes), and avoidance of contamination is most effectively achieved with good aseptic technique, correct use of class II safety cabinets (see Appendix 1), and maintenance of a clean and tidy cell culture laboratory [Freshney, 2005, Chapter 6]. The use of antibiotics may be helpful to avoid loss of cells in circumstances where the risk of contamination is high, for example, in primary mouse embryonic feeder cultures or in routine experimental work where environmental contamination is very high. However, routine use of antibiotics for cultures that should be "clean" is not

recommended and certainly not for the preparation of cell banks. Antibiotics can affect the function of cells, and routine use of specific antibiotics can encourage the development of resistance in microorganisms, leaving no fallback treatment for protection of critical cultures. They may also suppress but not eliminate the growth of mycoplasma, increasing the risk of false-negative results in mycoplasma testing (*see* Section 1.5).

Cultures can be tested by inoculation of the supernatant medium from a culture into bacteriological broth followed by incubation at both standard cell culture temperature (typically 35–37°C) and room temperature to reveal growth of contaminants with different optimal growth temperatures (see scheme in Fig. 1.5). Reliance on recognition of contamination by appearance of turbidity in inoculated broths can lead to difficulties because nonmotile organisms may not produce turbidity, while cell debris may cause turbidity. Accordingly, it is usual to subculture all broths onto solid nutrient agar media at the termination of their incubation period to detect any culturable organisms.

Detailed reference methods for this approach are published in national pharmacopoeia [European Pharmacopoeia, 2006a; US Food and Drug Administration, 2005a].

In addition to these standard culture methods, there are a number of kits available from tissue culture companies that may have useful applications in stem cell work. Such methods may also have valuable application alongside traditional culture methods because some are more rapid and may detect more fastidious organisms that can arise in cell cultures but would not be detected by the standard sterility tests described above. However, the range of organisms that could potentially be isolated in broth and agar cultures can be significantly expanded by supplementing these tests with additional growth media incubated in a CO_2-containing atmosphere [Cobo et al., 2005 and the references

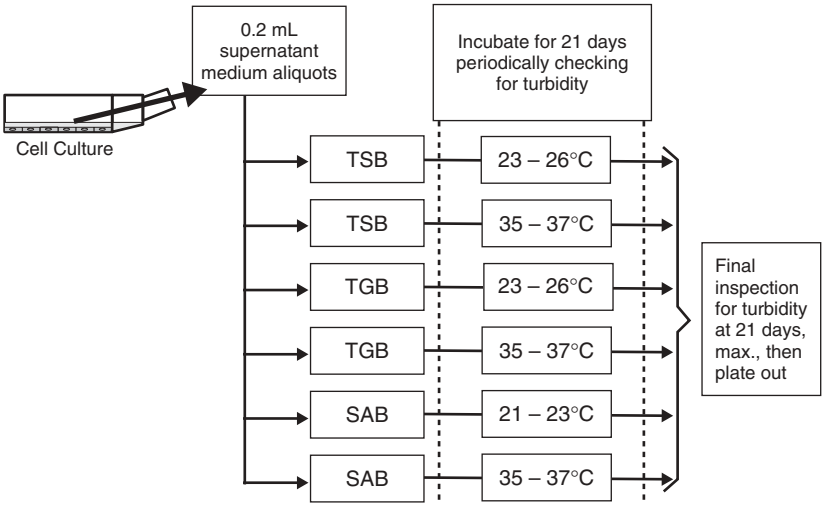

FIGURE 1.5. Example of a typical sterility test. TSB, trypticase soy broth used to isolate aerobic and facultative aerobic organisms. TGB, thioglycollate broth used to isolate anaerobic and microaerophilic organisms. SAB, Sabourard's broth used for isolation of fungi. Alternative media could include Todd–Hewitt broth (instead of TSB), brain-heart infusion broth (instead of TGB), and YM broth (instead of SAB). Additional media may be added that contain blood or serum (e.g., nutrient agar incorporating 5% defibrinated rabbit blood) [Cour, 2000].

therein]. By far the most common fastidious contaminants are mycoplasma species, and specific tests for these organisms are discussed in the next section.

1.5. MYCOPLASMA TESTING

Mycoplasmas are organisms of the order *Mollicutes*, which are much smaller than typical bacteria and, while similar to bacteria, have a number of distinct characteristics that give them special potential to cause problems in cell culture work. They have a degree of resistance to the antibiotics normally used in cell culture and can pass through standard bacteriological filters. Furthermore, they do not necessarily affect the growth rate of cells and do not usually produce visual turbidity in the supernatant medium of a contaminated culture, and thus may go unnoticed. Persistent contamination with mycoplasma can cause a diverse range of permanent deleterious effects on cell lines, and it is vital to carry out routine screening of new cell cultures coming into the laboratory to avoid potential spread to other cultures.

There are a number of techniques for mycoplasma detection that have been used widely, and examples are given in Table 1.4 [for reviews *see* Del Guidice and Gardella, 1984; Rottem and Naot, 1998]. The reference methods used in industry are Hoechst 33258 staining of Vero cells inoculated with culture supernate and culture using selective broth and agar media [European Pharmacopoeia, 2006b; US Food and Drug Administration, 2005b]. For routine screening, direct PCR or Hoechst 33258 staining (*see* Table 1.4) are useful, but these methods are generally not as sensitive in routine use as culture, which, in the absence of a more sensitive method, should be used for any important frozen stocks of cells that will be needed for future use. A scheme for a typical culture method and Hoechst 33258 staining is given in Table 1.5, which shows the added benefit of dual testing to give early screening results that will be confirmed some time later by the more sensitive culture method. It should be noted, however, that a considerable degree of skill is required to culture mycoplasma, and the need to include a positive control may not be acceptable in some laboratories without proper quarantine facilities. There are also a variety of proprietary methods available on the market, but, as indicated for sterility test kits (*see* Section 1.4), it is important to test these against a standard proven methodology before putting full confidence in them. This is important because the long-term consequences of missing positive cultures can be catastrophic in terms of wasted technical time and effort and invalidation of scientific data.

1.6. OTHER MICROBIAL CONTAMINANTS AND POTENTIAL BIOHAZARDS

A broad range of microorganisms could potentially contaminate human stem cell lines, and it would be impractical and too expensive to screen all cell lines for all of these organisms. While the future use of stem cell lines for therapy would require intensive investigation for microbial contamination, the use of these cells for research purposes should be based on sensible precautions that apply to any unscreened human cells. These precautions include a risk assessment based on any information available on the cells, use of aseptic technique, and good cell culture practice [Coecke et al., 2005]. Generally speaking, the risk associated with such cultures will be very low; however, there is no room for complacency because these cells could potentially carry a range of viruses due

TABLE 1.4 Comparison of Different Methods for Detection of Mycoplasma

Technique	Advantages	Disadvantages
Broth and agar subculture	Highly sensitive	Bacteria may grow on selective media
	Well-established method	Will not detect nonculturable strains
	Standard methods available in national pharmacopoeia	Long incubation periods (approx. 50 days total)
Vero cell culture inoculation and DNA stain	Results in 3 days Standard method available in national pharmacopoeia.	Vero test cells must be maintained and prepared High-power (×100 objective) UV-fluorescence microscopy required. Nuclear fragments from cells and small bacteria may give false positives with inexperienced workers.
PCR	Results within 1 day Large numbers of samples readily screened	Sensitivity needs to be demonstrated and monitored carefully. Nested PCR may give rise to false positives.
6-Methylpurine deoxyriboside (6-MPDR) Added to sample and indicator culture (e.g., 3T3, Vero). Mycoplasma contamination detected due to mycoplasmal adenosine phosphorylase. Converts 6-MPDR to toxic metabolites that kill indicator cells.	Simple end point (cell death)	Indicator cells must be maintained and prepared. Five days incubation required. False negatives have been observed when compared with other methods [e.g., Uphoff et al., 1992].
Mycoplasma RNA hybridization.	Sensitivity reported to be high but may vary.	Radioactive versions require scintillation counting equipment Difficult to discriminate between negative and low positive results.

to their origin in the human reproductive tract (e.g., herpes virus, HIV, hepatitis B), and a careful combination of risk assessment (to avoid use of cells with a significantly raised risk of contamination with serious human pathogens), containment (e.g., use of sealed culture vessels, use of a Class II safety cabinet [*see* Appendix 1]), treatment of cell culture waste as if infectious), and quarantine of cell cultures newly arrived in the

TABLE 1.5 Outlines of Typical Protocols for Reference Methods for Detection of Mycoplasma

Time (days)	Broth and Agar Culture	Hoechst stain
0	1. Inoculate 200-μL samples of supernatant medium into broth and onto an agar plate (contains thallous acetate and pig serum) and incubate in a anaerobic environment.	1. Replace medium on Vero cell monolayer (on glass coverslip) with supernatant medium to be tested and incubate in 5% CO_2:95% air.
Day 3		2. Remove medium and fix monolayer with fixative (1:3 acetic acid:methanol) for 3 min. Replace with fresh fixative for a further 3 min. 3. Drain fixative, air-dry slides and immerse in 2 mL of stain (0.1 μg/mL bisbenzimide Hoechst 33258 in Hanks' BSS without Phenol Red or PBSA) and incubate for 5 min at room temperature in the dark. 4. Remove stain, add nonfluorescent mountant, and apply a coverslip. 5. Scan the stained area (100× epifluorescence) for fluorescent cell nuclei (acts as a control for stain) and for small fluorescing particles over the cytoplasm.
Days 3–5	2. Inoculate 0.2 mL of sample from broth to a selective agar plate and observe original plate.	
Day 14	3. Inoculate selective agar plate from broth and observe plates inoculated at day 0 and days 3–5.	
Day 21	4. Check for pH change in broth and subculture if altered. Observe all plates and return to incubation conditions with final observation at 28 days for each plate.	
Day 42	5. Observe any remaining plates for colonies.	

Positive cultures should be discarded along with any media and reagents used for the affected cultures. It is possible to eliminate mycoplasma from cell lines with certain antibiotics [Uphoff and Drexler, 2004]. However, success rates for complete eradication are low and the toxic effects of the antibiotics used may alter the characteristics of the cell line.

laboratory until evaluations and basic quality control have been carried out should lower the risk.

1.7. QUALITY CONTROL OF CULTURE CONDITIONS, REAGENTS, AND MEDIA

In cultures of stem cells there is great potential for variability and instability. It is helpful in dealing with these issues to try to control the variation in the nutritional and environmental influences to which the cells are subjected. Calibration and monitoring of temperature and CO_2 levels are clearly important, especially in multi-user labs, where incubators may rarely reach standard culture conditions during the working day. Use of consistent sources and consistent composition of key media and supplements is also important. In addition, any critical reagents most likely to suffer from batch-to-batch variation, such as bovine serum and serum replacement formulations, should be tested before routine use, and a batch reserved, to ensure they provide acceptable growth of cultures.

It is also important to remember that feeder cells are also a potential cause of contamination and this is a particularly high risk with primary cell feeder cultures. To avoid this significant risk of contamination it is wise to establish large stocks of cryopreserved feeder cell preparations that can be quality controlled (i.e., viability, sterility, and mycoplasma as a minimum) before use (*see* Chapter 2).

1.8. CONCLUSIONS

The culture of hES cell lines is challenging, and they are prone to variation and instability. Culture methods and characterization of the stem cell nature of these cells are still developing, and standardization of some quality control methods can be difficult. Testing cell banks for the most common contaminants (bacteria, fungi, and mycoplasma) is essential to avoid spread of contamination in the laboratory, which can have a significant impact on efficiency and quality of laboratory work and scientific data. While characterization techniques in general will develop rapidly with time, there are current markers for confirming typical expression profiles of hES cells and some that give an indication of the level of cell differentiation. Genetic characterization is also important to confirm identity and also to check for chromosomal changes that indicate overgrowth of diploid cells by transformed cells that may no longer express all the characteristics of hES cells. As the basic science develops, it will be important to be responsive to review and update quality control methods and establish more quantitative methods for phenotypic analysis that may also become important factors in the future development of stem cell therapy.

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APPENDIX 1. SOME POINTS FOR CONSIDERATION IN THE USE OF CLASS II BIOSAFETY CABINETS

- Switch on the cabinet some time before use.
- Disinfect the work surface before you begin work.
- Disinfect bottles of media, etc. as they are passed into the cabinet.
- Do not clutter the cabinet or obstruct air grills (airflow).
- Separate waste and sterile reagents (e.g., on different sides of the work area) and manipulate cultures and reagents in the central zone.
- Handle only one cell line in the cabinet at one time.
- Remove reagents and disinfect surfaces after use.
- Leave cabinet switched on for a period after use (replace front cover).
- Periodically clean the cabinet thoroughly with an appropriate disinfectant.

CHAPTER 2

HUMAN EMBRYONAL STEM CELL LINES: DERIVATION AND CULTURE

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Culture of Human Stem Cells, Edited by R.I. Freshney, G.N. Stacey and J.M. Auerbach

2.1. INTRODUCTION

The development of human embryonic stem (ES) cell technology has been heralded as the dawn of a new era in cell transplantation therapy, drug discovery, and genomics [Odorico et al., 2001]. In particular, the potential to generate banks of specific cell types for cell therapy presents one means of circumventing the significant shortage of transplantable material for a wide range of human disorders. However, there have only been a handful of published results regarding human ES cell derivation, and the number of cell lines available for research purposes has, until recently, been very limited. In addition, the routine cultivation of human ES cells still remains technically demanding, and novel reagents and techniques will have to be developed before these cells can be grown under conditions suitable for the significant scale-up and rigorous quality control that will be required to generate the large banks of cells required for human cell therapy.

This review focuses on the derivation, characterization, and routine cultivation of human ES (hES) cells and some of the issues surrounding the growth, propagation, and cryo-preservation of these cells.

2.1.1. Derivation and Cultivation of Human ES Cells

There have been a small number of published reports on the derivation of human ES cell lines, but many of the purported hES lines derived worldwide have not been rigorously characterized and assessed. For example, of the 78 lines listed on the National Institutes of Health Stem Cell Registry [http://stemcells.nih.gov/research/registry/eligibilitycriteria.asp], only 21 have been formally characterized and are currently available to the wider scientific community. For the goal of human ES cell technology to be fully realized, there needs to be more published information regarding the derivation process and the limitations of the establishment of cell lines and the initial propagation process.

Human ES cell lines have generally been derived from 6- or 7-day-old, preimplantation human blastocysts by isolation of the inner cell mass and culturing on a feeder layer of fibroblasts [Thomson et al., 1998; Reubinoff et al., 2000; Park et al., 2003; Amit et al., 2003; Mitalipova et al., 2003; Cowan et al., 2004; Fang et al., 2005; Genbacev et al., 2005], although there is at least one report of successful hES derivation using embryos cultured for up to 8 days [Stojkovic et al., 2004]. In most cases, embryos have been obtained by donation from couples undergoing routine in vitro fertilization, although human ES cell lines have also been derived from embryos specifically created for this purpose [Lanzendorf et al., 2001]. In addition, pluripotent ES cell lines have been generated from human primordial germ cells obtained from first-trimester elective terminations [Shamblott et al., 1998; Turnpenny et al., 2003], but in most cases these cells have been shown to lose pluripotency gradually within 10–12 passages of inception [Turnpenny et al., 2003].

Alternative sources of relatively high-quality human embryos for ES cell derivation are embryos screened by preimplantation genetic diagnosis (PGD) that are certain or likely to have known genetic disorders [Pickering et al., 2003]. PGD was developed as an alternative to prenatal diagnosis in order to reduce the transmission of severe genetic disease for fertile couples with a reproductive risk [for a review, *see* Braude et al., 2002]. A small cellular biopsy is made from a cohort of cleavage-stage early human embryos that have been cultured in vitro and tested for the presence of a specific genetic or chromosomal defect, allowing suitable, unaffected embryos to be selected for transfer to the uterus. Many diseases are now amenable to this approach including spinal muscular atrophy type I, cystic fibrosis, sickle cell disease, Huntington disease, and a variety of reciprocal and Robertsonian translocations. Selection of embryos by sexing is also available for serious X-linked disorders where a specific genetic test is not possible. After clinical PGD, embryos identified as being at high risk for transmission of the disease or that have given ambiguous results are sometimes available for research (subject to informed patient consent). These are often of high quality as they are derived from fertile patients and could be useful for stem cell derivation. To date, several groups have derived disease-specific hES cell lines from PGD embryos, including those encoding cystic fibrosis [Pickering et al., 2005; Mateizel et al., 2006], Huntington disease [Mateizel et al., 2006; Verlinsky et al., 2005], myotonic dystrophy type I [Mateizel et al., 2006; Verlinsky et al., 2005], and a series of hES cell lines with X-linked disorders including fragile X syndrome, adrenoleukodystrophy, and Becker muscular dystrophy [Verlinsky et al., 2005].

The general method of initiating hES cells involves the segregation of the inner cell mass from the surrounding trophoblast either through complement-mediated lysis of the trophectoderm (referred to as immunosurgery) or occasionally by physical means through the use of a laser or with glass needles [*see*, e.g., Wang et al., 2005]. More recently, there have been several reports of the successful establishment of hES cell lines without the need to isolate the inner cell mass, by plating either intact blastocysts [Heins et al., 2004; Simon et al., 2005; Genbacev et al., 2005] or developmentally earlier morulae [Strelchenko et al., 2004] directly onto feeder cells.

2.1.2. Propagation of Human ES Cells

One of the major limitations to advancement in human ES cell biology is that propagation of human ES cells is still highly laborious and technically demanding and current conditions for the growth of these cells make it impossible for large-scale cultures to be established. Until recently, most human ES cell lines have been traditionally established on mouse embryonic feeder (MEF) layers in the presence of high concentrations of nonhuman serum, and most available hES cell lines have required coculture with MEFs to maintain pluripotency and cellular expansion and to inhibit differentiation.

One means of circumventing the requirement for coculture of hES on MEFs is to use MEF-conditioned medium, but this approach still requires large numbers of MEFs as well as the use of extracellular matrix proteins to inhibit ES cell differentiation completely [Xu et al., 2001]. The reliance on mouse embryo cells and nonhuman serum in the propagation of human ES cells offers the potential for the transmission of xenogeneic pathogens, thus limiting the therapeutic application of these cell lines. A potential alternative to MEFs is the use of human fibroblasts as feeder layers. Several recent published studies have shown that hES cell lines can be generated on human fibroblast feeder layers either in the presence of animal or human serum or by using synthetic serum replacement.

For example, it appears that human fetal muscle- and adult skin-derived fibroblasts are both as effective as MEFs in supporting the proliferation and pluripotency of human ES cells previously established on mouse feeders [Richards et al., 2003]. More importantly, human fetal fibroblasts and human-derived serum were also shown to support the de novo generation of human ES cells [Richards et al., 2002], although spontaneous differentiation of hES cell colonies was accentuated after the tenth passage when they were maintained in human serum [Richards et al., 2003]. Human foreskin fibroblasts have also been shown to provide an alternative source of human feeder cells capable of maintaining proliferation and pluripotency of human ES cells previously established on MEFs [Amit et al., 2003]. In addition, unlike MEFs (used after 4–6 passages) or human fetal fibroblasts (used after 4–16 passages), human foreskin fibroblasts are capable of supporting hES cell expansion without differentiation for up to 40 passages [Amit et al., 2003]. After these reports, several groups successfully established new hES cell lines on a number of different human fibroblast populations, including those obtained from neonatal foreskin [Inzunza et al., 2005; Lysdahl et al., 2006; Ellerstrom et al., 2006] and placenta [Simon et al., 2005; Genbacev et al., 2005].

Another alternative is to derive fibroblast-like feeder cells from hES cell lines themselves. Not only have these cells been shown to promote the proliferation and maintenance of pluripotency of lines previously established on MEFs [Stojkovic et al., 2005; Wang et al., 2005], but they have also been shown to support the derivation of new hES cell lines [Wang et al., 2005].

Finally, an alternative to the use of animal serum for hES propagation is the use of various synthetic serum replacements, for example, Invitrogen's Knock-Out Serum Replacer. Many groups have shown that hES lines can be established and propagated in this medium [*see*, e.g., Lysdahl et al., 2006; Simon et al., 2005; Inzunza et al., 2005; Wang et al., 2005], but the exact formulation of this and related reagents is generally unobtainable, they are very expensive, many still contain animal products, and either mouse or human feeders or feeder-conditioned media are still required to maintain pluripotency.

However, the optimal conditions for generating and expanding hES cells to the numbers required for research and therapeutic applications will require these cells to be grown with relatively simple culture conditions, in the absence of feeders, serum additives, and extracellular matrix substrates. Unlike hES, a number of mouse ES cell lines can be grown on gelatin-coated plates without feeder layers, as long as sufficient concentrations of leukemia inhibitory factor (LIF) are provided. Human ES cells do not proliferate in the presence of LIF alone, and so identification of the mitogens and growth factors secreted by the fibroblast and other feeder cells that maintain hES pluripotency will be required. However, despite a number of biochemical and molecular approaches to identify these factors [e.g., Lim and Bodnar, 2002], to date the identity of the pluripotency growth factor(s) remains elusive, although Pyle et al. [2006] have shown that MEFs secrete neurotropins that seem to support pluripotent expansion of hES cells, even in the absence of MEFs.

Nevertheless, some progress in identifying chemically defined media that support the propagation of hES cell has been made. An extracellular matrix (ECM) such as Matrigel™ [Xu et al., 2001] or laminin and conditioned medium from MEFs or human fibroblasts can be used to propagate hES cells under feeder-free culture conditions. This reduces carryover of feeder cells for protocols that may require the cells to be feeder-free, such as for karyotyping or differentiation studies.

Yao et al. [2006] showed that hES cell lines previously derived on MEFs could subsequently be propagated feeder-free on Matrigel in defined medium containing N2 and B27 neuronal stem cell supplements and 20 ng/mL FGF-2. The limitation of this approach is that the B27 supplement is of unknown composition and Matrigel is derived from a mouse tumor cell line, so this still represents a xenogeneic system. Amit et al. [2004] found that a combination of TGF-β1, LIF, FGF-2, and fibronectin in a synthetic serum replacement induced pluripotent expansion of preexisting hES cell lines. Similarly, Xu et al. [2005] found that a combination of the bone morphogenetic protein (BMP) antagonist Noggin and FGF-2 in serum replacement and on Matrigel was similarly potent in maintaining preexisting hES cell lines in a pluripotent state, whereas other groups have found that FGF-2 synergizes with activin or Nodal in serum-free chemically defined medium to retain pluripotency [Vallier et al., 2005]. Pebay et al. [2005] discovered that a combination of sphingosine-1-phosphate with platelet-derived growth factor (PDGF) could promote hES cell expansion in completely defined medium, but this still required propagation of cells on Matrigel. Ludwig et al. [2006] recently reported the derivation of two new hES cell lines under feeder-free conditions with a fully disclosed chemically defined medium. However, one of the cell lines had an abnormal chromosomal complement (XXY) when analyzed after 4 months in culture, and the other cell line become trisomic for chromosome 12 after 7 months, suggesting that this medium formulation may not be completely competent to support long-term normal hES cell proliferation. Whether any or all of these formulations will permit derivation of new cell lines that remain karyotypically normal still remains to be determined.

Similar to mouse ES cells, human ES cell colonies are density dependent and will spontaneously differentiate when the colony gets too large. Unlike most mouse ES cell lines, however, which are passaged by protease digestion and dilution in new ES cell medium, optimal culture conditions for many human ES cell lines require passaging every 4–7 days by manual cutting of hES cell colonies into 4–8 pieces with finely drawn glass pipettes and subsequent transfer of pieces to new MEFs. Although this process is physically demanding and technically challenging, many groups including our own have found that this procedure is ideal for growing very high-quality colonies of completely undifferentiated hES cells, with fewer than 20% of the resultant colonies undergoing spontaneous differentiation after passaging. Another potential advantage of manual versus enzymatic passaging of cell lines is that the former approach appears to place considerably less stress on hES cells. In all cases where genetic instability has been reported, it may be due in part to the fact that the cell populations have been passaged with proteolytic enzymes [Draper et al., 2004], whereas manual passaging has not been reported to induce karyotypic abnormalities in hES cells, even after extended passaging [Buzzard et al., 2004; Mitalipova et al., 2005]. This highlights the need to assess routinely the karyotype of each hES cell line to ensure that the cells have not adapted to culture by undergoing chromosomal alterations. This is particularly important when hES cells that have been grown under one set of standard conditions are then subjected to a significant change in culture conditions (e.g., new medium formulation, new sources, or changes in feeder species).

2.1.3. Differentiation of Human Embryonic Stem Cells

Pluripotent ES cells are capable of generating a wide variety of somatic cell types both in vitro and in vivo. In vitro, pluripotency can be demonstrated by assessing the phenotype of cells within colonies that have differentiated spontaneously, or by growing the cells to confluence, thus promoting forced differentiation. Most differentiation protocols, however, rely on the formation of embryoid bodies, three-dimensional cell aggregates that form when ES cells are removed from either feeder layers or extracellular matrix substrate. An immunocytochemical or molecular examination of cell phenotypes within differentiated colonies or embryoid bodies will usually reveal cells from all three germ layers, endoderm, mesoderm, and ectoderm, thus verifying the pluripotent nature of individual cell lines. Routine immunocytochemical and PCR-based analyses of hES cells represent simple, rapid, and relatively inexpensive means of assessing pluripotency and multilineage differentiation of ES cells, and should be performed whenever culture conditions have been significantly altered.

An additional standard in vivo test for demonstrating pluripotency of human ES cell lines relies on the formation of small, benign solid tumors (teratomas) after the injection of ES cells into immunocompromised SCID mice. On subsequent examination, if the tumors contain cells from all three germ layers, the cell line is considered to be pluripotent. With mouse ES cells, a more instructive test is to introduce marked cells into developing mouse blastocysts, implant the embryos into surrogate carriers, and then determine the contribution of marked cells to various organs and tissues in the resulting chimeric offspring. With human ES cells, this latter approach has been avoided for ethical reasons, and so the teratoma test has been used by some, but not all, research groups to demonstrate the pluripotent nature of individual cell lines. Nevertheless, the teratoma test and several other reports of direct transplantation of ES cells highlight the potential deleterious effect of injecting undifferentiated ES cells, namely the generation of fast-growing tumors.

These observations point out the need to have a means of selecting out any latent ES cells in the cells to be transplanted so that the risk of teratoma formation in transplanted recipients is low.

2.2. CELL CULTURE MEDIUM COMPONENTS

2.2.1. Human Embryonic Stem (hES) Cell Medium

BRL-conditioned medium (*see* Section 2.2.5)	60 mL
BRLM/20FB (s*ee* Section 2.2.4)	40 mL
LIF (ESGRO), 1×10^6 U pack	100 μL to give 1000 U/mL

2.2.2. Media for Vitrification and Thawing of hES Cells

2.2.2.1. hES-HEPES Medium.

DMEM with Glutamax, no sodium pyruvate	16 mL
ESFBS [ES-grade fetal bovine serum (FBS)]	4 mL
HEPES, 1 M	0.5 mL

2.2.2.2. Sucrose Solution, 0.2 M.

Sucrose, 1 M	1 mL
hES-HEPES medium	4 mL

2.2.2.3. Sucrose Solution, 0.1 M.

Sucrose, 1 M	0.5 mL
hES-HEPES	4.5 mL

2.2.2.4. Vitrification Medium, 10%.

hES-HEPES medium	2 mL
Ethylene glycol	0.25 mL
DMSO	0.25 mL

2.2.2.5. Vitrification Medium, 20%.

hES-HEPES medium	0.75 mL
Sucrose solution, 1 M	0.75 mL
Ethylene glycol	0.5 mL
DMSO	0.5 mL

2.2.3. Mouse Embryonic Feeder Cell Medium (MEFM)

DMEM with Glutamax, no sodium pyruvate	230 mL
FBS	25 mL (10%)
Nonessential amino acids, 100×	2.5 mL
2-Mercaptoethanol, 50 mM	0.5 mL (0.1 mM)
L-Glutamine, 200 mM	2.5 mL (2 mM)

2.2.4. Buffalo Rat Liver (BRL) Medium (BRL-CM)

DMEM (high glucose, no L-glutamine)	240 mL
ESFBS (ES-grade FBS)	60 mL (20%)
Nonessential amino acids, 100×	3 mL
2-Mercaptoethanol, 14.3 M	2.2 μL (0.1 mM)

Filter through a 250-mL PES membrane filter system.

2.2.5. BRL-Conditioned Medium

BRL cells are grown to confluence in BRL-CM and the medium replaced with fresh BRL-CM, which is collected 3 days later.

All media should be filtered through a sterile PES filter of pore diameter 0.2 μm, especially conditioned-medium from other cell types. ***Note:*** growth factors and other macromolecules that may be used in differentiation studies should be prepared aseptically but should not be filtered in case they are depleted by binding to the filter.

2.3. PREPARATION OF MOUSE EMBRYONIC FEEDER (MEF) CELLS

The feeder layer cells most widely used to support hES cells are derived from mouse embryos [Thomson et al., 1998; Reubinoff et al., 2000] and referred to as mouse embryo fibroblasts (MEFs), although they are probably a more primitive mesenchymal precursor cell.

It must be noted that each hES cell line may grow differently on different feeder layers, some being more suitable than others for efficient propagation, and it generally takes 5–10 passages to ensure that the cells have adapted to the new feeders. As always, cells should be rigorously assessed for karyotypic stability and evidence of pluripotency whenever standard culture conditions are altered. Protocol 2.1 outlines the derivation of primary MEF cells.

2.3.1. Primary Culture

Mouse embryonic feeder cells can be isolated from gestational day 15.5–16.5 mouse embryos (E15.5–E16.5) and easily grown in culture, providing a reliable and consistent source of feeders that can be maintained for extended periods in liquid nitrogen. MEFs are typically ready for use within 2–5 days after thawing. Protocol 2.1 has been adapted from Nagy et al. [2003].

Protocol 2.1. Primary Culture of Mouse Embryo Feeder (MEF) Cells

Reagents and Materials

Sterile or aseptically derived

- ❑ MEFM (*see* Section 2.2.3)
- ❑ Phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBSA)

- ❑ Trypsin, 0.25% in GIBCO Solution A (*see* Section 2.9)
- ❑ Alcohol, 70%
- ❑ Culture flasks, 75 cm^2
- ❑ Petri dish, plastic, non-tissue culture grade, 10 cm
- ❑ Screw-topped centrifuge tubes, 15 mL and 50 mL
- ❑ Forceps and scissors for dissection (sterilize before use by autoclaving and store in 70% ethanol)
- ❑ Disposable scalpels, #11

Nonsterile

- ❑ Timed pregnant mouse, 15.5–16.5-day (typical strains of mice used include SV129 or C57BL)

Procedure

(a) Sacrifice the 15.5–16.5-day pregnant mouse.
(b) Wash the mouse abdomen thoroughly with 70% alcohol.
(c) Cut through the skin and tissue to expose the uterus.
(d) Remove the uterine horns and place in a 10-cm plastic Petri dish containing PBSA.
(e) Remove the embryos from the embryonic sac with a sterile scalpel and discard all other tissue including the placenta and membranes.
(f) Remove the top of the head containing the brain and discard.
(g) Dissect the embryos by making a slit along the central axis from head to tail along the front of each pup, exposing and discarding the internal organs.
(h) Place the remainder of the embryos in a 50-mL centrifuge tube and wash 4 times with PBSA.
(i) Transfer the embryo remnants to a clean 10-cm Petri dish, removing the PBSA, and mince repeatedly with a fresh sterile scalpel down to approximately 2-mm pieces.
(j) Place the minced embryos into a 15-mL centrifuge tube, add approximately 10 mL of 0.25% trypsin, and incubate at 37°C for 10–20 min.
(k) Allow the pieces to settle, remove a 5-mL aliquot of the trypsin and dispersed cells from the incubated tube, and place in a sterile 50-mL centrifuge tube.
(l) Add an equal volume of MEFM to inactivate the trypsin.
(m) Add another 5 mL of 0.25% trypsin to the original 15-mL centrifuge tube containing the embryos and incubate at 37°C for a further 10–20 min.
(n) Repeat steps (k), (l), and (m) for approximately 5 incubations, adding all aliquots of trypsin and dispersed cells to the same 50-mL centrifuge tube. Only insoluble cartilage should be left in the original 15-mL tube that contained the embryos.
(o) Triturate the trypsinized cell suspension vigorously and allow remaining pieces to settle.
(p) Remove and save the supernate, discarding the sediment.
(q) Centrifuge the supernate at 1000 *g* for 5 min.
(r) Resuspend the pellet in 10 mL of MEFM, and count the viable number of cells with Trypan Blue.

(s) Seed cells at a density of 5×10^6 cells per 160-cm^2 flask, and add a further 10 mL of MEFM.
(t) Incubate flask overnight at 37°C in a 5% CO_2 incubator.
(u) The next day, replace the medium with 20 mL of fresh MEFM to remove cellular debris.
(v) Grow to 80–90% confluence before freezing (*see* Section 2.6.1).

Note: To ensure that MEFs are completely free from any microbial contamination, each new preparation of MEFs from mouse embryos should be expanded sequentially for at least 15 continuous passages in antibiotic-free medium. If no evidence of microbes is observed at this time, then the early-passage cells can be expanded, frozen down in large numbers of aliquots, and safely used for routine hES cell propagation.

2.3.2. Subculture of MEFs

Noting the passage number of MEFs is critical to maintain stock supplies and to prevent the cells from becoming senescent as previously discussed. Briefly, cells at lower passage number p0–p2 should be used to maintain stocks, and higher passage number p3 and p4 cells should be used for inactivation and hES cell support. It is essential that MEFs are not used after passage 4, as the cells can become senescent at this point and may be more difficult to expand.

MEFs should be grown in tissue culture flasks of 75 cm^2 or 225 cm^2 depending on how many feeder cells are needed. A 75-cm^2 flask of MEFs is useful when making feeder plates and should provide between two and six 4-well plates if inactivated at around 80% confluence. A 225-cm^2 flask of MEFs should be used for bulking up stock cells or for inactivation to produce a larger number of feeder plates. It is therefore essential that a good stock supply be made before hES cell work to ensure there is no shortage of feeder cells. Passaging in larger quantities with 225-cm^2 flasks will provide a good stock of MEFs more quickly; Protocol 2.2 therefore discusses volumes for passaging a 225-cm^2 flask of MEFs. For working volumes for passaging a 75-cm^2, divide all components by a factor of 3. Each 225-cm^2 flask of MEFs should be split at a ratio of 1:3 to generate a further three 225-cm^2 flasks of stock MEFs.

Protocol 2.2. Passaging of Mouse Embryo Feeder (MEF) Cells

Reagents and Materials

Sterile or aseptically prepared

- ❑ MEFs passages 0–3 of approximately 80% confluence, 75-cm^2 or 225-cm^2 flask.
- ❑ MEFM (*see* Section 2.2.3)
- ❑ Gelatin (autoclaved), 0.1%, w/v
- ❑ Trypsin, 0.25%, in GIBCO Solution A (*see* Section 2.9)
- ❑ PBSA
- ❑ Trypan Blue viability stain
- ❑ Centrifuge tube, 15 mL

- ❑ Tissue culture flasks, 3 × 225 cm^2
- ❑ Hemocytometer counting chamber

Procedure

(a) Aspirate medium from a 225-cm^2 flask and wash cells once with 10 mL of PBSA.
(b) Add 3 mL of trypsin and incubate cells at 37°C for 4 min.
(c) Remove flask from incubator and gently tap to further dislodge the MEFs.
(d) Add 6 mL of MEFM and triturate to ensure all cells are unattached and to prevent clumping.
(e) Transfer medium and suspended cells to a 15-mL centrifuge tube.
(f) Centrifuge at 1000 *g* for 5 min.
(g) Add 5 mL of 0.1% gelatin to each of the 3 fresh sterile 225-cm^2 flasks into which the MEFs will be passaged. Incubate with gelatin for a minimum of 5 min at room temperature.
(h) Remove MEFs from the centrifuge and aspirate the medium. Tap the tube to disperse the pellet and resuspend in 3 mL of fresh MEFM. Triturate with a pipette to ensure a single-cell suspension; essential for consistent cell growth in the passaged flasks.
(i) Remove the 5 mL of 0.1% gelatin from each of the new flasks and replace with 25 mL of MEFM.
(j) Split the 3 mL of MEF cells into three 225-cm^2 flasks.
(k) Each 225-cm^2 flask should also subsequently be split at a 1:3 ratio at the next passage.

From each passage (p0–3) MEFs can be frozen to maintain a stock of cells or can be passaged to provide feeder cells for hES cell propagation (*see* Section 2.3.5).
After passage 4, MEFs should be discarded as they are likely to senesce or transform.

2.3.3. Freezing MEF Cells

Maintaining a large frozen stock of high-quality competent MEFs at varying passage numbers (p0–p4) is essential if they are to be used in hES cell culture. Early passage number cells (p0–p2) should be used as the main seed stocks, and these can be subsequently passaged and frozen again to provide a large number of higher passage using stocks (p3 and p4) for the day-to-day maintenance of hES cells.. A 75-cm^2 culture flask of 80% confluent MEFs will produce enough cells for cryostorage in one vial of stock cells, and a 225-cm^2 flask will produce three cryovials.

Protocol 2.3. Cryopreservation of Mouse Embryo Feeder Cells

Reagents and Materials

Sterile or aseptically prepared

- ❑ A 75-cm^2 or 225-cm^2 tissue culture flask containing MEFs, p0–p4, 80–90% confluence.

- ❑ MEFM (*see* Section 2.2.3)
- ❑ Trypsin, 0.25%, EDTA, in GIBCO Solution A (*see* Section 2.9)
- ❑ ESFBS
- ❑ DMSO
- ❑ PBSA
- ❑ Centrifuge tube, 15 mL
- ❑ Cryovials

Procedure

(a) Remove medium from a flask containing MEFs and wash cells once with 5 mL of PBSA per 75-cm^2 flask.

(b) Add 1 mL of trypsin per 75-cm^2 flask, ensuring that the whole monolayer is covered, and incubate at 37°C for 4 min.

(c) Gently tap the flask to further dislodge the cells. Neutralize the trypsin with 5 mL of MEFM and triturate 5–6 times to ensure cells are dislodged and to prevent clumping. Transfer medium containing MEFs to a 15-mL centrifuge tube.

(d) Centrifuge at 1000 *g* for 5 min.

(e) Label and prepare cryovials for freezing. Labeling should include the new passage number of MEFs, the date, and any other information that may be needed. Prepare cryovials for freezing by adding 100 μL of DMSO in sterile conditions.

(f) Aspirate the supernate from the cells, taking care not to disturb the pellet.

(g) Resuspend the pellet in 900 μL of ESFBS per 75-cm^2 flask, making sure the pellet is evenly mixed by triturating with a plastic pipette.

(h) Transfer the ESFBS containing the MEFs to the cryovial containing DMSO and mix thoroughly before immediately storing at −80°C overnight.

(i) The next day, move the cryovial into liquid nitrogen for long-term storage.

2.3.4. Thawing MEF Cells

MEFs should be thawed from long-term liquid nitrogen storage and used to replenish stocks of MEFs or for inactivation in the support of undifferentiated hES cells.

Protocol 2.4. Thawing Cryopreserved Mouse Embryo Feeder Cells

Reagents and Materials

Sterile or aseptically prepared

- ❑ Single cryovial of MEFs
- ❑ MEFM (*see* Section 2.2.3)
- ❑ Gelatin, 0.1% (autoclaved and sterile)
- ❑ Tissue culture flask, 75 cm^2

- ❑ Water bath set at 37°C
- ❑ Pipettor, 1 mL

Procedure

(a) Add 3 mL of 0.1% gelatin to a 75-cm^2 flask to cover the surface to which the cells will attach. Incubate at room temperature for a minimum of 5 min.
(b) Remove 1 cryovial of MEFs from liquid nitrogen storage.
(c) Thaw the cells rapidly at 37°C in a water bath.
(d) Aspirate the 0.1% gelatin from the 75-cm^2 flask and replace with 7 mL of MEFM.
(e) Using a 1-mL pipettor, transfer the ESFBS/DMSO containing the MEFs from the cryovial into the 75-cm^2 flask containing MEFM.
(f) Incubate cells overnight at 37°C.
(g) The next morning change the medium for 7 mL of fresh MEFM to remove traces of DMSO and cellular debris.
(h) Grow the MEFs for up to 5 days or until 80% confluent, replacing the MEFM every 2 days.

2.3.5. Chemical-Based Inactivation of MEF Cells

MEFs (p0–p4) are rapidly dividing cells and thus need to be mitotically inactivated before use as a feeder layer to prevent MEF overgrowth. Inactivation of the dividing cells can be brought about through irradiation or chemical blockade, the most commonly used being mitomycin C, which is a simple and reliable method of preparing MEFs for routine hES cell culture.

Safety note: Care must be taken when handling mitomycin C as it is genotoxic. Gloves must always be worn, and the compound should only be handled in a Class II laminar flow hood (microbiological safety cabinet).

For inactivation of cells for hES cell propagation, a 75-cm^2 flask of approximately 80% confluent MEFs will generally provide between two and four 4-well plates of MEFs depending on the yield of the cells. Protocol 2.5 discusses the inactivation of a 75-cm^2 flask of MEFs or a 225-cm^2 flask; multiply reagent and medium volumes by 3.

Protocol 2.5. Growth Arrest of Mouse Embryo Feeder Cells

Reagents and Materials

Sterile or aseptically prepared

- ❑ Flask of 80–90% confluent MEFs, 75 cm^2 (or 225 cm^2)
- ❑ MEFM (*see* Section 2.2.3)
- ❑ Trypsin, 0.25%, in GIBCO Solution A (*see* Section 2.9)
- ❑ PBSA
- ❑ Gelatin, 0.1%
- ❑ Mitomycin-C (MMC), 50 μg/mL, in DMEM (filter sterilized)
- ❑ Multiwell plates, 4-well: typically 1–4 plates required per 75-cm^2 flask of MEFs

- ❑ Pipettor
- ❑ Centrifuge tubes, 15 mL

Procedure

(a) Dilute 50 μg/mL MMC 1:10 with MEFM to give 5 μg/mL.
(b) Add 0.5 mL of gelatin to each well of the 4-well plates.
(c) Remove a 75-cm^2 flask of p3 or p4 80% confluent MEFs from the incubator and wash once with 5 mL of PBSA.
(d) Incubate MEFs with 10 mL of 5 μg/mL MMC at 37°C for 2 h.

(***Note***: As the preparation of MEFs may vary, new users should determine the optimal concentration and length of MMC incubation time needed for complete inactivation of cell division).

(e) During the MMC incubation, gelatinize the 4-well plates with 0.1% gelatin for at least 5 min before use.
(f) After MMC treatment, wash the cells once with 5 mL of PBSA.
(g) Incubate the flask at 37°C with 1 mL of trypsin for 4 min.
(h) Gently tap the flask to dislodge all the cells and inactivate the trypsin with an equal volume of MEFM. Transfer to a 15-mL centrifuge tube.
(i) Make the total volume of MEFM in the 15-mL containing MEFs up to 6 mL and centrifuge at 1000 *g* for 5 min.
(j) Aspirate the supernate and suspend the MEF pellet in 5 mL of MEFM, taking care to ensure a single-cell suspension.
(k) Count the cells with a hemocytometer.
(l) Aspirate the gelatin from the 4-well plates.
(m) Seed the MEFs at 7.5×10^4 cells per well and make up the total volume of MEFM to 500 μL per well. The MEFs will attach within 6 h.
(n) Return the newly seeded plates to the incubator, ready for use the next day.

Note: MEF plates can be used to support hES cells for up to 7 days after inactivation before the cells begin to die. For best results, passage hES cells onto MEF plates within 1–3 days of inactivation.

2.4. HUMAN EMBRYONIC STEM CELL DERIVATION

Information on the use of human embryonal stem cells for research in the USA is available on the National Institutes of Health website: http://stemcells.nih.gov. In the UK the Human Fertilisation and Embryology Authority (HFEA: www.hfea.gov.uk) licenses all human embryonic stem cell derivation research projects. This regulatory body not only is responsible for granting licenses, but also oversees all ongoing human embryo research projects to ensure compliance with the strict guidelines. Human embryonic stem cell derivation requires a close partnership with an assisted conception unit in order to obtain embryos. Current legislation forbids donors to be offered financial or medical inducement for embryo donation and requires fully informed and written donor consent from both parents.

2.4.1. Embryos

To date, only a handful of laboratories worldwide have been able to derive human stem cell lines. This may be due in part to the quality of the embryos available for research and stem cell derivation. During assisted reproduction treatment, good-quality embryos are used in fertility treatment of the patients requiring treatment. Often the second-grade embryos are frozen for later patient use, and only then will the poorest-quality embryos that would not have been used for patient fertilization be donated for research [Pickering et al., 2003].

An alternative approach to obtaining high-quality embryos is the use of embryos screened for known genetic disorders with preimplantation genetic diagnosis (PGD). PGD can be used to diagnose, with a high degree of certainty, embryos containing monoallelic genetic disorders and often relies on the use of polymerase chain reaction (PCR) to amplify DNA from a single cell obtained from in vitro fertilized (IVF) eggs when they are at the 8-cell stage [Braude et al., 2002]. If affected and then donated for research, PGD embryos can thus provide a source of high-quality but genetically mutant embryos for the derivation of disease-specific cell lines.

2.4.2. Summary of Embryo Development

A more detailed account of human embryo development in vitro can be found elsewhere [Pickering et al., 2003]. Briefly; the human embryo rapidly grows from the fertilized oocyte, reaching the blastocyst stage approximately 5–6 days after fertilization. It is at the blastocyst stage that the inner cell mass can be isolated and used for hES cell derivation [Bongso et al., 1994].

2.4.3. Immunosurgery

One of the most efficient ways of increasing the probability of generating an hES cell line is to isolate the inner cell mass (ICM) from the surrounding trophectoderm by immunosurgery. This is a skilled method that is usually carried out by an embryologist and involves the use of animal-derived enzymatic products to lyse the trophectoderm and thus liberate the ICM. An alternative way of isolating the ICM is through the use of a laser, which avoids the exposure of the embryo to animal products [De Vos and Steirteghem, 2001].

The ICM can then be cultured in vitro on MEFs until a putative stem cell line emerges. Protocol 2.6 describes the technique used in the Stem Cell Biology Laboratory at Kings College London to derive five new hES cell lines.

Protocol 2.6. Derivation of Human Embryonic Stem Cell Lines

Reagents and Materials

Sterile or aseptically prepared

- ❑ Day 5–6 human blastocyst obtained through an HFEA license and with full patient consent as discussed above. Full details of HFEA requirements can be found on the HFEA website: www.hfea.gov.uk

- ❑ Pronase, 0.5%, in DMEM
- ❑ Anti-human antibody, 30–50%
- ❑ DMEM with Glutamax
- ❑ hES medium (*see* Section 2.2.1)
- ❑ Guinea pig complement diluted 1:1 with DMEM
- ❑ A single well of freshly inactivated MEFs seeded at 7.5×10^4 per well of a 4-well plate containing 500 μL of hES medium
- ❑ Wide-bore pipette

Procedure

(a) Allow the embryo to reach blastocyst stage, usually around day 5.
(b) If embryo has not hatched from the zona pellucida, incubate at 37°C with 5–10 μL of 0.5% Pronase until the zona is dissolved.
(c) The zona-free blastocyst should be exposed to 30–50% anti-human antibody diluted in DMEM with Glutamax for 10 min.
(d) After incubation, rinse the blastocyst briefly in hES medium to inactivate the anti-human antibody.
(e) Incubate the blastocyst at 37°C for 5–15 min with 5–10 μL of 20% guinea pig complement in order to lyse the trophectoderm. The embryo can be gently passed through a wide-bore pipette to help the process at this stage.
(f) When the trophectoderm is fully lysed, gently remove the intact inner cell mass with a pipette and transfer immediately onto one well of the MEFs in 500 μL of hES medium.
(g) The ICM should attach within 2–5 days and should be observed daily for outgrowth. It can be left in situ for up to 15 days, with freshly inactivated MEFs added to the well as required (only when the colony is large enough to passage should it be transferred to a fresh MEF plate).
(h) Cells with stem cell-like morphology from the ICM usually appear from the center of the colony.
(i) When the colony reaches around 0.1–0.5 mm in size it should be dissected into 2–10 pieces with a pulled glass pipette and transferred onto 2–4 wells of fresh inactivated MEF plates (*see* Protocol 2.7). This process should be repeated every 5–7 days.
(j) After the first few passages of the newly derived hES cell line, the protocols of hES cell propagation can be followed (*see* Section 2.5).

Note: As soon as there is more than a single colony, clumps of the nascent hES cell line should be frozen at every passage until large numbers of cryo-straws from each cell line have been successfully frozen (*see* Section 2.6). Minimally 50% of the first 10–15 passages should be frozen until the cell line is well-established.

2.5. HUMAN EMBRYONIC STEM CELL PROPAGATION

Most of the hES cell lines available to date have been derived and maintained on a feeder layer of support cells. As discussed above, MEFs are the most commonly used feeder

layers, but some human feeder layers are also routinely used to support the cells. Some artificial matrices can be used for derivation and support, although to date most matrices used to support hES cells are not totally free of animal products.

Propagation of hES cells is a fairly easy procedure. If cells are grown on a feeder layer, then good timing of growth-inactivated feeder plate production is essential to maintaining the hES cell colonies in the best possible undifferentiated state. Typically, hES cells should be passaged every 5–7 days onto fresh feeder cells. This can be performed mechanically, using a pulled glass pipette or incubation with glass beads or by chemical treatment with enzymes such as collagenase IV or 0.25% trypsin. The hES cells will need to have the medium changed every other day and supplemented on days in between.

The simplest way to coordinate hES cell propagation is to construct a weekly feeding and passaging plan. The following group of protocols discuss the daily maintenance of hES cells.

2.5.1. Culture on MEF Cells and Feeding Routines

Culturing hES cells on MEFs requires timing and coordination to ensure MEF plates are inactivated and ready for use when the hES cells require passaging. Each batch of MEFs can behave differently in culture and should be grown and tested before use with hES cells for full characteristics to be appreciated. Typically, MEFs can be thawed on Thursday for inactivation on Monday or Tuesday the following week, allowing the passage day for hES cells to be Tuesday or Wednesday accordingly. An example of a weekly plan can be seen in Table 2.1.

2.5.1.1. Feeding of hES Cells. The feeding routine may vary depending on individual cell lines and the conditions in which they are grown. If cultured on MEFs in four-well plates the medium should be replaced with 500 μL of fresh hES medium per well (*see* Section 2.2.1). On days between feeding, 250 μL of fresh hES medium should be added to each well.

TABLE 2.1 An Example of a Weekly Cell Maintenance Routine

	Cell maintenance		
Day	MEF cells	hES cells	
		Passaging	Feeding routine
Monday	Inactivate & plate	—	Feed
Tuesday	—	Passage	Supplement
Wednesday	—	—	Feed
Thursday	Thaw	If required	Supplement
Friday	Refeed	—	Feed
Saturday	—	—	Double Feed
Sunday	—	—	—

Adaption of this routine should be undertaken to suit individual laboratories, ensuring fresh feeder plates are inactivated ready for hES cell passaging and that all cells have a regular feeding routine. The lab requirements of hES cell numbers will determine how often passaging should be performed. Feeding of cells will typically requires complete change of fresh media (i.e., 500 μL per well) and supplementing will require the addition of half the volume of media to each well (i.e., 250 μL per well).

On the first day after passage, 250 μL of fresh medium should be added without removing the old medium to allow the hES cells to attach to the MEFs. Cells should be double fed (i.e., 1000 μL of fresh hES medium per well) if they cannot be fed or supplemented the following day, for example, on a Saturday to support the cells until Monday.

2.5.1.2. Passaging hES Cells. Passaging hES cells involves the division of undifferentiated colonies into smaller pieces and transfer onto a new support layer or matrix to allow further outgrowth of the cells. This culture method maintains the cells in an undifferentiated state by encouraging self-renewal. This can be performed with manual techniques such as cutting the colonies with a pulled glass pipette, or dislodging the colonies with glass beads (*see* Chapters 3, 6) or by using chemicals or enzymes such as collagenase IV (*see* Protocol 2.8) to dislodge the cells from the feeder layer.

Manual Passaging. This protocol uses pulled glass pipettes to cut the hES cell colonies into smaller pieces, allowing for precise selection of undifferentiated cells within a colony. Manual passaging also reduces the potential for genetic alteration, which may be seen with repeated exposure to enzymes or chemicals [Suemori et al., 2006]. However, it is laborious and highly skill-dependent, qualities that do not lend themselves to large-scale cell culture.

Protocol 2.7. Manual Passage of hES Cells

Reagents and Materials

Sterile or aseptically prepared

- ❑ Undifferentiated hES cell colonies on MEF plates
- ❑ Fresh MEF plate (ideally within 1–3 days postinactivation)
- ❑ hES medium (*see* Section 2.2.1)
- ❑ Pipettor tips for 50 μL
- ❑ Glass Pasteur pipettes

Nonsterile

- ❑ Pipettor 100 μL, adjustable, set at 50 μL
- ❑ Gas burner with naked flame or similar
- ❑ Dissecting phase-contrast microscope, preferably with a heated stage set at 37°C housed within a category II laminar flow hood

Procedure

(a) All work must be done under sterile conditions, using surgical masks, gloves, and lab coats.

(b) First, change the MEFM on the fresh MEF plates for 500 μL of hES medium.

(c) Light the gas burner and use a blue flame to pull the glass pipettes. Gently rotate the glass pipette and pull when soft to produce a sealed narrow end no thicker than a human hair (Fig. 2.1).

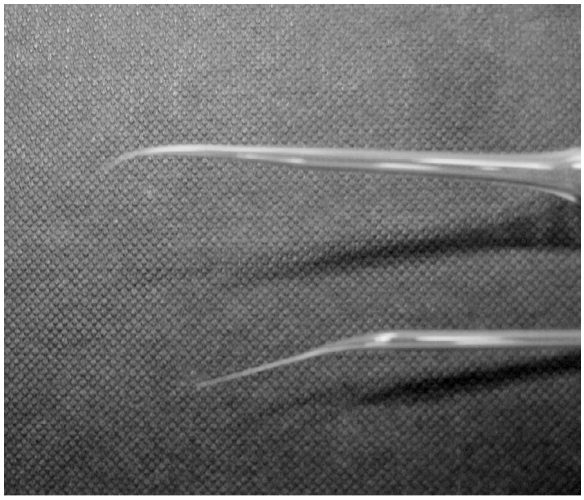

FIGURE 2.1. Examples of pulled glass pipettes. Various different pulled tips should be explored to find a preference, for example, a straight-edged or rounded-edged pipette tip.

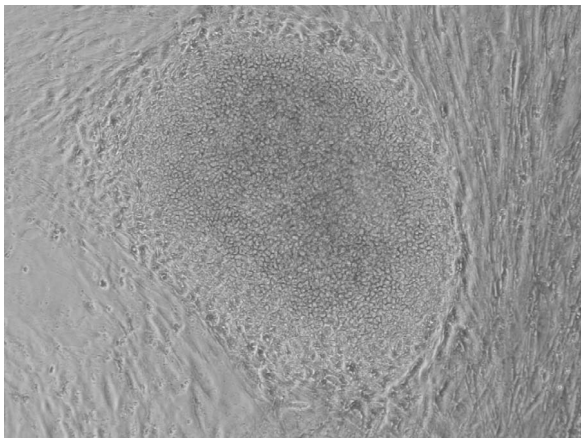

FIGURE 2.2. Typical hES cell colony. Undifferentiated cells around the edge of the colony surround more differentiated cells in the center with a brownish appearance. The colony is surrounded by growth-inactivated feeder cells.

(d) Place all pulled pipettes immediately in the laminar flow hood to keep sterile. Pipettes should only be pulled at the time of passaging, to reduce the risk of contamination.

(e) Remove hES cell plate from the incubator and place on the heated stage.

(f) Select areas of the colonies that are undifferentiated by their appearance. Undifferentiated hES cells are small, round, and compact with a translucent color. Areas of brown cells that are spontaneously differentiating should be avoided (Fig. 2.2).

(g) Using the tip of a pulled pipette, cut around the area of undifferentiated cells and cut into smaller equal chunks. Typically a colony of size similar to that in Fig. 2.2 should be cut into 6–10 pieces depending on the rate of regrowth of that particular hES line.

(h) When the colony has been cut, lift the free-floating pieces with a pipettor and suitable tip.

(i) Transfer the pieces of colony onto new MEF plates. As a guide, equally distribute around 4–6 smaller pieces within a fresh MEF well.

(j) Carefully transfer the old and new hES plates back into the incubator. The pieces should be allowed to settle overnight without disturbing the plate, to allow cells to attach and not clump in the center of the plate.

(k) The following day, supplement the hES medium in both old and new plates with another 250 μL of hES medium to reduce the risk of aspirating unattached hES cells in a full feed.

Note. A fresh sterile pulled pipette should be used each time you enter a well to avoid contamination.

Enzymatic Passaging of hES Cells. This method is good for large-scale culture of hES cells, although it must be noted that genetic changes may occur to the cells under repeated exposure to enzymes.

Protocol 2.8. Subculture of hES Cells with Collagenase

Reagents and Materials

Sterile or aseptically prepared

- ❑ Undifferentiated hES cell colonies on MEF plates
- ❑ Fresh MEF plate (ideally within 1–3 days postinactivation)
- ❑ PBSA
- ❑ hES medium
- ❑ Collagenase IV solution in PBSA (200 U/mL)
- ❑ Cell scraper or pulled glass pipette (*see above*)
- ❑ Mechanical pipette and tips
- ❑ Centrifuge tubes, 15 mL

Procedure

(a) Aspirate the medium from hES plate and wash cells twice in 500 μL of PBSA per well of a 4-well plate.

(b) Add 500 μL of collagenase IV solution to each well and incubate at 37°C for 8–10 min.

(c) Use a pulled glass pipette to gently lift the colonies.

(d) Transfer the cells with a mechanical pipette into a sterile 15-mL centrifuge tube.

(e) Rinse the hES plate with 750 μL of fresh hES medium, placing any cell colonies into the same 15-mL tube.
(f) Break up the large clumps of hES cells in the 15-mL tube with a pipettor, aiming to break the colonies into 10 or more pieces.
(g) Centrifuge the cells at 750*g* for 2 min.
(h) Carefully aspirate off the supernate and resuspend the cells in 5 mL of fresh hES medium.
(i) Seed around 4–6 cell clumps per well of a fresh MEF plate.
(j) Return newly seeded hES cells to the incubator and allow attachment overnight, supplementing the medium with fresh hES medium the following day.

2.6. CRYOPRESERVATION OF hES CELLS

Human embryonic stem cells, like many other cells, can be stored long-term in liquid nitrogen. While other stem cells have been frozen successfully by the conventional slow freezing (1°C/min) and rapid thawing (*see* Chapters 6, 9, 10), to date the most common process of preparing hES cells for freezing is by vitrification.

2.6.1. Vitrification of hES Cells

This method uses a stepwise increasing concentration of sucrose and DMSO freezing solutions to help preserve the cells and to reduce ice crystal formation within the cells. The thawing process is very similar and uses a stepwise concentration to reduce sucrose and DMSO concentration, thereby thawing the cells slowly back into standard hES medium.

Protocol 2.9. Cryopreservation of hES Cells by Vitrification

Reagents and Materials

Sterile or aseptically prepared

- ❑ Undifferentiated hES cells on MEF feeder layers
- ❑ hES-HEPES medium (*see* Section 2.2.2.1)
- ❑ 10% Vitrification solution (*see* Section 2.2.2.4)
- ❑ 20% Vitrification solution (*see* Section 2.2.2.5)
- ❑ Multiwell plate, 4-well (Fig. 2.3a)
- ❑ Open pulled vitrification straws (Fig. 2.3b)
- ❑ Cryovial, 4.5 mL
- ❑ Pulled glass pipettes (*see* Protocol 2.7, Manual Passaging of hES Cells)
- ❑ Pipette tips for use at 80 μL
- ❑ Syringe with wide-bore needle (14 gauge)

Nonsterile

- ❑ Pipettor, set at 80 μL
- ❑ Phase-contrast dissecting microscope within a category II laminar flow hood, with a heated stage set at 37°C

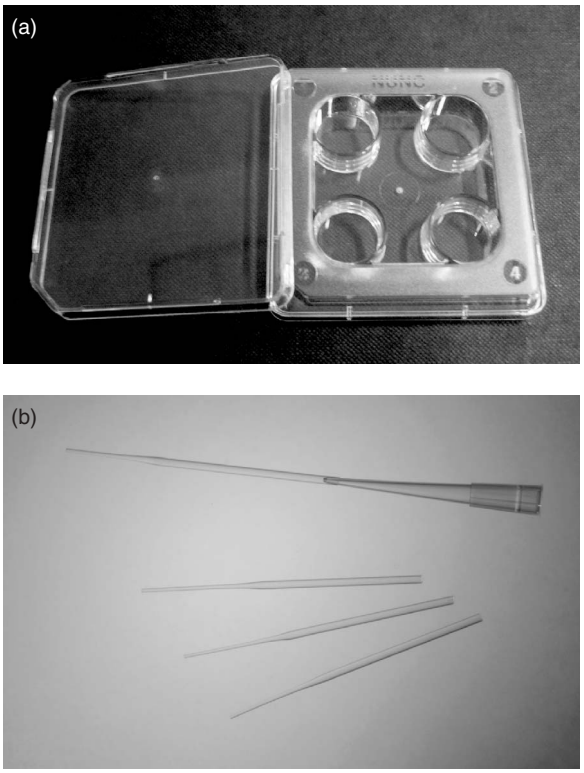

FIGURE 2.3. Vitrification plate and straws. (**a**) Four-well plate used to hold the vitrification solutions. (**b**) Vitrification straws, one attached to a pipettor tip. (Straws courtesy of LEC Instruments Pty).

- ❑ Wide-necked vacuum flask filled with liquid nitrogen
- ❑ Long-handled forceps
- ❑ Appropriate safety equipment for handling liquid nitrogen

Procedure

(a) Prepare a vitrification plate (*see* Fig. 2.3a).
Well 1: hES-HEPES medium, 1 mL.
Well 2: empty
Well 3: 10% vitrification solution, 1 mL
Well 4: 20% vitrification solution, 1 mL
Upturned lid: 10 μL of 20% vitrification solution

(b) Prewarm the plate in an incubator for 2 min before use.

(c) Pre-label the 4.5-mL cryovial with the hES cell line name, passage number, date, and any other relevant information.

(d) Carefully using a wide-bore syringe needle gently heated over a gas burner, poke a hole in the top and bottom of the 4.5-mL cryovial to allow free flow of liquid nitrogen through the tube. This will help to keep the cells consistently frozen during storage.

(e) Cut the hES cell colonies into pieces roughly twice the size required for passaging. Small pieces of colony don't tend to survive the thawing process, and therefore the correct size for each hES cell line should be determined before bulk freezing.
(f) Transfer 6–8 pieces of colony into well 1 for 60 s.
(g) Using a pipettor, transfer all of the colony pieces from well 1 into well 3 and time precisely for 60 s.
(h) Transfer the colony pieces from well 3 to well 4 for precisely 30 s. Care must be taken with timing, as the DMSO solution is toxic to cells when not frozen.
(i) Transfer the colony pieces into the 10-μL droplet.
(j) Using a pipettor, place the vitrification straw on the end of the tip (*see* Fig. 2.3b) and carefully but quickly draw up the solution containing the pieces of colony.
(k) Carefully remove the pipette tip and, using a pair of long-handled forceps, submerge the straw at a slight angle into the liquid nitrogen to snap freeze the cells.
(l) Once frozen (after around 5–10 s) place the straw in a 4.5-mL cryovial and place back in liquid nitrogen.
(m) When full, transfer the 4.5-mL cryovial containing the straws into long-term liquid nitrogen storage for recovery later.

2.6.2. Thawing hES Cells

Thawing rates of hES cells can be very variable; it is therefore advisable to have "practice runs" to determine the optimum size of colony to freeze to obtain maximum regrowth after thawing.

Protocol 2.10. Thawing hES Cells Cryopreserved by Vitrification

Reagents and Materials

Sterile or aseptically prepared

- ❑ One straw of frozen hES cell colony pieces, preferably held in a transportable liquid nitrogen container.
- ❑ Plate of MEFs (ideally inactivated 1–3 days before use)
- ❑ hES-HEPES medium (*see* Section 2.2.2.1)
- ❑ Sucrose solution, 0.1 M (*see* Section 2.2.2.3)
- ❑ Sucrose solution, 0.2 M (*see* Section 2.2.2.2)
- ❑ Multiwell plate, 4-well
- ❑ Pipette tips for 80 μL

Nonsterile

- ❑ Pipettor, set at 80 μL
- ❑ Phase-contrast dissecting microscope within a category II laminar flow hood, with a heated stage set at 37°C

- ❑ Insulated container filled with liquid nitrogen
- ❑ Long-handled forceps
- ❑ Appropriate safety equipment for handling liquid nitrogen

Procedure

(a) Prepare and prewarm a thawing plate as shown in Fig. 2.3:
Well 1, sucrose, 0.2 M _____ 1 mL
Well 2, sucrose, 0.1 M _____ 1 mL
Well 3 and well 4, hES-HEPES medium _____ 1 mL per well

(b) Collect a cryovial from the long-term liquid nitrogen store and transfer into a portable liquid nitrogen container.

(c) Using forceps, remove a single straw of hES cells and take to laminar flow hood.

(d) Working quickly, place a finger over the top and submerge the narrowed end into well 1 containing the 0.2 M sucrose.

(e) As soon as the frozen contents thaw, the hES cell clumps should be drawn into the sucrose solution (expel any remaining liquid with a pipettor).

(f) Incubate the cells for precisely 60 s, before transferring into well 2 (0.1 M sucrose).

(g) Incubate the cells in well 2 (0.1 M sucrose) for 60 s.

(h) Transfer the cells into well 3 (hES-HEPES) for 5 min.

(i) The cells can then be transferred to the last step of thawing, well 4 for 5 min.

(j) After the last step collect the hES cells with a pipettor and seed onto MEFs, as previously discussed (*see* Protocol 2.7).

2.7. CHARACTERIZATION OF hES CELLS

Molecular and biochemical characterization of hES cell lines is essential to validate and confirm the pluripotent and normal (i.e., nontransformed) properties of hES cell lines. Initial characterization should be performed as soon as is feasible after derivation once the new line has been established, and routine characterization is advisable during routine culturing of all cell lines when culture conditions are altered or to confirm that no major genetic adaptive changes, which might be indicative of chromosomal alterations, have occurred to the cells because of long-term passage.

Several factors have been shown to affect the properties of stem cells in culture including long-term passage, treatment with enzymes and chemicals, and changes in medium components. Characterization of established hES cell lines should therefore be undertaken routinely approximately every 10 passages or after changes to culture conditions.

Full characterization of hES cell lines includes an examination of cell surface marker expression and expression of known hES genes and an analysis of the chromosomal complement of each cell line. The following subsections outline three of the main methods of characterizing hES cells: immunocytochemistry for presence of cell surface markers, PCR for pluripotent gene expression patterns, and karyotyping for chromosomal complement. However, it should noted that there are additional methods of characterization that

are also often used including telomere repeat length, alkaline phosphatase studies, and teratoma formation in immunodeficient animals [for further discussion *see* Carpenter, Rosler, and Rao, 2003].

2.7.1. Immunocytochemical Characterization of hES Cells

The panel of surface markers used to characterize undifferentiated hES cells recognize specific proteins or carbohydrates expressed on the cell surface. Common markers of undifferentiated hES cells include the globoseries glycolipid antigens designated stage-specific antigen-1 (SSEA-1), SSEA-3, and SSEA-4 [Kannagi et al., 1983] and the keratin sulfate-related antigens (Trafalgar antigens) TRA-1-60 and TRA-1-81 [Andrews et al., 1984]. Other markers such as the POU transcription factor OCT-4 are also expressed in pluripotent cell populations including undifferentiated hES cells [Rosner et al., 1990]. Furthermore, it has been shown that OCT-4 expression is necessary to maintain pluripotency in ES cells [Nichols et al., 1998; Boyer et al., 2005], and therefore the loss of OCT-4 expression can be used as a marker of differentiation [Loh et al., 2006].

Stem cell markers can be identified by immunocytochemical (ICC) staining using chromogenic or fluorescent antibodies and microscopy or flow cytometry. Protocol 2.11 outlines the use of ICC as a simple screening method as it is a relatively inexpensive and rapid way of characterizing of hES cells and can be performed without the need of a flow cytometer.

Protocol 2.11. Characterization of hES Cells by Fluorescence Immunocytochemistry

Reagents and Materials

Sterile or aseptically prepared

- ❑ hES cells grown on 0.1% gelatinized glass coverslips

Nonsterile

- ❑ 4% Paraformaldehyde
- ❑ PBSA
- ❑ Tris-buffered saline (TBS^-)
- ❑ Tris-buffered saline with 0.5% Triton X-100 added (TBS^+)
- ❑ Powdered milk, 5% w/v, in deionized H_2O (dH_2O)
- ❑ Primary antibodies; SSEA-1, -3, -4, TRA-1-60, -1-81, and OCT-4
- ❑ Suitable secondary antibodies
- ❑ Mounting reagent, such as Fluorosave
- ❑ Aspirator and pipettes
- ❑ Aluminum foil
- ❑ Cardboard staining tray(s)
- ❑ Glass coverslips to fit a 24-well plate
- ❑ Plate shaker (optional)
- ❑ Fluorescent microscope and camera

Procedure

Day 1

(a) hES cells can be stained on the MEF feeder layer, because the MEFs should not express any of the hES cell markers. A 24-well plate should be prepared by seeding 5×10^5 mitomycin C-inactivated MEFs on 0.1% gelatinized 13-mm glass coverslips, as previously described (*see* Protocol 2.5).

(b) hES cells should ideally be passaged onto the coverslipped MEF cells 2–3 days before fixation to allow cells to attach and grow out as a monolayer. Allow at least 2 wells of hES cells per antibody used for staining.

(c) Remove the hES medium from coverslipped hES cells and wash once with 500 μL of PBSA.

(d) Fix the cells with 4% paraformaldehyde for 30–40 min.

(e) Remove fixative and wash cells once with 500 μL of PBSA.

(f) Add 500 μL of TBS^+ to each well and incubate at room temperature for 15 min, preferably on a plate shaker, taking care not to dislodge the cells when pipetting.

(g) Aspirate TBS^+. Repeat step (f) 4–5 times, to ensure cells are thoroughly washed. The TBS^+ will also permeabilize the cells and allow antibody to stain fully.

(h) At the final wash, nonspecific antibody binding should be blocked by incubating the cells with 500 μL of 5% powdered milk dissolved in dH_2O for 30–60 min.

(i) During this incubation period the primary antibody dilutions can be made up in 5% milk solution according to the supplier's instructions.

(j) Aspirate the milk solution and repeat the washing process from step (f) 4–5 times to ensure all nonbound antibody is removed.

(k) Incubate each well with 250–500 μL of appropriate primary antibody-5% milk solution at room temperature overnight, preferably on a plate shaker or making sure the cells are submerged.

Note: Care must be taken when pipetting and aspirating to avoid cross-contamination of antibodies.

Day 2

(a) Aspirate the primary antibody solutions, using a different pipette tip for each antibody to avoid cross-contamination.

(b) Wash the cells with 500 μL of TBS^- per well.

(c) Incubate each well with 500 μL of TBS^- for 15 min on a plate shaker.

(d) Aspirate TBS^- and repeat step (c) 4–5 times to ensure removal of the primary antibody.

(e) During the wash incubations appropriate secondary antibodies can be made up in TBS^+. Allow for 250–500 μL of antibody-TBS^+ solution per well, depending on whether a plate shaker is used. ***Note:*** if a DNA fluorochrome is being used to visualize the nucleus, step (g) should be read at this point.

(f) At the final wash, aspirate off the TBS^- and incubate the cells with 250–500 μL of antibody-TBS^+ solution per well at room temperature on a plate shaker for a minimum of 1 h.

(g) Hoechst 33258 or DAPI can be used to visualize the nucleus of the hES cells, may be added to the secondary antibody solution at an appropriate concentration, and can be incubated alongside the secondary antibody.
(h) Mount the cells in an appropriate mounting reagent, such as Fluorosave.
(i) Allow cells to dry fully before visualizing with a fluorescence microscope and camera equipment.

Note: All secondary antibodies and plates when being incubated with secondary antibodies should be wrapped in aluminum foil to prevent bleaching by daylight.

2.7.2. Characterization of hES Cells by Polymerase Chain Reaction Analysis

PCR enables a small amount of DNA to be amplified to a sufficient quantity to enable electrophoresis. This method of characterization allows the detection of genes such as OCT-4 and Nanog that are expressed only in undifferentiated hES cells. Reverse transcriptase-PCR (RT-PCR) is a way of using isolated messenger RNA (mRNA) to construct complementary DNA (cDNA). The most straightforward way to isolate mRNA easily from hES cells is to use an RNA extraction kit. RT-PCR uses a mixture of reverse transcriptase, free nucleotides, reaction buffer, and extracted RNA as a template to make cDNA, which can then be used in a standard PCR with appropriate primers for undifferentiated hES cell genes.

Protocol 2.12. RT-PCR Synthesis of cDNA in hES Cells

Reagents and Materials

Sterile of aseptically prepared

- ❑ Several pieces of hES cell colony prepared the same as for routine passaging (*see* Section 2.5.1.2)

Nonsterile

- ❑ RNA extraction kit, such as RNeasy
- ❑ 5× AMV-RT Superscript enzyme
- ❑ Buffer: AMV-RT with 10 mM $MgCl_2$
- ❑ Primers: oligo$(dT)_{18}$, 2 μg/μL
- ❑ Primers: oligo$(dN)_{10}$, 2 μg/μL
- ❑ Nucleotides: dNTPs, 2 mM
- ❑ Ribonuclease inhibitor: RNasin
- ❑ Template: extracted RNA, 2 μg
- ❑ RNAse-free water
- ❑ Heated block set at 70°C
- ❑ PCR thermal cycler
- ❑ Pipettor and tips
- ❑ Ice

Procedure

(a) Prepare an RNA template from hES cells, using an RNA extraction kit according to the manufacturer's instructions.

(b) Make a RT-PCR premixture:

dNTPs, 2 mM	1 μL
Xoligo$(dT)_{18}$	1 μL
oligo$(dN)_{10}$	1 μL
RNA template	2 μg
RNAse-free water	5 μL
Total volume	10 μL

(c) Heat the premixture to 70°C for 3 min.

(d) Immediately after heating, cool the premix rapidly on ice to prevent stable secondary structures being formed.

(e) Add to the premix:

5× AMV-RT buffer	4 μL
RNasin (to prevent RNA degradation)	1 μL
Total premix volume	15 μL

(f) Remove 2-μL aliquot of the final premix to use as a negative control in the standard PCR.

(g) Add 1 μL of AMV-RT enzyme to the premix.

(h) Transfer immediately to the thermal cycler, applying the following single cycle:

37°	10 min
42°C	30 min
52°C	20 min
80°C	10 min

(i) After heated incubations, the newly synthesized cDNA template should be diluted 5-fold with RNase-free water and is ready to use in standard PCR.

(j) The final cDNA sample can be stored at −20°C.

Protocol 2.13. Analysis of Gene Expression in hES Cells by PCR

Reagents and Materials

Sterile or aseptically prepared

- ❑ Several pieces of hES cell colony prepared the same as for routine passaging (*see* Section 2.5.1.2)

Nonsterile

- ❑ 10× *Taq* buffer (standard 25 mM $MgCl_2$)
- ❑ *Taq* DNA polymerase
- ❑ dNTPs, 2 mM

- ❑ cDNA template from RT-PCR synthesis
- ❑ Autoclaved dH_2O
- ❑ Agar powder
- ❑ Ethidium bromide
- ❑ 5× Loading dye
- ❑ Ethidium bromide buffer, 50 mM:
  dH_2O _____ 380 mL
  TBE, 1 M _____ 20 mL
  Ethidium bromide _____ 20 μL
- ❑ DNA ladder, band sizes 200 base pairs (bp) to 700 bp
- ❑ PCR thermal cycler
- ❑ Electrophoresis tank and power pack, set at 100 volts (V)
- ❑ Human undifferentiated gene primers:
  OCT-4 sense primer: 5′-GAA GGT ATT CAG CCA AAC-3′
  OCT-4 antisense primer: 5′-CTT AAT CCA AAA ACC CTG G-3′
  Predicted DNA band size for OCT-4: 650 bp
  Nanog sense primer: 5′-CAG AAG GCC TCA GCA CCT AC-3′
  Nanog antisense primer: 5′-CTG TTC CAG GCC TGA TTG TT-3′
  Predicted DNA band size for Nanog: 216 bp
- ❑ Housekeeping gene, human β-actin primers:
  Sense: 5′-ATT GGC AAT GAG CGG TTC CG-3′
  Antisense: 5′-AGG GCA GTG ATC TCC TTC TG-3′
  Predicted DNA band size for β-actin: 211 bp

Procedure

(a) Make a premix to a total volume of 20 μL per set of primers, typically:
  10× Taq buffer _____ 2 μL
  2 mM dNTPs _____ 2 μL
  cDNA template _____ 2 μL
  Taq polymerase _____ 0.5 μL
  Sense primer _____ 1 μL
  Antisense primer _____ 1 μL
  dH_2O _____ 11.5 μL
  Total volume: _____ 20 μL

(b) Place samples in PCR thermal cycler and apply the following cycle conditions:
  94°C _____ 40 s (denaturation)
  55–60°C _____ 40 s (annealing, check melting temperatures of primers)
  72°C _____ 60 s (extension)
  Cycle 30–40 times
  72°C _____ 5 min (to minimize secondary structures)

(c) While waiting for cycling to complete, make a 1.5% agar gel with ethidium bromide:
   i) Add 0.75 g of agar gel powder to 50 mL of dH_2O.
   ii) Heat carefully in a microwave until the agar has dissolved. ***Warning***: The gel gets extremely hot. Protective clothing including gloves and goggles must be worn.
   iii) Carefully add 5 μL of ethidium bromide to the mixture.
   iv) Placing a comb into a PCR gel mold, gently pour the heated mixture in. Take care not to make air bubbles as these may interfere with DNA migration through the gel.
   v) Allow the gel to cool at room temperature for 30 min until firm before removing the comb and placing in an electrophoresis tank covered with 50 mM ethidium bromide buffer.

(d) After the PCR cycles, remove the samples from the cycler.

(e) To each sample add 5 μL of loading dye and load into the gel, making sure to include the β-actin positive control sample and appropriate DNA ladder markers.

(f) Run the gel at 100 V for 30–40 min or until the DNA ladder markers have clearly separated and can easily be distinguished by UV illumination.

(g) Visualize the gel with a UV-transilluminator (wavelength 315 nm).and photograph with a camera if required.

(h) Compare any DNA fragments made in each of the samples to the DNA ladder for expected band sizes (stated above). The β-actin positive control sample should also be confirmed to have worked.

(i) DNA bands of the expected size can be easily extracted with a gel cleaning kit such as GENECLEAN® and should be confirmed by sequencing.

2.7.3. Karyotyping hES Cells

Karyotyping of hES cells is essential to determine the sex and gross chromosomal complement of any newly derived line as well as a means of determining whether chromosomal abnormalities have occurred after extended passaging or a change in culture conditions. This is usually performed by Giemsa (G)-banding of chromosome spreads that have been exposed briefly to proteinase, and can be used to detect chromosomal aberrations including translocations, deletions, and gain or loss of individual chromosomes. Like most other human cells, hES cells are diploid and should be either 46 XX or 46 XY. Because karyotype analysis is highly specialized [*see*, e.g., Rooney and Czepulkowski, 2001] it is usually contracted out to a specialist genetic research laboratory or local hospital cytogenetic laboratory and will not be detailed further here (*see* Chapter 1).

2.8. DIFFERENTIATION STUDIES USING hES CELLS

Human embryonic stem cells are derived from the inner cell mass and thus have the potential to be induced to differentiate into any cell type [further discussed by Asahara

et al., 2000]. This property of hES cells makes them an ideal cell population to further understand the control of differentiation, providing insight as to how individual cell types are specified during development and what signals or mechanisms control cell fate decisions, together with the ultimate aim of providing source populations of cells for a wide variety of human therapies.

To induce hES cells that have been grown on MEFs to differentiate, they are first configured into embryoid bodies (EBs) by manually cutting the hES colonies and then propagating the cell clusters as free-floating three-dimensional balls of cells for 3–5 days before applying inducing conditions. Protocol 2.14 describes EB formation from hES cells; specific differentiation protocols are described elsewhere (*see* Chapters 3–6).

2.8.1. Outline of hES Cell Differentiation

Directing the differentiation of hES cells can be achieved through epigenetic or genetic methods. Epigenetic manipulation uses the addition of growth factors (mitogens), contact factors such as extracellular matrices, or coculture with other cell types, to encourage fate decisions in the hES cells [Schuldiner et al., 2000 and Watanabe et al., 2005]. Genetic manipulation of hES cells by transfection with a viral vector can be used to introduce specific genes that are thought to regulate a particular fate decision [Kanda et al., 2004; Vicario and Schimmang, 2003]. In normal embryo development a combination of genetic and epigenetic patterning mechanisms act to specify regional fate and cell identity, generating distinct cell types [Pevny, 1998]. It is therefore likely that a combination of genetic and epigenetic factors will increase the yield of, for example, ES-derived neuronal cells in vitro.

Whichever methods are attempted, the first step of all differentiation protocols is the formation of EBs. This not only removes hES cells from the feeder layer (although it must be noted that some feeder layer carryover is likely), it also helps to initiate differentiation by allowing the cells to float without the contact with MEFs that may influence cell fate.

2.8.2. Embryoid Body Formation

hES cells that have been grown on a MEF feeder layer do not survive well as single cells. To begin a differentiation protocol the cells must be removed from the feeder layer and floated as a cluster of cells, typically around 300–500 cells, without being allowed to attach to a surface. When the cells are transferred onto an appropriate extracellular matrix (ECM), differentiation will be initiated within the cluster of cells, the nature depending on the growth factors that have been added or the genes that have been inserted. The outgrowth of cells that appear are atypical of hES cells.

Protocol 2.14. Generation of Embryoid Bodies from hES Cells

Reagents and Materials

Sterile or aseptically prepared

- ❑ Undifferentiated hES cell colonies on MEFs
- ❑ Pipettor, set at 50 μL

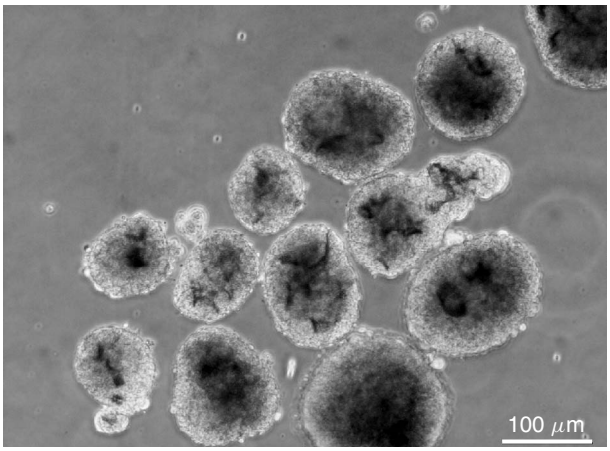

FIGURE 2.4. Free-floating EBs. Typically 300–500 cells per cluster.

- ❑ Ultra-low-attachment Petri dishes (specially coated dishes that prevent cell attachment; *see* Section 2.9), 2.6 cm
- ❑ Pulled glass pipettes (*see* Protocol 2.7)

Nonsterile

- ❑ Dissecting microscope with a 37°C heated stage

Procedure

(a) Put 2–3 mL of hES medium into an ultra-low-attachment Petri dish and pre-warm to 37°C for at least 10 min before use.

(b) Remove the undifferentiated hES cell plate from the incubator and place on the heated stage of the dissecting microscope.

(c) Using a pulled glass pipette (*see* Protocol 2.7), cut the colony into small pieces approximately the same as for manually passaging hES cells (300–500 cells). Care must be taken to dissect only the undifferentiated cells.

(d) When all the available regions of the colony have been dissected and enough cell pieces have been made, collect all the floating pieces and transfer to the low-attachment dish. The cell clumps will then form a free-floating ball of cells, embryoid bodies (EBs) (Fig. 2.4).

(e) The following morning check that the EBs have not attached to the bottom of the dish. If they have, they can be lifted gently with a pulled glass pipette and be made to float free.

(f) Incubate the EBs for 3–5 days in standard hES medium, supplementing it with 1 mL every day.

(g) To initiate differentiation of the cells, transfer the cells with a pipettor into 6-well plates or 75-cm^2 flasks containing the appropriate differentiation medium and ECM coating.

2.9. SOURCES OF MATERIALS

Item	Supplier
Agar	Sigma
Antibodies: SSEA-1, -3, -4, TRA-1-60, -1-81, OCT-4	Chemicon; Santa Cruz (OCT-4)
Antibodies, secondary	Abcam; Chemicon; Santa Cruz
Anti-human antibody	Sigma
BRL cells	ECACC
AMV-RT buffer with 10 mM $MgCl_2$	Promega
Centrifuge tubes	Corning
Collagenase IV	Invitrogen (GIBCO), Sigma
Cryovial, 4.5 mL	Corning
Culture flasks	Corning
DMEM	Invitrogen (GIBCO)
dNTPs, 2 mM	Promega
Dulbecco's modified Eagle's medium (DMEM) with Glutamax, no sodium pyruvate	Invitrogen (GIBCO)
ES-grade FBS (ESFBS)	Autogen Bioclear
ESGRO (10^6 units) murine LIF	Chemicon
Ethidium bromide	Sigma
Fetal bovine serum (FBS)	Autogen Bioclear
Fluorosave	Calbiochem-Novabiochem
Gel cleaning kit	Q-BIOgene
Gelatin	Sigma
GENECLEAN®	Q-BIOgene
Glutamax	Invitrogen
Guinea pig complement	Sigma
HEPES	Invitrogen(GIBCO)
L-Glutamine	Invitrogen (GIBCO)
Loading dye, 5×	Promega
Matrigel™	BD Biosciences
2-Mercaptoethanol	Invitrogen (GIBCO)
Mitomycin C (MMC)	Sigma
Multiwell plates	Nunc, VWR
Nonessential amino acids	Invitrogen (GIBCO)
Nucleotides: dNTPs, 2 mM	Promega
$Oligo(dN)_{10L}$	Promega
$Oligo(dT)_{18}$	Promega
PES Membrane filter system	Corning
Primers: $oligo(dT)_{18}$; $oligo(dN)_{10L}$	Promega
Pronase	Sigma
Ribonuclease inhibitor: RNasin	Promega
RNA extraction kit	Q-BIOgene
RNA extraction kit, such as RNeasy	Q-BIOgene

(*continued*)

Item	Supplier
RNase-free water	Qiagen
Sterile filters, PES	Corning
Superscript enzyme: 5× AMV-RT Superscript enzyme	Promega
Taq buffer, 10×	Promega.
Taq DNA polymerase	Promega
Tris-buffered saline	
Trypan blue	Invitrogen
Trypsin, 1:250, 0.25%, in Solution A	Invitrogen (GIBCO) 25050-014
Ultra-low-attachment Petri dishes	Corning
Vitrification straws	LEC Instruments

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CHAPTER 3

TECHNIQUES FOR NEURAL DIFFERENTIATION OF HUMAN EC AND ES CELLS

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Culture of Human Stem Cells, Edited by R.I. Freshney, G.N. Stacey and J.M. Auerbach

3.1. INTRODUCTION

Embryonic stem (ES) cells, and their malignant counterparts, embryonal carcinoma (EC) cells, satisfy many of the criteria required of cell lines for studying neuronal differentiation. They divide rapidly, are susceptible to transfection, and are able to differentiate to yield a multitude of cell types including neurons. Because of their pluripotency and uncontrolled spontaneous differentiation, ES cell cultures are typically heterogeneous. Moreover, the culture conditions for ES cells can be quite demanding, often requiring the presence of feeder cells or expensive growth factors. By contrast, EC cells, with reduced potency, are considerably easier to culture in large amounts, mostly without feeder cells, and in relatively simple medium, and their cultures may also be much less heterogeneous and so easier to standardize (*see also* Chapter 6). Consequently, studies with EC cells may be more readily reproducible, robust, and interpretable than corresponding work with ES cells. The disadvantages of EC cells are that they are tumor cells, adapted to tumor growth, genetically abnormal, and often with reduced capacity for differentiation, so that some caution is necessary in drawing wider conclusions. Nevertheless, EC cells can offer a simplified system in which to develop tools and strategies for investigating the biology of ES cells.

Since EC cells are tumor cells with grossly abnormal karyotypes and often with reduced pluripotency compared to ES cells, it could be thought that the value of EC cells as an experimental tool vanished once ES cells became available. But an alternative view is that they retain distinct experimental advantages, remaining complementary to ES cell research. The pluripotency of ES cells provides significant disadvantages as well as advantages for their use as experimental tools. The starting population of ES cells before differentiation can affect the outcome of induced differentiation. If the undifferentiated cell population contains many unnoticed spontaneously differentiated cells, this will produce discrepancies in the outcome of repeated experiments. In contrast, little spontaneous differentiation is found in EC cultures, making experimental reproducibility more feasible.

The NTERA2 cell line has been frequently studied in relation to neural differentiation, primarily because on exposure to retinoic acid it reliably yields postmitotic neurons, which develop elaborate neurite processes similar to those produced by primary neurons in culture [Andrews, 1984; Pleasure et al., 1992]. Research into mouse ES cells has often provided valuable insights into how their human counterparts behave, but there are many important species differences, highlighting the importance of using animal models as a guide rather than basing assumptions on them.

When studying the progression of human ES cells to neural progenitors and finally to terminally differentiated cells, it is useful to monitor the expression of both intracellular and cell surface markers. With respect to cell surface antigens, undifferentiated human ES cells express SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81, which are all downregulated on differentiation [Draper et al., 2004a, b]. This profile of expression is also shared by the NTERA2 EC cells. Also expressed are epitopes associated with alkaline phosphatase (readily detected by the antibody TRA-2-54 [Andrews et al., 1984]), human Thy-1, and HLA-A,B,C (Class 1 major histocompatibility complex antigens). On differentiation induced by retinoic acid, dimethyl sulfoxide (DMSO), or hexamethylenebisacetamide (HMBA), the derivative cells exhibit a very different marker profile [Draper et al., 2002], often associated with increased immunostaining with the antibodies VIN-IS-56 (GD3 and GD2), VIN-2PB-22 (GD2), and A2B5 [Eisenbarth et al., 1979]

(GT3), which are all ganglioside glycolipids. This expression profile is similar to that seen in differentiating NTERA2 cells [Draper et al., 2002; Andrews et al., 1990]. An international Stem Cell Initiative is currently underway to characterize data collected from human ES cell lines globally and to provide a resource for those wishing to work with these cell lines [Andrews et al., 2005].

Among the cell surface marker antigens that may be used to follow neural differentiation, the ganglioside glycolipids recognized by several antibodies, notably A2B5 [Eisenbarth et al., 1979; Fenderson et al., 1987] (GT3), VIN-IS-56 (GD3 and GD2), and VIN-2PB-22 [Andrews et al., 1990] (GD2) can be used to isolate prospective neural precursors. In particular, during NTERA2 differentiation, A2B5 expression has been utilized to obtain a cell population that possesses high levels of *NeuroD1* and *NSE* expression, in comparison to A2B5-negative cells [Przyborski et al., 2000]. Another surface marker, N-CAM, is a cell surface glycoprotein believed to be involved in neural cell–cell adhesion [Rutishauser et al., 1982], and antibodies to N-CAM provide useful tools for identifying cells of neural lineage.

Other markers, which are intracellular, are not so useful for purifying cell populations but are nevertheless invaluable for monitoring the progress of neural differentiation. One such marker is the class VI intermediate filament protein nestin, which is expressed during neurogenesis [Hockfield et al., 1985] and in adult neuroepithelial stem cells [Lendahl et al., 1990]. It was first identified in rat with the Rat-401 monoclonal antibody [Hockfield et al., 1985] and has been localized to many areas, such as the subventricular zone [Doetsch et al., 1997], the hippocampal dentate gyrus [Yamaguchi et al., 2000], and even the pancreas [Street et al., 2004]. It is mainly expressed in neuroepithelial progenitor cells but is also seen in other cell types such as post-injury astrocytes [Clarke et al., 1994] and is downregulated in mature neurons and mature oligodendrocytes [Gallo et al., 1995]. However, nestin is not an ideal marker for the identification of neural progenitors since it is abundantly expressed by undifferentiated ES and NTERA2 cells.

β-Tubulin III (also called β-6 tubulin) is another cytoskeletal protein that is found exclusively in neurons of higher vertebrates (except for transient expression in some embryonic structures) and is a marker of neuronal differentiation and neurite outgrowth [Schachner et al., 1981], [*see* for review Katsetos et al., 2003]. The monoclonal antibody TUJ1 is specific for this isotype and binds to the C-terminus, allowing immunostaining [Lee et al., 1990].

A series of studies on the highly conserved gene Musashi-1 have shown that it plays an important role in cell fate decision, including the maintenance of the stem cell state, differentiation, and tumorigenesis [Okano et al., 2005]. It has also been used as a marker for neural stem and progenitor cells in the human brain [Okano et al., 2005]. Rat monoclonal antibodies (Mab 14H1 and 14B8) that recognize Musashi-1 but not the Musashi-1-related protein Musashi-2 have been generated [Kanemura et al., 2001].

Oligodendrocytes can be detected by use of the mouse IgM monoclonal antibodies O1–O4, which exclusively detect cells of oligodendrocyte lineage but not neurons, astrocytes, or fibroblasts [Sommer et al., 1981]. Cells expressing these antigens can be classified as those found only on galactocerebroside-expressing oligodendrocytes, in the case of O1 and O2, or those that are present on both galactocerebroside-positive (usually after prolonged time in culture) and -negative cells, in the case of O3 and O4 [Schachner et al., 1981]. O3 and O4 are expressed a day earlier than O1 and O2 in cells cultured from the mouse pons, and they are also detected at birth in mouse cerebellum, as opposed to O1 and O2, which do not appear until 1 week postnatally [Schachner et al., 1981].

These two distinct groups also show morphological differences, as O1/O2-expressing cells are mostly of the "hairy eyeball" oligodendrocyte type, whereas O3/O4-expressing cells show simpler morphology and fewer processes [Schachner et al., 1981].

3.2. THE APPROACH OF OTHERS

Cell aggregation is a familiar method for inducing the differentiation of human EC and ES cells. The use of cell aggregation to induce the differentiation of mouse EC cells was first utilized by Martin and Evans [1975]. Since then, cell aggregation has been incorporated in differentiation protocols for many different EC (F9, P19, and NTERA2) [Cheung et al., 1999] and ES [Itskovitz-Eldor et al., 2000] cell lines. The benefit of using cell aggregation is thought to derive from increased cell contacts, promoting intercellular signaling, an important aspect of in vivo development. Other early techniques for inducing EC and ES cell differentiation utilized retinoic acid (RA) in standard serum-containing medium, but these are being replaced by better defined conditions, based on serum-free medium in the absence of RA. Such serum-free conditions have proven to be permissive for successful neuronal differentiation of murine and human ES and EC cells [Okabe et al., 1996; Zhang et al., 2001; Marchal-Victorion et al., 2003].

With the use of serum-free medium and cell aggregation, conditions originally utilized to maintain neural stem cells as "neurospheres" [Carpenter et al., 1999], it has been possible to derive NTERA2 cells that exhibit characteristics of radial glial cells (possessing BLBP, Pax6, and 3CB2 expression) [Marchal-Victorion et al., 2003]. Use of these conditions significantly boosts the number of NTERA2 neurons yielded from a traditional ~5% to over 30%.

Efforts to drive neural differentiation efficiently have also produced protocols that utilize the coculture of undifferentiated cells with other cell lines [Kawasaki et al., 2000; Saporta et al., 2004], although the neuralizing factors produced by those cells remain unidentified. Notably, NTERA2 responds in a very similar fashion to human ES cells to differentiation factors secreted by the PA6 stromal cell line, with both cell lines yielding dopaminergic neurons expressing tyrosine hydroxylase [Schwartz et al., 2005]. Thus these similarities indicate that the simpler NTERA2 system can indeed complement work with human ES cells to develop useful technologies for eventual applications in regenerative medicine.

Inducing cell differentiation through the use of conditioned medium [Schulz et al., 2004; Zeng et al., 2004] is not ideal. In order to standardize experiments, two cell lines need to be constantly monitored and maintained. Furthermore, standardizing a protocol is particularly difficult if the differentiation-inducing factor within the conditioned medium remains unknown.

To date several methods of deriving neural progenitor cells from human ES cells have been described. As mentioned above, one such method involves differentiating human ES cells into neurospheres in a multistage process. The spheres can then be maintained indefinitely if supplemented with fibroblast growth factor (FGF)-2 in suspended culture, but they can be induced to differentiate further into astrocytes, neurons, and oligodendrocytes on FGF-2 withdrawal [Zhang et al., 2001].

Other methods have been based on monolayer differentiation of both human and mouse ES cells [Conti et al., 2005; Shin et al., 2005]. One of these studies in mouse ES cells relied upon a combination of both epidermal growth factor (EGF) and FGF-2

to promote continuous proliferation, with differentiation being induced by growth factor withdrawal [Conti et al., 2005]. It also showed evidence that the resultant neural stem cell (NS) has a close relationship to radial glia, a type of neural stem cell found transiently in fetal development. Previous studies had suggested that culture in FGF-2 alone ultimately resulted in glial-restricted cell lines that could not be described as true neural stem cells [Li et al., 2001].

Another monolayer technique used in neural differentiation of human ES cells involves the use of the bone morphogenic protein (BMP) inhibitor Noggin [Gerrard et al., 2005]. Several studies have now shown that addition of Noggin to human ES cell cultures results in derivation of both mature neural and glial lineages [Pera et al., 2004] as well as neural progenitor lines [Gerrard et al., 2005]. The method used involved growing human ES cells on Matrigel in conditioned medium before EDTA treatment and culture in specified neuralizing medium supplemented with Noggin. The neural progenitor cells were then maintained for several passages before being replated as single cells, and differentiation was induced by addition or removal of growth factors.

Ultimately it can seem that human ES cells have attributes, such as pluripotency and self-renewal, that are highly desirable in relation to study and eventual therapeutic applications. Although the cellular mechanisms that govern the processes of self-renewal and differentiation are far from being understood, a great deal of information has been gathered on EC cells, murine ES cells, and stem cells and progenitors of other tissues, which can be used as a basis for study. By applying this information and further investigating processes of differentiation into neural and other tissues the potential of stem cells to provide insight into development of tissues and commitment of cells to certain lineages can be exploited.

3.3. PRINCIPLES AND ADVANTAGES OF METHODS USED

The study of ES and EC differentiation, in parallel, facilitates the development of experimental techniques. ES cells are very sensitive to induction of differentiation and cell death, so that establishing experiments can be a prolonged and frustrating process. The robust nature of EC cells and their undifferentiated phenotype similar to ES cells make them ideal for establishing experimental techniques before their ES experimentation. We have found this to be particularly true for establishing techniques such as RNAi [Matin et al., 2004].

Retinoic acid (RA) is a classic inducer of EC cell differentiation [Strickland and Mahdavi 1978]. Exposure of NTERA2 human EC cells to RA induces differentiation, marked by a loss of stem cell markers (Oct4, SSEA3, TRA-1-60) and upregulation of characteristic neural markers such as neuroD, β-III tubulin, and neurofilaments [Pleasure et al., 1992; Fenderson et al., 1987; Przyborski et al., 2000; Andrews et al., 1984, 1987]. Neuronal differentiation of RA-treated NTERA2 cells is very predictable and consistent, providing a positive control for analysis of ES neuronal differentiation.

By following a 4-week RA treatment with successive replating in the presence of mitotic inhibitors, it is possible to isolate a pure population of neurons [Leypoldt et al., 2001]. These NTERA2-derived neurons have been extensively analyzed, and it appears that they are functionally mature, to the extent that they express a variety of neurotransmitter phenotypes (catecholinergic, cholinergic, GABAergic, and serotonergic) [Squires et al., 1996; Guillemain et al., 2000] and form functional synapses [Hartley et al., 1999].

By utilizing the dependable neuronal differentiation of NTERA2 cells it is possible to establish and optimize novel techniques.

3.4. PREPARATION OF MEDIA AND REAGENTS

3.4.1. Media

3.4.1.1. DMEM/10FB and DMEM/20FB. DMEM/10FB: DMEM, 4.5 g/L glucose, with 10% fetal bovine serum.

DMEM/20FB: DMEM, 4.5 g/L glucose, with 20% fetal bovine serum.

3.4.1.2. Human Embryonic Stem Cell (hES) Medium. For approximately 500 mL:

Knockout DMEM	400 mL
Knockout serum replacement	100 mL
FGF-2	4 ng/mL
Nonessential amino acids, 100×	5 mL
β-Mercaptoethanol	3.5 µL
L-Glutamine	1.0 mM

3.4.1.3. Neurosphere Expansion Medium. For 100 mL:

DMEM/F12	99 mL
N2 supplement	1 mL
FGF-2	20 ng/mL
Insulin	20 µg/mL
Heparan sulfate sodium salt	2 µg/mL

3.4.1.4. Neurosphere Medium. For 100 mL:

DMEM/F12	97 mL
B27 supplement	2 mL
Insulin-transferrin-selenium supplement	1 mL
FGF-2	20 ng/mL

3.4.1.5. EC Cell Differentiation Medium. DMEM/10FB (*see* Section 3.4.1.1) supplemented with 10 µM all-*trans* retinoic acid (ATRA).

All-*trans* retinoic acid is light sensitive and should be handled in reduced lighting. Stocks of ATRA are conveniently stored at −20°C as 10 mM solutions in DMSO and diluted 1:1000 just before use.

3.4.1.6. EB Medium. For approximately 500 mL:

Knockout DMEM	400 mL
Knockout serum replacement	100 mL
Nonessential amino acids, 100×	5 mL
β-Mercaptoethanol	3.5 µL
L-Glutamine	1.0 mM

3.4.2. Enzymes

3.4.2.1. Collagenase. Collagenase IV, 10 mg, in 10 mL of DMEM/F12 to give 1 mg/mL final concentration. Sterilize by filtration.

3.4.2.2. Dispase. Dilute dispase stock 1:4000 in phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBSA)

3.4.2.3. Trypsin/EDTA. Trypsin, 0.05% (w/v), EDTA, 0.5 mM, in PBSA.

3.4.3. Substrates

3.4.3.1. Agarose. Agarose, 1% (w/v), in DMEM/F12 (*see* Section 3.4.1.1).

3.4.3.2. Gelatin Coating. Gelatin, 0.1% (w/v), in PBSA.

Add gelatin solution to flask or dish, leave for 5–10 min, remove, and allow dish/flask to dry.

3.4.4. Other Reagents

3.4.4.1. Flow Cytometry Wash Buffer.

(a) Fetal bovine serum, U.S. origin, 5%, in PBSA.
(b) Fetal bovine serum, U.S. origin, 5%, Triton X-100, 0.1% (v/v), in PBSA

3.4.4.2. Paraformaldehyde, 4% (w/v). Paraformaldehyde, 4 g, in 100 mL of PBSA. Mix and heat until solution is clear; store at 4°C.

3.4.4.3. Triton Solution. Triton X-100, 0.1% (v/v), fetal bovine serum, 5%, in PBSA.

3.4.4.4. Buffered Triton X-100 with Goat Serum. PBSA containing Triton X-100, 1% (v/v), glycine, 1 M, 5% normal goat serum.

3.4.4.5. Blocking Solution. FBS, 5%, Triton X-100, 0.1% (v/v), in PBSA.

3.5. CELL CULTURE

Note: All protocols should be carried out under sterile conditions in a class II laminar flow hood (microbiological safety cabinet) unless otherwise noted.

3.5.1. Maintenance of Human ES Cell Lines

Details of passaging of human ES cells can be found in Chapter 2.

3.5.2. Maintenance of NTERA2 Cell Line

NTERA2 cells are grown in Dulbecco's modified Eagle's medium (DMEM), high-glucose formulation, supplemented with 10% fetal bovine serum, in either 25-cm^2 or 75-cm^2 tissue culture flasks and maintained at 37°C in a humidified atmosphere of 10% CO_2 in air. Passage cells routinely on reaching confluence by means of mechanical detachment. Depending on cell proliferation and cell density, NTERA2 cells require passaging every 3 days by splitting a confluent flask of cells at a ratio of 1:3.

Protocol 3.1. Subculture of NTERA2 Cells

Reagents and Materials

Sterile

- ❑ DMEM/10FB (*see* Section 3.4.1.1)
- ❑ Glass beads, 3 mm
- ❑ Culture flasks, 25 cm^2 or 75 cm^2

Procedure

(a) Aspirate growth medium, leaving a small volume (~2 mL).

(b) Place sterile 3-mm glass beads in the flask and gently move from side to side, covering all the available growth surface, to detach the cells.

(c) Suspend the detached cells in 5 mL of medium and replate in 3 new flasks.

Cell passage number is limited to P55, to minimize any genotypic or phenotypic alterations.

3.6. CRYOPRESERVATION

3.6.1. NTERA2

Protocol 3.2. Cryostorage and Retrieval of NTERA2 Cells

Reagents and Materials

Sterile

- ❑ DMEM/10FB (*see* Section 3.4.1.1)
- ❑ Freezing medium: 90% FBS, 10% DMSO (v/v)
- ❑ Glass beads, 3 mm

Procedure

(a) Mechanically detach cell cultures from their flasks using glass beads and resuspend in DMEM/10FB (*see* Protocol 3.1).

(b) Place the suspension in a sterile tube and centrifuge at 1200 rpm (277 *g*) for 3 min at 4°C.

(c) Aspirate the medium and resuspend the cell pellet in freezing medium.
(d) Aliquot the cell suspension into 1-mL cryotubes.
(e) Place within a Nalgene cryo-freezing container and leave at −80°C for a minimum of 24 h.
(f) When ampoules have reached −80°C, transfer to liquid nitrogen storage.
(g) To retrieve frozen stocks from liquid nitrogen, rapidly thaw at 37°C.
(h) Wash cells in 10 mL of DMEM/10FB to remove traces of DMSO and centrifuge at 1200 rpm (277 *g*) for 3 min.
(i) Resuspend the cells in 5 mL of fresh medium and reseed into a 25 cm^2 flask.

3.6.2. Storage and Retrieval of Human ES Cells

Harvest colonies of cells in the usual manner from a confluent 25-cm^2 flask (*see* Chapter 2) and transfer to a 15-mL tube in 10 mL of medium. After this, follow the same freezing protocol as for NTERA2 cells except that centrifugation should be carried out at 800 rpm (123 *g*) for 3 min at 4°C. The same applies to thawing. *See* Chapter 2 for more details about freezing and thawing human ES cells.

3.7. DIFFERENTIATION

3.7.1. EC Cells

Protocol 3.3. Induction of Human EC Cell Differentiation by Retinoic Acid

Reagents and Materials

Sterile or aseptically prepared

- ❑ Confluent culture of NTERA2 cells
- ❑ DMEM/10FB
- ❑ ATRA, 10 mM in DMSO; from −20°C freezer
- ❑ Differentiation medium: DMEM/10FB supplemented with 10 μM ATRA (*see* Section 3.4.1.5)
- ❑ Trypsin/EDTA (*see* Section 3.4.2.3)
- ❑ Culture flasks, 75 cm^2

Procedure

(a) Incubate cells in 2 mL of trypsin-EDTA at 37°C until cells detach from the bottom of the flask.
(b) Inactivate trypsin by the addition of 8 mL of DMEM/10FB.
(c) Centrifuge at 1200 rpm (277 *g*) for 3 min.
(d) Seed cells at 1×10^6 cells per 75-cm^2 flask in differentiation medium.
(e) Replenish differentiation medium every 7 days.

The cells commit to differentiation within 2–3 days and are quite distinct in morphology and surface antigen phenotype by 7 days; neurons begin to appear during the second week.

3.7.2. ES Cells

Protocol 3.4. Human ES Cell Differentiation in Embryoid Bodies (EBS)

Reagents and Materials

Sterile

- ❑ DMEM/10FB (*see* Section 3.4.1.1)
- ❑ EB medium: human ES cell medium without FGF-2 (*see* Section 3.4.1.6)
- ❑ Collagenase IV solution, 1 mg/mL (*see* Section 3.4.2.1)
- ❑ Glass beads, 3 mm
- ❑ Petri dish, bacteriological grade, 10 cm (78.5 cm^2)
- ❑ Centrifuge tubes, 15 mL
- ❑ Gelatin-coated flasks or dishes (*see* Section 3.4.3.2)

Procedure

(a) Grow human ES cells to confluence in 25-cm^2 tissue culture flask.

(b) Aspirate the medium from the flask and add 1 mL of collagenase. Ensure that the collagenase covers the area of cell growth before placing in 5% CO_2 incubator at 37°C for 15 min.

(c) Remove the flask from the incubator and check that the colonies are curling around the edges before adding around 20 sterile glass beads to the flask.

(d) Gently shake the flask from side to side to remove the cells from the surface.

(e) Add 9 mL of DMEM/10FB and resuspend the cells by triturating 5–10 times.

(f) Remove the cell suspension from the flask and place in a sterile 15-mL tube.

(g) Centrifuge at 800 rpm (123 *g*) for 3 min at 4°C.

(h) Resuspend the pellet in 10 mL of EB medium and place the suspension in a 3.5-cm bacterial culture dish. It is essential that a bacterial dish is used to prevent attachment of the embryoid bodies (EBs) to the bottom of the dish.

(i) Place the dish in a 5% CO_2 incubator at 37°C.

(j) Continue to culture in suspension, feeding with new EB medium every 2 days.
  (i) Transfer the cells and medium to a 15-mL tube.
  (ii) Allow the EBs to settle under gravity for 10 min.
  (iii) Aspirate the spent medium and resuspend the EBs in 10 mL of fresh medium.
  (iv) Return the EBs to the dish.

(k) Plate out after 21 days of culturing in suspension, when the EBs should be sufficiently differentiated. Spontaneous differentiation is seen earlier than 21 days, but human ES cells may also be seen to persist at earlier times. Gelatin is a sufficient substrate for attachment and initial outgrowth of differentiated cells from the EBs.

(i) Remove the EBs from the dish as for feeding.
(ii) Replate the EBs on a gelatin-coated dish/flask in EB medium. The density of replating will depend on the size of the EBs (which normally varies within the culture), but about 50 EBs per 25 cm^2 is usually sufficient.
(iii) Spontaneous differentiation should be visible as outgrowths from the attached EBs as early as the day after replating.

3.7.3. Neurospheres

Protocol 3.5. Derivation of Neurospheres from Human ES Cells

(After Zhang et al. [2001])

Reagents and Materials

Sterile
- ❑ DMEM/10FB (*see* Section 3.4.1.1)
- ❑ EB medium (*see* Section 3.4.1.6)
- ❑ Neurosphere expansion medium (*see* Section 3.4.1.3)
- ❑ Collagenase IV solution (*see* Section 3.4.2.1)
- ❑ Dispase, 0.1 mg/mL (*see* Section 3.4.2.2)
- ❑ Glass beads, 3 mm
- ❑ Culture flasks, 25 cm^2
- ❑ Culture flasks, 25 cm^2, gelatin coated (*see* Section 3.4.3.2)
- ❑ Centrifuge tubes, 15 mL

Nonsterile
- ❑ Agarose

Procedure

Stage 1 (Day 0).
(a) Grow human ES cells to confluence in a 25-cm^2 tissue culture flask (*see* Chapter 2, Section 2.5).
(b) Aspirate medium from the flask and add 1 mL of collagenase IV solution, ensuring that the collagenase covers all the area of cell growth.
(c) Place the flask in a 5% CO_2 incubator at 37°C for 15 min.
(d) Remove the flask from the incubator and check that the colonies are curling around the edges.
(e) Add around 20 sterile glass beads to the flask, then shake from side to side to remove the cells from the surface.
(f) Add 9 mL of DMEM/10FB to the flask and resuspend the cells by triturating 5–10 times.
(g) Remove the cell suspension from the flask and place in a sterile 15-mL tube before centrifuging at 800 rpm (123 *g*) for 3 min at 4°C.

(h) Aspirate the supernate and resuspend the pellet in 5 mL of EB medium.
(i) Place in a non-gelatin-coated 25-cm^2 tissue culture flask and incubate in a 5% CO_2 incubator at 37°C for 4 days.
(j) Change medium daily by transferring the floating cell clusters and medium to a 15-mL tube.
(k) Allow the cell clusters (EBs) to settle by gravity for about 15 min.
(l) Aspirate the old medium and exchange it for new.
(m) Return the cell suspension to the tissue culture flask and replace it in the incubator.

Stage 2 (Day 4).
(a) After 4 days of culture in suspension, remove the embryoid bodies to a 15-mL tube and allow to settle under gravity for about 15 min.
(b) Aspirate the old medium and replace with 5 mL of neurosphere expansion medium.
(c) Place approximately 50 EBs into each of several gelatin-coated 25-cm^2 flasks and make the total amount of neurosphere expansion medium up to 10 mL per flask.
(d) Place the flasks in a 5% CO_2 incubator at 37°C for 4 days.
(e) Aspirate the old medium and replace it with 10 mL of new neurosphere expansion medium every 2 days.

Stage 3 (Day 14).
(a) After 10 days at stage 2, check the flasks under a phase-contrast microscope for the presence of neural rosettes (Fig. 3.1).
(b) Aspirate the supernate from each flask and add 1 mL of 0.1 mg/mL Dispase to each. Ensure that the dispase covers the cell layer before placing the flask in a 5% CO_2 incubator at 37°C for 15 min.
(c) Remove the flask from the incubator and gently sway from side to side in order to encourage disaggregated neural rosettes to detach.
(d) Gently add 9 mL of DMEM/F12 to each flask to dilute the dispase and remove the 10 mL (total) with neural rosette clusters to a 15-mL tube.
(e) Centrifuge the tube at 800 rpm (123 *g*) for 3 min at 4°C, then remove the supernate and resuspend the pellet in 5 mL of neurosphere medium.

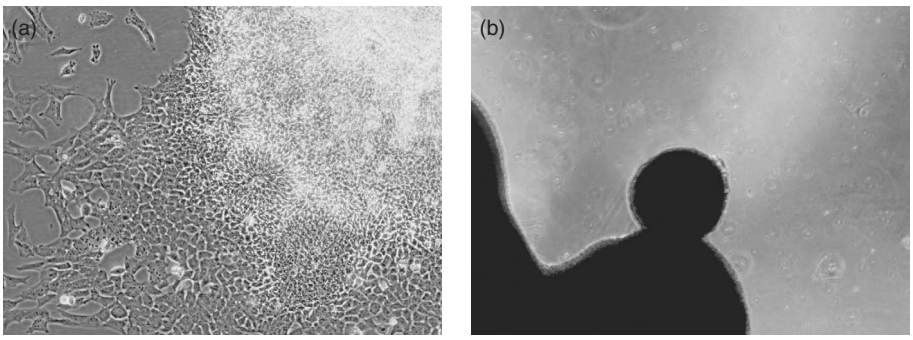

FIGURE 3.1. Neural rosettes on monolayer (**a**) and neurospheres in suspension (**b**).

Agarose Coating.

(a) Agarose coating is a cost-effective method (when compared to alternatives) of preventing unwanted attachment of suspended cultures to tissue culture flasks. While cells are incubating, prepare a 1% (w/v) agarose solution in DMEM/F12 and microwave on full power for 30–40 s or until the agarose is in solution.

(b) Allow to cool at room temperature for no longer than 10 min before coating the lower surface of the tissue culture flask with agarose. It is important that the agarose is still hot enough that it has not begun to set but not so hot that it warps the plastic of the flask.

(c) Allow this to set at room temperature for 20–30 min.

(d) Add 5 mL of neurosphere medium containing neural rosettes to each of the agarose-coated flasks.

(e) Place in a 5% CO_2 incubator at 37°C.

Protocol 3.6. Maintenance of Neurosphere Cultures

Reagents and Materials

Sterile

- ❑ Neurosphere expansion medium (*see* Section 3.4.1.3)
- ❑ Culture flasks, 25 cm^2, agarose coated (*see* Protocol 3.5, Agarose Coating)
- ❑ Centrifuge tubes, 15 mL

Procedure

Feeding

(a) Feed the neurospheres with fresh neurosphere medium every 3–5 days (depending on size of neurospheres):

  (i) Transfer the neurosphere suspension to a 15-mL tube and allow the spheres to settle under gravity for 10–20 min.

  (ii) Aspirate the old medium and add 10 mL of new neurosphere medium.

  (iii) Add the neurospheres back to the agarose-coated flask and return the flask to the 5% CO_2 incubator at 37°C.

(b) The neurospheres will tend to aggregate into a large mass over time, making replating and passaging difficult. It is therefore advisable to break up any clumps thoroughly when feeding or passaging (*see below*) and to agitate the flasks on a regular basis.

Passaging Neurosphere Cultures.

(a) Passage the neurospheres once every 2–3 weeks or when their size determines. As mentioned, the neurospheres will tend to aggregate over time and so it is important not to let too many spheres occupy the same flask.

(i) Remove the spheres from flask, allow to settle under gravity and replace with 10 mL of fresh neurosphere medium, as for routine feeding.
(ii) Prepare 2 new agarose-coated flasks for each flask being passaged.
(iii) Transfer the neurospheres in fresh medium from one tube into two 25-cm^2 flasks, making each flask up to 10 mL of medium total per 25-cm^2 flask.
(iv) Incubate in 5% CO_2 incubator at 37°C.

Protocol 3.7. Replating Neurospheres for Differentiation

Reagents and Materials

Sterile

- ❑ Neurosphere culture or agarose-coated flasks
- ❑ Neurosphere medium (*see* Section 3.4.1.4)
- ❑ Trypsin, 0.05% (w/v), in 0.5 mM EDTA
- ❑ Gelatin-coated 25-cm^2 flask (*see* Section 3.4.3.2)

Procedure

Replating

(a) Remove the required number of neurospheres from an agarose-coated 25-cm^2 flask, depending on their size and the size of the container into which they are to be plated. For example, 20 neurospheres of about 1-mm diameter would be appropriate for a 25-cm^2 flask.
(b) Place the neurospheres in a gelatin coated 25-cm^2 flask and leave to attach and grow out for 3–4 days, feeding every other day with fresh neurosphere medium. Significant neuronal differentiation can be observed at this time from individual spheres, while single cells detach, settle and proliferate throughout the flask, showing a variety of morphologies. (Fig. 3.2; Plate 1)

Passaging the Replated Neurospheres.

(a) After 3–4 days, when there is significant outgrowth of processes and coverage of the flask, aspirate the medium and add 1 mL of trypsin/EDTA for 5 min.
(b) After 5 min the cells will be seen mostly to be floating in suspension. Add a further 9 mL of DMEM/10FB to the flask and triturate the suspension several times in order to remove cells from the surface and disaggregate further any remaining clumps.
(c) Transfer the suspension to a 15-mL tube and centrifuge at 800 rpm (123 *g*) for 3 min at 4°C.
(d) Remove the supernate from the tube and resuspend the pellet in 9 mL of neurosphere medium.
(e) Add 3 mL of the cell suspension to each of 3 gelatin-coated flasks (a 1:3 split) and make up the medium in each flask to 10 mL total.
(f) Place the flasks in a 5% CO_2 incubator at 37°C.

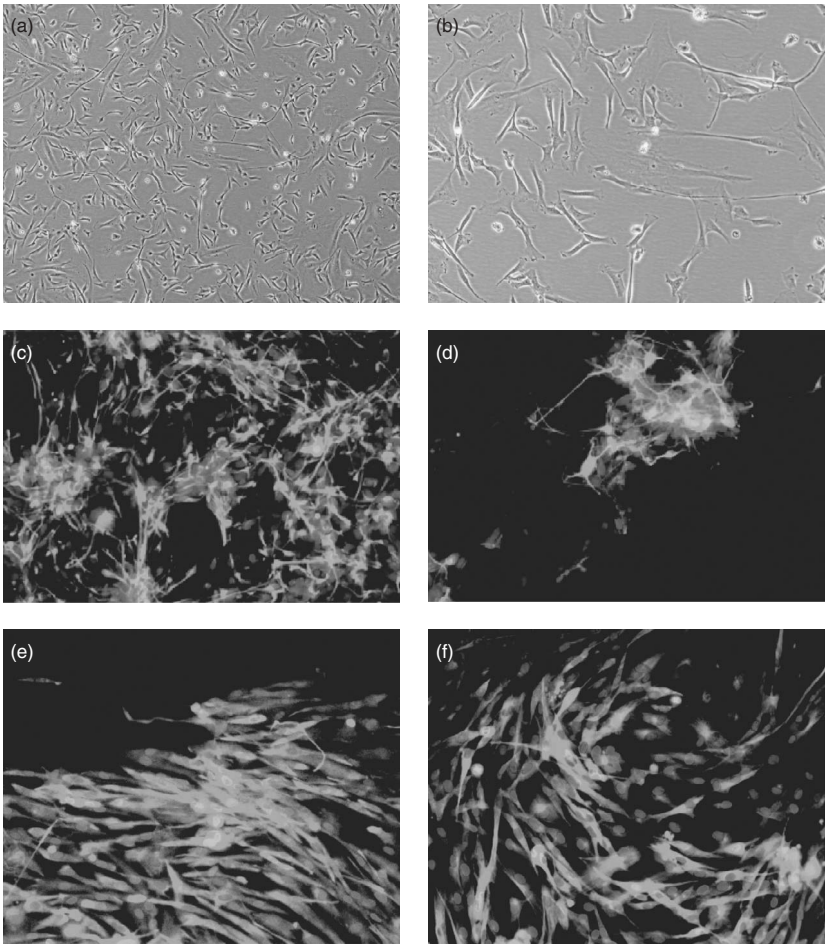

FIGURE 3.2. HUES-1 NS cells phase 4× (**a**), phase 10× (**b**), immediately after replating TUJ1 (**c**), nestin (**d**), after 3 passages TUJ1 (**e**), nestin (**f**). (*See also* Plate 1.)

(g) For the first 3 passages, or so, remnants of neurospheres will still be present in the culture. Eliminate these gradually by continued passaging until only a monolayer is present.

Maintenance of Replated Neurospheres (NS Cells).
After several passages, neurosphere-derived (NS) cells will be dominant in the culture. Although these cells cannot be described as neuronal in their morphology, they are positive for several markers of early neural cells, such as musashi-1 and nestin (*see* Fig. 3.2).

(a) Feed the cells every other day by aspirating old medium and adding 10 mL of fresh medium.

(b) Passage using 1 mL of 0.05% trypsin in 0.5 mM EDTA for 5 min every 3–4 days, using the same method as for replating neurospheres. A 1:3 split is usually sufficient for the NS cells.

3.8. ANALYSIS

3.8.1. Indirect Immunofluorescence

3.8.1.1. Intracellular Antigens.

Protocol 3.8. Antibody Staining for Intracellular Antigens

Reagents and Materials

Nonsterile

- ❑ PBSA
- ❑ Paraformaldehyde (*see* Section 3.4.4.2)
- ❑ Blocking solution: 1% (v/v) sheep serum and 0.1% (w/v) Triton X-100 in PBSA
- ❑ Primary antibody in blocking solution
- ❑ Secondary antibody in blocking solution

Procedure

(a) Wash cells briefly with PBSA.
(b) Fix with 4% paraformaldehyde at room temperature for 20 min.
(c) Wash the cells again with PBSA before a 10-min incubation with blocking solution.
(d) Aspirate the blocking solution and add blocking solution containing the relevant primary antibody at an appropriate dilution determined by titration.
(e) Incubate for 1 h at room temperature or overnight at 4°C.
(f) After the incubation with primary antibody, wash the cells 3 times in PBSA.
(g) Incubate with blocking solution containing the relevant secondary antibody for 1 h at room temperature.
(h) Stain cell nuclei with Hoechst 33342 (10 μg/mL) for 5 min.
(i) Wash in PBSA and keep in PBSA for visualization.

3.8.1.2. Bromodeoxyuridine (BrdU) Staining. An important aspect of characterizing and monitoring a pathway of differentiation is to determine whether specific cell types are postmitotic, or remain proliferative. Through the incorporation of bromodeoxyuridine (BrdU), an analog of thymidine, it is possible to assess accurately whether a cell is proliferating, that is, whether it continues to replicate DNA. With Protocol 3.9, it is possible to assess the incorporation of BrdU incorporation, in parallel with fluorescence staining for protein expression (Fig. 3.3; Plate 2). The length of BrdU exposure determines how results can be interpreted: A brief exposure of 1 hour of BrdU enables the determination of whether particular cell phenotypes are proliferative (labeling index), whereas longer exposures (24 h) allow estimation of the number of actively dividing cells (growth fraction).

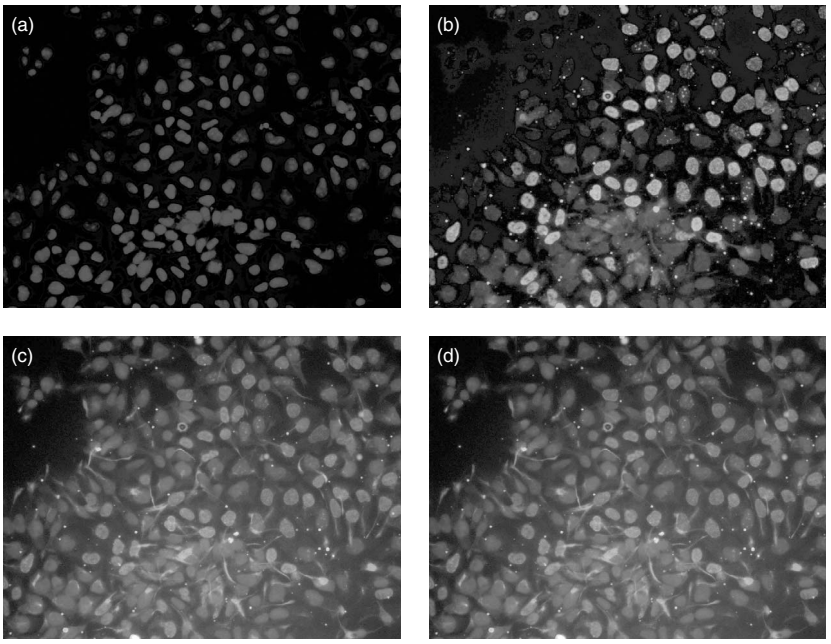

FIGURE 3.3. Undifferentiated EC cells. Exposed to BrdU for 1 h, the cells were fixed and stained for BrdU incorporation and nestin expression. (**a**) Blue, Hoechst 33342, (**b**) green, BrdU, (**c**) red, nestin, and (**d**) overlay of all three. (*See also* Plate 2.)

Protocol 3.9. Determination of Labeling Index and Growth Fraction of Differentiating Neural Cells

Reagents and Materials

Sterile

- ❑ Growing cultures of neurospheres or replated neurospheres
- ❑ PBSA
- ❑ BrdU, 10 mM, in appropriate growth medium

Nonsterile

- ❑ HCl, 1 M and 2 M
- ❑ PBSA Triton X: PBSA containing 0.1% (v/v) Triton X-100
- ❑ Borate buffer, 0.1 M
- ❑ PBSA containing Triton X-100, glycine, and normal goat serum (*see* Section 3.4.4.4)
- ❑ Anti-BrdU antibody
- ❑ Hoechst 33342, 10 μg/mL in PBSA

Procedure

(a) Remove the growth medium and replace with BrdU-containing medium.

(b) Incubate for the required period (1 h for labeling index, 24 h for growth fraction).

(c) Wash the cells in PBSA.
(d) Fix with 4% paraformaldehyde for 30 min at 4°C.
(e) Wash the cells in PBSA containing 1% Triton X-100 (3 × 5 min).
(f) Incubate the cells in 1 M HCl for 10 min on ice, to break open the DNA structure of the labeled cells.
(g) Treat with 2 M HCl for 10 min at room temperature.
(h) Incubate for 20 min at 37°C.
(i) Add borate buffer to neutralize the acid.
(j) Incubate cells for 12 min at room temperature.
(k) Wash the samples in PBSA/Triton X-100 three times for 5 min each, at room temperature.
(l) Incubate with PBSA containing Triton X-100, glycine, and goat serum for 30 min.
(m) Incubate the cells overnight at 4°C with anti-BrdU antibody or a combination of anti-BrdU and other antibodies (Nestin or TUJ1, etc.).
(n) Wash in PBSA/Triton X-100 three times for 5 min each.
(o) Cell samples can then be treated with a variety of secondary antibodies to visualize the anti-BrdU-labeled cells.
(p) Incubate cells with PBSA containing Hoechst 33342, 10 μg/mL, for 5 min.
(q) Wash and store cells in PBSA for subsequent analysis by flow cytometry.

3.8.2. Flow Cytometry

3.8.2.1. Cell Surface Antigens. A single-cell suspension of unfixed cells can be analyzed by flow cytometry after staining by indirect immunofluorescence.

Protocol 3.10. Analysis of Cell Surface Antigens of Neural Cells by Flow Cytometry

Reagents and Materials

Sterile
- ❑ Trypsin/EDTA (*see* Section 3.4.2.3)

Nonsterile
- ❑ Primary and secondary antibodies
- ❑ Flow cytometry wash buffer (*see* Section 3.4.4.1a)
- ❑ FACS buffer
- ❑ FACS tubes

Procedure

(a) Treat cells with 1 mL of trypsin/EDTA for approximately 2 min to obtain a single-cell suspension.

(b) Add 9 mL of wash buffer to the cell suspension and triturate until all small aggregates have been reduced to single cells.
(c) Determine cell concentration with a hemocytometer.
(d) Centrifuge at 1200 rpm (277 *g*) for 3 min at 4°C.
(e) Aspirate the supernate and resuspend the cells in a volume of flow cytometry wash buffer to give a cell concentration of 5×10^6 cells/mL.
(f) Add 50 μL of cell suspension to each well of a 96-well plate.
(g) Dilute antibodies to the relevant working volume (with flow cytometry wash buffer) and add 50 μL of diluted antibody to the wells containing cells. For a negative control, either omit the primary antibody or replace with the non-specific antibody produced by the parental myeloma cell line, P3X63Ag8. Alternatively, some may feel it necessary to use a class-matched antibody that does not bind to the cells.
(h) Seal the plate with a plate sealer and place on a shaker at 4°C for 1 h.
(i) After primary incubation, centrifuge the plate at 1200 rpm (277 *g*) for 3 min at 4°C.
(j) Remove the plate sealer and aspirate the antibody-containing solution, leaving the pellet in place.
(k) Wash cells three times in 100 μL of wash buffer.
(l) Dilute the secondary antibody and add 50 μL to each well containing a cell pellet, before placing back on the shaker at 4°C for 1 h.
(m) After incubation, centrifuge and wash the cells three times, before resuspending in 500 μL of FACS buffer in FACS tubes.
(n) Analyze by flow cytometry, omitting non-single cells and debris on the basis of forward scatter and dead cells on the basis of side scatter. A minimum of 10,000 cells are analyzed per sample.

3.8.2.2. Intracellular Antigens. The technique for detecting intracellular antigens (e.g., Sox2) by flow cytometry is very similar to that of analyzing cell surface antigens except for the requirement of fixing and permeabilizing the cells.

Protocol 3.11. Analysis of Intracellular Antigens of Neural Cells by Flow Cytometry

Reagents and Materials

Sterile
- ❑ Trypsin/EDTA (*see* Section 3.4.2.3)
- ❑ PBSA

Nonsterile
- ❑ Paraformaldehyde, 4% (w/v) (*see* Section 3.4.4.2)
- ❑ Blocking solution (*see* Section 3.4.4.5)
- ❑ Flow cytometry wash buffer (*see* Section 3.4.4.1b)

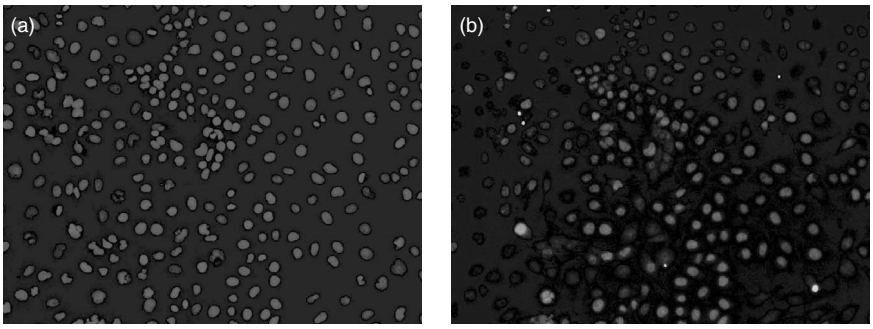

FIGURE 3.4. Undifferentiated NTERA2 cells, stained for the expression of Sox2. (**a**) Hoechst. (**b**) Sox2. (*See also* Plate 3.)

Procedure

(a) Trypsinize the cells, resuspend, and then fix in 4% paraformaldehyde for 20 min at room temperature.
(b) Wash the cells twice in PBSA and pellet by centrifugation at 1200 rpm (277 *g*) for 3 min.
(c) Resuspend the cells thoroughly in blocking solution at a concentration of 5×10^5 cells/ml, and incubate at room temperature for 10 min.
(d) Add 50 μL of antibody, appropriately diluted, to 50 μL of the cell suspension, producing a 100-μL suspension.
(e) Hold at 4°C for 1 h.
(f) Cell washes and secondary staining are identical to Protocol 3.10, Steps (i)–(n), except for the inclusion of Triton X-100 (0.1%, v/v) in the antibody staining solution (*see* Section 3.4.4.1b).
(g) At this stage stained cells may be visualized on a fluorescence microscope (Fig. 3.4; Plate 3).

3.8.2.3. Fluorescence-Activated Cell Sorting. Purification of cell types can be achieved by fluorescence-activated cell sorting (FACS), on the basis of cell surface antigen expression (NCAM, A2B5 etc). We have found FACS to be valuable for the purification of undifferentiated cells, before differentiation, as well as for the purification of differentiated cell populations. Once obtained, live sorted cells can be subsequently analyzed by PCR or Western blot or maintained alive for further analysis at a later date.

Protocol 3.12. Fluorescence-Activated Cell Sorting of Neural Cells

Reagents and Materials

Sterile

❑ Trypsin/EDTA (*see* Section 3.4.2.3)
❑ Primary and secondary antibodies

- ❑ DMEM/10FB (*see* Section 3.4.1.1)
- ❑ DMEM/20FB: DMEM with 20% FBS
- ❑ Gentamicin, 50 mg/mL (stock)
- ❑ FACS buffer (PBSA with 10% FBS)
- ❑ FACS tubes, 5 mL, 75 × 12 mm

Procedure

(a) Trypsinize cells in to a single-cell suspension.

(b) Stain with primary and secondary antibodies as described for flow cytometry analysis (*see* Protocol 3.10), increasing the number of cells and volumes of antibody in proportion, except that normal growth medium should be used to dilute the antibodies and to wash the cells.

(c) After staining with antibodies, analyze the cells and sort with a suitable flow cytometer.

(d) Collect sorted cells in medium supplemented with 20% FBS.
Once collected, cells are used for molecular analysis or plated in normal growth medium containing antibiotic (gentamicin 50 μg/mL) when staining can be visualized by fluorescence microscopy (Fig. 3.5; Plate 4). The medium used during FACS depends on the cell type. NTERA2 cells can be handled in

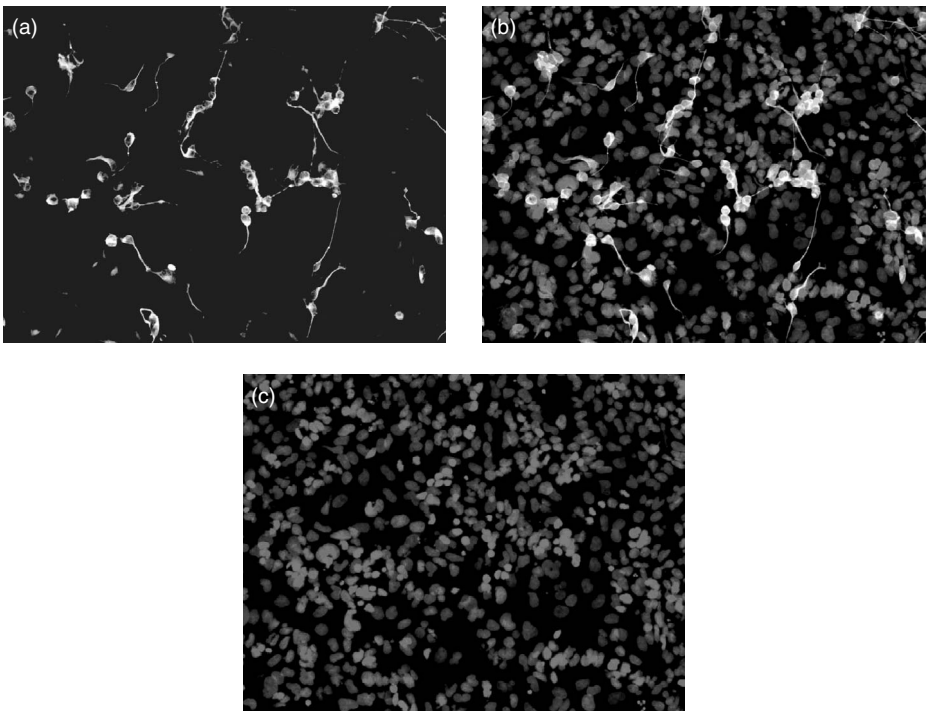

FIGURE 3.5. A2B5-positive NTERA2 cells were FACS purified after 18 days of RA exposure. Cells were then replated and stained 48 h later: (**a**) TUJ1, (**b**) Hoechst 33342, (**c**) overlay. (*See also* Plate 4.)

serum-containing medium, whereas human ES cells should be handled in their regular growth medium.

We have found the ability to sort cells very useful, especially for assessing the association of cell surface marker expression to other markers of the neural pathway (Table 3.1). Thus the presence or absence of a cell surface antigen can be correlated with the expression of multiple transcription factors, with the use of PCR analyzing FACS-sorted cells.

3.8.2.4. Neuronal Counts. EC and ES cells are typically differentiated at high density, which makes quantifying neuronal yield by eye very difficult. Thus to facilitate neuronal counting cells we replate cells at lower densities before immunofluorescent staining.

Protocol 3.13. Counting Cultured Neurons

Reagents and Materials

Sterile

- ❑ DMEM/10FB
- ❑ Trypsin/EDTA (*see* Section 3.4.2.3)
- ❑ TUJ1 antibody
- ❑ Multiwell plates, 12-well (3 cm^2/well)

Nonsterile

- ❑ Paraformaldehyde, 4% (w/v)
- ❑ Hoechst 33342 DNA fluorochrome, 50 ng/ml

Procedure

(a) After 21 days of retinoic acid-induced differentiation (*see* Protocol 3.3), trypsinize NTERA2 cells and resuspend in DMEM/10FB.

(b) Seed the cells at a density of 1×10^5 cells/cm^2 in 12-well plates and incubate for 48 h to allow attachment to the plate and adequate neurite outgrowth.

(c) Fix with 4% paraformaldehyde for 20 min at room temperature.

(d) Stain for TUJ1 (*see* Protocol 3.11).

(e) Once stained for TUJ1, counterstain with Hoechst 33342, and perform a nuclei count to obtain total cell number.

(f) Identify the neurons by reactivity to TUJ1. Each neuron is identified on the basis of possessing a neuronal morphology (small soma and the presence of at least one process).

At least five separate fields of view should be chosen for total cell number and neuronal counts, with a minimal cell count of 500.

TABLE 3.1 Antibodies Used

Antibody	Supplier	Technique used
Sox2	BD Bioscience	Western, flow cytometry and immunohistochemistry
Nestin	Abcam	Flow cytometry and immunohistochemistry
TUJ1 (βIII-tubulin)	Covance	Immunohistochemistry and Western
MAP2	Sigma	Immunohistochemistry
NF-68	Sigma	Immunohistochemistry and Western
NCAM (CD56)	Dakocytomation	Flow cytometry and immunohistochemistry
A2B5	Chemicon, ATCC	Flow cytometry and immunohistochemistry
Musashi-1	Abcam	Immunohistochemistry
GFAP	Dakocytomation	Flow cytometry and immunohistochemistry
O4	R&D Systems	Flow cytometry and immunohistochemistry
SSEA1	Chemicon, Santa Cruz, DSHB	Flow cytometry and immunohistochemistry
SSEA3	Chemicon, Santa Cruz, DSHB	Flow cytometry and immunohistochemistry
SSEA4	Chemicon, Santa Cruz, DSHB, Abcam	Flow cytometry and immunohistochemistry
TRA-1-60	Chemicon, Santa Cruz, DSHB, Abcam	Flow cytometry and immunohistochemistry
TRA-1-81	Chemicon, Santa Cruz, DSHB, Abcam	Flow cytometry and immunohistochemistry
TRA-1-85	Chemicon, Santa Cruz, DSHB	Flow cytometry and immunohistochemistry
VIN-IS-56	Chemicon, Santa Cruz, DSHB	Flow cytometry and immunohistochemistry
P3X		Flow cytometry and immunohistochemistry

3.9. HIGH-THROUGHPUT SCREENING

The ease with which NTERA2 cells can be grown and their reliability to yield a predictable percentage of neurons makes the cell line an ideal candidate for studying cell differentiation with a high-throughput system. Pharmaceutical companies have, for a long time, utilized high-throughput techniques for screening compound libraries to identify potential drugs. It is now becoming increasingly feasible for academic laboratories to use automated techniques [Abraham et al., 2004; Fennell et al., 2006].

The real advantage of automated systems and a 96-well format is the ease with which multiple cell culture parameters (substrate adherence, type of basal medium, medium additives, growth factors, etc.) can be explored. During processes such as neural differentiation, cellular analysis can be performed through the use of fluorescence indicators and documented by automated imaging systems with rapid image analysis provided by algorithm software. Multiple cell parameters can be quantified quickly, including cell counts, neurite outgrowth, nuclear vs. cytoplasm localization of proteins, and cell viability assays.

Multiple factors are known to increase the noise and variability of cell-based assays used for high-throughput assays. In particular, it has been observed that the peripheral

wells of 96-well plates generate results inconsistent with internal wells of the plate, the phenomenon being termed the "edge effect." The edge effect experienced within 96-well plates is thought to derive from excessive evaporation at the edges of the plates, and methods for its reduction have been reported [Lundholt et al., 2003]. Rather than attempting to eliminate the edge effect, we have found it best to exclude the use of peripheral wells, effectively reducing it to a 60-well plate (6×10).

3.9.1. Microtitration Assays

To determine cell line tolerance to test compounds and medium conditions, plate cells at 3000 cells/well in 96-well plates (collagen I coated) in 100 μL of standard growth medium. Once cells are sufficiently attached (after 4 h), exchange standard growth medium for compound containing medium (150 μL). Change medium every other day.

To assay the effects of compounds on NTERA2 cells, substitute standard growth medium (10% serum growth medium) for medium containing 5% serum. Reducing serum concentration will reduce any masking effects by serum-derived proteins. NTERA2 cells can be maintained long-term in 5% medium, but die after approximately a week in medium supplemented with only 2.5% serum.

3.9.2. Viability Assays

Vital stain cells with cell health profiling solution (*see* Table 3.2) and then examine within 20 min. Count cells in around 10 fields per well and record the total cell number. Identify cell nuclei and count them with an algorithm capable of performing object segmentation, resolving densely packed cells. Segmentation should be based on object (nuclei) boundary and indentation, rather than fluorescence intensity.

Assess cell health on the basis of nucleus shape and size with Hoechst 33342 staining, ideally with an automated system for consistency and high throughput. Identify dead or dying cells by loss of plasma membrane integrity, subsequently becoming permeable to TOTO-3 (1,1′-[1,3-propanediylbis[(dimethyliminio)-3,1-propanediyl]]bis[4-[(3-propen-2(3*H*)-benzoxazolylidene) methyl]]-tetraiodide), alongside loss of mitochondrial transmembrane potential, assayed by tetramethylrhodamine methyl ester (TMRM). Observation of increases in fluo 4-AM (4-(6-acetoxymethoxy-2,7-difluoro-3-oxo-9-xanthenyl)-4′-methyl-2,2′-(ethylenedioxy)dianiline-*N*, *N*, *N*′, *N*′-tetraacetic acid tetrakis(acetoxymethyl) ester) fluorescence indicates rises in intracellular calcium levels, experienced by dying cells.

TABLE 3.2 Cell Viability Profiling Solutions

Fluorescent Dye	Concentration	Fluorescence
Hoechst (33342) (blue)	0.8 μM	Nuclei size decreases in volume due to cytotoxicity.
TMRM (red)	20 nM	TMRM signal in mitochondria decreases upon permeabilization and membrane depolarization
Fluo-4, AM (green)	1 μM	Fluo-4 signal increases as intracellular Ca^{++} levels increase.
TOTO3 (far red)	1 μM	TOTO-3 fluorescence increases upon loss of plasma membrane integrity.

3.9.3. Cytotoxicity Assay

Use a colorimetry-based assay (Cytotox 96 nonradioactive cytotoxicity assay, Promega) that quantifies lactate dehydrogenase (LDH) activity to determine the level of cytotoxicity induced by compounds and medium conditions. Released on cell lysis, LDH converts a tetrazolium salt in the medium to a red formazan product, the amount of which is proportional to the number of cells lysed. This should be confirmed for each type of experimental variable being analyzed because such metabolic assays are prone to error [*see*, e.g., Kendig and Tarloff, 2006], and significant effects should be backed up by an alternative cytotoxicity assay, for example, MTT assay a few days after removal of test compound [Plumb et al., 1989].

Plate cells at 8000 cells/well in a 96-well plate, expose to 100 µl of the relevant test compound or medium, and analyze 24 or 48 h later, following the manufacturer's protocol. Measure absorbance with a plate reader.

3.9.4. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Assessment of the expression of multiple genes can be conveniently performed by RT-PCR analysis. The benefit of using RT-PCR is that gene expression can be analyzed in populations of cells grown as either monolayers or suspended cell aggregates, providing cells are well triturated to ensure successful RNA isolation. PCR avoids problems that are experienced with attempting to stain large cell aggregates (EBs or neurospheres) with antibody.

3.9.4.1. RNA Extraction. Harvest the cells with trypsin and suspend in PBSA. After pelleting by centrifugation at 1200 rpm ($277g$) for 3 min at 4°C, remove the supernate and either use the cells immediately or store at −70°C. Carry out RNA extraction with one of a variety of standard techniques, such as use of the RNeasy Mini kit (Qiagen, U.K.), adhering to the manufacturer's instructions.

3.9.4.2. cDNA Production with Reverse Transcription (Table 3.3). Determine RNA concentration both by optical densitometry at 260 nm and by electrophoresis on a 1% (w/v) agarose gel. Perform the RT reaction used to synthesize single-stranded cDNA for 3 h at 37°C in a final reaction volume of 40 µL. Add the following components to RNase-free 1.5-mL Eppendorf tubes: 5 µg of total RNA, 2 µL of oligo(dT) (0.5 µg/µl; MWG Biotech), and dH_2O to a final volume of 29 µL. Heat the tube to 70°C for 8 min and then place on ice for 10 min. After this, add the following reagents: 8 µL of reverse transcriptase buffer (Promega), 1 µL of Moloney murine leukemia virus (MMLV) reverse

TABLE 3.3 PCR Reagents

	Per 50 − µL Reaction	Mastermix
PCR Buffer (10×)	5 µL	125 µL
$MgCl_2$	1.5 µL	37.5 µL
dNTPs	0.5 µL (25 mM stock)	12.5 µL
Taq polymerase	0.4 µL	10 µL
Primers	1.5 µL (×2) of 10 pM	-
H_2O	38.6 µL	965 µL

TABLE 3.4 Primer Sequences Used

Primer	Sequence	Tm (°C)	Size (bp)
β-Actin	ATCTGGCACCACACCTTCTACAATGAGCTGCG CGTCATACTCCTGCTTGCTGATCCACATCTGC	65	838
NeuroD	AAGCCATGAACGCAGAGGAGGACT AGCTGTCCATGGTACCGTAA	55	579
Pax6	GCTGCCAGCAACAGGAAGGAG GTGCCCATTGGCTGACTGTTC	62	600
Sox-2	CCCCCGGCGGCAATAGCA TCGGCGCCGGGGAGATACAT	60	448
Sox-1	CTCACTTTCCTCCGCGTTGCTTCC TGCCCTGGTCTTTGTCCTTCATCC	58	848
Nestin	CACTCCCCTGGGCTTCTACC AGGGGACGCTGACACTTACA	60	508
Hash1	CGCGTGTGCTGCTCCCTTCT GGCTCGCCGGTCTCATCCTA	60	404
GFAP	TCATCGCTCAGGAGGTCCTT CTGTTGCCAGAGATGGAGGTT	60	400

transcriptase (Promega), and 2 μL of 25 mM dNTPs (Promega). After brief vortexing and centrifugation, incubate the reaction at 37°C for 2–3 h, then heat for 5 min at 95°C. Use negative controls lacking MMLV to ensure that no gDNA contamination occurred during RNA extraction. Analyze subsequent PCR reactions (containing 1 μL of cDNA solution from the RT reaction) by electrophoresis in 1% agarose gel. Use primers hybridizing to the mRNA encoding the β-actin cytoskeletal protein as an internal standard for RT-PCR. (Table 3.4)

3.10. SOURCES OF MATERIALS

Item	Catalog No.	Supplier
Agarose	15510027	Invitrogen
Antibodies (*see* Table 3.1)		
B27 supplement	10889-038	Invitrogen (GIBCO)
Collagenase IV	17104-019	Invitrogen (GIBCO)
Cryo-freezing container		Nalge Nunc
Cryotubes		Nalge Nunc
Cytotoxicity assay	Cytotox 96	Promega
Dispase	354235	BD Bioscience
DMEM, high glucose	41965-039	Invitrogen (GIBCO)
DMEM/F12	21331-020	Invitrogen (GIBCO)
EDTA	E 5134	Sigma
FACS tubes	55.476	Sarstedt
Fetal bovine serum, US origin	16000-044	Invitrogen (GIBCO)
FGF-2	500-P18	Peprotech

(*continued*)

Item	Catalog No.	Supplier
Flasks, 25 cm^2 and 75 cm^2	430168 430720	Corning Life Sciences
Fluo 4-AM	Fluka 93596	Sigma
Gelatin	G 1890	Sigma
Gentamicin, 50 mg/mL	15750-045	Invitrogen (GIBCO)
Heparan sulfate, sodium salt	H 7640	Sigma
Hoechst 33342 *bis*benzimide	B 2261	Sigma
Insulin, recombinant, human, stock (10 mg/ml)	I 9278	Sigma
Insulin-transferrin-selenium supplement	51300-044	Invitrogen (GIBCO)
Knockout DMEM	10829-018	Invitrogen (GIBCO)
Knockout serum replacement	10829-028	Invitrogen (GIBCO)
L-Glutamine	21051-016	Invitrogen (GIBCO)
Microtitration plates, 96-well, collagen I coated	354649	BD Biosciences
N2 supplement	17502-048	Invitrogen (GIBCO)
Nonessential amino acids, 100×	11140-035	Invitrogen (GIBCO)
PA6 stromal cell line		
Paraformaldehyde	P 1213	Sigma
Phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBSA)	D-PBS 14190	Invitrogen (GIBCO)
Plate reader	Wallac 1420 Victor V2	Perkin Elmer
Retinoic acid, all-*trans*	R 4632	Sigma
Tetramethylrhodamine methyl ester (TMRM)	T 5428	Sigma
TOTO3		
Triton X-100	T 9284	Sigma
Trypsin	15090-046	Invitrogen (GIBCO)
β-Mercaptoethanol	M 7154	Sigma

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CHAPTER 4

CARDIOMYOCYTE DIFFERENTIATION IN HUMAN ES CELLS

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Culture of Human Stem Cells, Edited by R.I. Freshney, G.N. Stacey and J.M. Auerbach

4.1. INTRODUCTION

Human embryonic stem cells (hESCs) have the potential to differentiate to cardiomyocytes in culture. They are easily identifiable first as areas of rhythmically contracting cell clumps. Further detailed electrophysiological and immunohistochemical analysis shows that these clumps contain individual cardiac cells with a spectrum of phenotypes comparable to those found in the normal human fetal heart at 8–10 weeks of gestation [reviewed in Boheler et al., 2002; Passier and Mummery, 2005]. They are therefore of interest for studying differentiation of cells during early human heart development, as well determining the physiological and pharmacological properties of human cardiomyocytes, including their responses to cardiac drugs. At some point in the future, they may also represent a source of transplantable cells for cardiac muscle repair by replacement of cardiomyocytes lost during ischemic damage.

The differentiation protocols that are effective in inducing hESCs to differentiate to cardiomyocytes depend in part on the individual hESC cell line used and in part on the methods used for propagation before differentiation. Here several methods for generating and characterizing cardiomyocytes from hESCs are described, as well as methods for dissociation of cardiomyocytes into single-cell suspensions that are useful for both characterizing cells by antibody staining and electrophysiological measurements as well as preparing cells for transplantation into (animal) hearts.

4.2. OVERVIEW OF METHODS

Many of the methods in current use are based on those developed for mouse embryonic stem cells (mESCs), namely, the formation of aggregates in suspension called embryoid bodies (EBs). Many mESCs will start to beat spontaneously between 4 and 10 days of initial aggregation depending on the number of cells in the aggregate or the cell line. It is believed that the formation of an outer layer of (extra)embryonic endoderm on the EBs may be important as a source of differentiation signals since it is known that in normal development of multiple species endoderm is essential for and signals to the anterior mesoderm during heart formation. The conversion of undifferentiated stem cells to cardiomyocytes is generally a low-efficiency process. Much of the literature on improving these efficiencies concerns activating specific developmentally relevant signaling pathways while the cells are growing as EBs. The *wnt* and bone morphogenetic protein (BMP) signaling pathways have proved most potent in this context.

The first report of cardiomyocytes derived from hESC [Kehat et al., 2001] appeared almost three years after hESC were first derived from blastocyst stage embryos [Thomson et al., 1998]. To induce cardiomyocyte differentiation, hESCs (cell line H9.2) were dispersed using collagenase IV into small clumps (3–20 cells) and grown for 7–10 days in suspension to form EB-like structures, comparable to mESCs but apparently without the distinct outer layer of endoderm cells. After these EBs were plated onto gelatin-coated culture dishes, beating areas were first observed in the outgrowths 4 days after plating (11–14 days after the start of the differentiation protocol). A maximum in the number of beating areas was observed 20 days after plating (27–30 days of differentiation), with 8.1% of 1884 EBs scored as beating. Spontaneous differentiation to cardiomyocytes in aggregates was also observed by others using different cell lines, for example, H1, H7, H9, H9.1, and H9.2 [Xu et al., 2002]. However, in this report approximately 70%

of the EBs displayed beating areas after 20 days of differentiation. On day 8 of this differentiation protocol (growth in suspension followed by plating in culture dishes) 25% of the EBs were beating. A third group also demonstrated spontaneous derivation of cardiomyocytes from hESC lines H1, H7, H9, and H14, but in this case 10–25% of the EBs were beating after 30 days of differentiation [He et al., 2003]. The reasons for these apparent differences in efficiency are not clear. In addition, counting beating EBs may not accurately reflect the conversion of hESC to cardiomyocytes since individual EBs may contain significantly different numbers of cardiac cells. Recently, the differentiation of two independent hESC lines, BG01 and BG02, has been described [Zeng et al., 2004; Denning et al., 2006]. After dissociation of hESC by collagenase IV into small clumps, cells were grown for 7 days as EBs and cultured on adherent plates for another 7 days. In this case immunoreactivity was demonstrated for the cardiac marker cardiac troponin I.

An alternative method for the derivation of cardiomyocytes from hESCs was described by Mummery et al. [Passier et al., 2005; Mummery et al., 2002, 2003]. Beating areas were observed after coculture of hES2 cells [Reubinoff et al., 2002] with a mouse visceral endoderm-like cell line (END2). Endoderm plays an important role in the differentiation of cardiogenic precursor cells that are present in the adjacent mesoderm in vivo. Earlier coculture of END2 cells with mouse P19 embryonal carcinoma (EC) cells, a mouse embryonal carcinoma cell line with pluripotent differentiation properties, and with mESCs had already shown that beating areas appeared in aggregated cells and that culture medium conditioned by the END2 cells contained cardiomyogenic activity [van den Eijnden-vanRaaij et al., 1991]. For the derivation of cardiomyocytes from hESCs, mitotically inactivated END2 cells were seeded on a 12-well plate and cocultured with the hESC line hES2. This resulted in beating areas in approximately 35% of the wells after 12 days in coculture [Mummery et al., 2003].

While these methods appear to be effective, all produce cardiomyocytes at low efficiency. Several potential cardiogenic factors have been tested in hESCs. No significant improvement in cardiomyocyte differentiation has been achieved by adding DMSO, retinoic acid [Kehat et al., 2001; Xu et al., 2002], or BMP-2 [Mummery et al., 2003; Pera et al., 2004]. It is not clear whether these factors lack a role in cardiac differentiation of hESCs, or whether differentiation protocols are not optimal. One factor that has been described as enhancing cardiomyocyte differentiation of hESCs is the demethylating agent 5′-deoxyazacytidine. Treatment of hESC aggregates with 5′-deoxyazacytidine enhanced cardiomyocyte differentiation and upregulated the expression of cardiac α-myosin heavy chain, as determined by real time RT-PCR, up to twofold [Xu et al., 2002].

The presence of fetal calf serum during differentiation also has important effects on differentiation efficiency. In most reports to date, serum has been present in the culture medium and differentiation efficiency has been described as being dependent on serum batch. Serum may contain inhibitory factors. For example, Sachinidis et al. [2003] observed a 4.5-fold upregulation in the percentage of beating mouse EBs after changing to a serum-free differentiation medium. We also recently observed a greater than 20-fold increase in cardiomyocyte yield in hES2–END2 cocultures in serum-free medium [Passier et al., 2005], which was further enhanced by ascorbic acid. The phenotype of the majority ($\sim$90%) of cardiomyocytes derived with this protocol show greatest similarity to human fetal ventricular cells, although atrial and pacemaker-like cells are also observed [Mummery et al., 2003]. This serum-free protocol is described here for both hES2 and hES3 cells.

4.3. PREPARATION OF MEDIA AND REAGENTS

4.3.1. Feeder Cell Media

4.3.1.1. MEF Medium.

(i) GMEM (BHK-21), 1×	500 mL
(ii) L-Glutamine, 200 mM (100×)	5 mL
(iii) Sodium pyruvate, 100 mM	5 mL
(iv) Nonessential amino acids, 100×	5 mL
(v) Fetal bovine serum (FBS)	50 mL

Note: FBS is always batch tested for optimal growth of ESCs; one supplier is Perbio.

(vi) Penicillin-/streptomycin, 100×	5 mL

4.3.1.2. END2 Culture Medium. DMEM/F12 (1:1) with L-glutamine 2.5 mM, penicillin 10 U/mL, streptomycin 10 μg/mL, FBS 7.5%

4.3.2. hESC (hES2 and hES3) Medium

4.3.2.1. Standard Medium. DMEM (high glucose) with:

	v/v	Final
(i) L-Glutamine, 200 mM	1:100	2 mM
(ii) Penicillin-streptomycin	1:200	25 U/mL, 25 μg/mL
(iii) Nonessential amino acids	1:100	
(iv) Insulin, transferrin, selenium (ITS-G)	1 :100	
(v) 2-Mercaptoethanol	1.8 μL/mL medium	
(vi) Fetal calf serum	20%	

(vii) Once mixed, filter medium through Stericup-GV filter unit

4.3.2.2. Serum-Free hESC Medium. As for standard medium but with serum omitted.

4.3.3. Dissociation Buffers for hESC Cardiomyocytes

Three buffers are needed; volumes given in Table 4.1 are for making 100 mL of each buffer.

Sterilize by filtration and store at $-20^{\circ}C$

Notes:

(1) When making buffer 3 (KB), leave out the glucose, otherwise a precipitate forms at $-20^{\circ}C$; add glucose just before use.

(2) When making buffer 3, add K_2HPO_4 as the last step; otherwise a precipitate forms.

(3) The efficiency of dissociation of the beating clusters into individual cardiomyocytes may depend on the batch of collagenase A, used and batch testing may be advisable. We use collagenase from Roche.

TABLE 4.1 Preparation of Buffers

Buffer	1. Low-Ca	2. Enzyme	3. KB
NaCl, 1.0 M	12 mL	12 mL	—
$CaCl_2$, 1.0 M	—	3 μL	—
K_2HPO_4, 1.0 M	—	—	3 mL
KCl, 1.0 M	0.54 mL	0.54 mL	8.5 mL
Na_2ATP	—	—	2 mM
$MgSO_4$, 1.0 M	0.50 mL	0.50 mL	0.50 mL
EGTA, 1.0 M	—	—	0.1 mL
Na pyruvate, 1.0 M	0.50 mL	0.50 mL	0.50 mL
Glucose, 1.0 M	2 mL	2 mL	2 mL
Creatine, 0.1 M	—	—	5 mL
Taurine, 0.1 M	20 mL	20 mL	20 mL
Collagenase A	—	1 mg/mL	—
HEPES, 1.0 M	1 mL	1 mL	—
pH (corrected with NaOH)	6.9	6.9	7.2

4.3.4. Gelatin-Coated Flasks, Plates, or Dishes

(i) Gelatin 0.1%: dissolve 0.5 g gelatin in 25 mL of distilled H_2O and autoclave. Add the hot gelatin solution to 475 mL of distilled H_2O and store at room temperature.

(ii) Add to flasks, plates, or dishes at 1 mL/cm^2, leave 5 min at room temperature, and remove.

(iii) Allow to dry overnight in sterile laminar flow and store at 4°C until required.

4.4. PROTOCOLS FOR HESC DIFFERENTIATION TO CARDIOMYOCYTES

The "cut-and-paste" method has been reported to be the most supportive of karyotypic stability, but most hESC lines can be adapted to bulk culture methods (trypsinization, collagenase) and can be cultured on mouse embryonic fibroblasts (MEFs; *see* Protocol 4.4) or human feeder cells (e.g., foreskin fibroblasts). In our hands, hES2 and hES3 cells are passaged routinely once a week on MEFs. At passage, colonies are usually 0.5–2 mm in diameter but will not become confluent.

Protocol 4.1. Passaging hESC (hES2 and hES3) with the "Cut-and-Paste" Method

Reagents and Materials

Sterile or aseptically prepared

- ❑ Cultures of hES2 or hES3 cells in organ culture dishes
- ❑ MEF feeder layer in an organ culture dish, newly prepared (*see* Protocol 4.5)
- ❑ Standard hESC medium (*see* Section 4.3.2.1)
- ❑ Dulbecco's PBS complete with Ca^{2+} and Mg^{2+} (D-PBS)
- ❑ Dispase solution, 10 mg/mL in standard hESC medium, freshly prepared and filter sterilized

- ❑ Petri dishes, 3.5 cm, 2, with D-PBS, maintained at 37°C in incubator
- ❑ Glass needles heated over a flame, pulled and broken to give two cutting ends. Sterilized by autoclaving just before passage
- ❑ Sterile filter, 0.22 μm
- ❑ Yellow pipettor tips (10–200 μL)

Nonsterile

- ❑ Pipettor, 100 μL
- ❑ Stereo dissecting microscope at 4× magnification (with heated stage if possible)

Procedure

(a) Inspect cultures and select dishes with undifferentiated hESC colonies.

(b) Using the glass needles, cut colonies (as if slicing a pizza) and select undifferentiated ''pieces'' for passage to new dishes (Fig. 4.1). Removal of the differentiated areas is essential for long-term propagation of undifferentiated cells. The most convenient working enlargement is 4× magnification on a stereo dissecting microscope..

(c) Remove medium and add 0.5 mL of dispase solution.

(d) Place the dish in an incubator for approximately 2 min, or leave on a heated microscope stage if available.

(e) Remove prewarmed dishes containing D-PBS from incubator.

(f) Pick up undifferentiated colony pieces from the dispase-treated dishes; using a 100 μL pipettor with yellow tip, transfer to the first dish of D-PBS and subsequently to the second dish. This results in two washes with D-PBS.

(g) Distribute the colony pieces evenly over a newly prepared MEF feeder layer in an organ culture dish (*see* Protocol 4.5) with 1 mL of standard hESC medium (*see* Section 4.3.2), with approximately 9 pieces per dish. Pieces should not be placed too close to the side of the dish or to one another, to provide sufficient space for the colonies to grow and to allow access for the next passage.

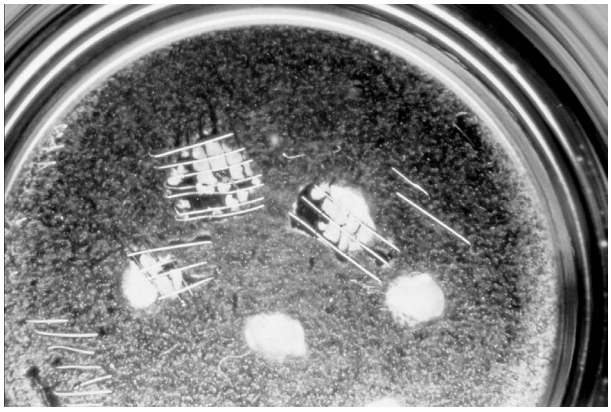

FIGURE 4.1. Colonies of HES2 cells "cut" for passage by dispase treatment. Each colony contains ~5000 cells. Intact colonies are still visible as white disks. (*See* Protocol 4.1 for hESC culture.) Photograph courtesy of Dorien Ward-van Oostwaard.

(h) Place the organ culture dish carefully in an incubator at 37°C with 5% CO_2.
(i) Refresh daily with standard hESC medium.

Notes on Feeder Cells for hESC

(1) Gamma-irradiated feeders (irradiated in suspension with 25 Gy) can also be used and frozen and stored. For hESC lines passaged by cut-and-paste for best karyotypic stability, mitomycin C-treated feeders are used fresh. Feeder dishes and plates should be used after 48 h but not used for transferring hESC colonies if older than 4 days. The passage number up to which MEFs can be used differs per lab. In general, low-passage (p)—e.g., p4 (split ratio 1:2; max 1:4)—cells are preferable, although some labs are able to use feeder cells up to p7.
(2) We generally use one feeder density ($1.70–1.75 \times 10^5$ per organ culture dish, $\sim 7 \times 10^4$ cells/cm^2), although lower densities have been described. Feeder density has been suggested to influence colony morphology—on high density feeders, colonies may appear "domed" rather than flat.
(3) Preparation of MEF feeders for hESC culture may depend on breeding efficiency and the number of embryos per mouse and initial growth of the isolated primary fibroblast cells. Cell growth rate determines feeder quality (faster growth generally indicates better ability to support self-renewal of hESC) and the number of confluent flasks that is converted into MEF cryovials up to p4–p5. For hESC culture, these vials are thawed weekly and grown up to confluence before mitomycin C treatment, and subsequently used for hESC passaging.
(4) To avoid the use of feeders some hESC lines have also been cultured on Matrigel, or extracellular matrix layers, e.g. laminin or fibronectin, using MEF-conditioned medium containing basic fibroblast growth factor (bFGF) [Rosler et al., 2004; Xu et al., 2001]. Cells for conditioning medium can be used repeatedly for 7–10 days and the conditioned medium frozen and stored until use.

Protocol 4.2. hES2 and hES3 Cardiomyocyte Differentiation by END2 Coculture

Reagents and Materials

Sterile or aseptically prepared

- ❑ Starting material for two 12-well hESC-END2 coculture plates: 12 organ culture dishes each with 9–10 colonies of either hES2 or hES3 (*see* Protocol 4.1)
- ❑ Mitomycin C-treated END2 cells (*see* Protocol 4.6) in 12-well plates
- ❑ Serum-free hESC medium (*see* Section 4.3.2.2)
- ❑ Standard hESC medium (*see* Section 4.3.2.1)
- ❑ D-PBS
- ❑ Dispase solution, 10 mg/mL in standard hESC medium, freshly prepared and filter sterilized
- ❑ Blue pipettor tips (200–1000 μL)

Nonsterile

❑ Pipettor, 1 mL

Procedure

(a) Refresh mitomycin C-treated END2 cells with hESC medium without FBS at least 1.5 h before plating the hESC cell pieces.

(b) Prepare dishes for washing undifferentiated hESC colony pieces:

(i) Fill six 3.5-cm-diameter dishes with D-PBS.

(ii) Add 1 mL standard hESC medium (i.e., with FBS) to two organ culture dishes.

(iii) Place all dishes in incubator at 37°C with 5% CO_2.

(c) Detach hESC colonies from MEFs in organ culture dishes by adding 0.5 mL of dispase and placing in the incubator for 7 min.

(d) Collect all undifferentiated hESC colonies from the 12 organ dishes with a 1-mL pipettor with a blue tip and distribute them for washing among 3 dishes of D-PBS prepared previously.

(e) Transfer the colonies to 3 new D-PBS dishes (to remove MEFs attached to the colonies).

(f) Transfer the colonies to 2 organ dishes containing 1 mL of standard hESC medium (*see* Section 4.3.2.1).

(g) Break colonies into pieces (*see* Protocol 4.1) by firmly pipetting up and down (2–3 times depending on the size of the colonies) against the bottom of the dish with a 1-mL pipettor.

(h) Transfer small *clumps* of hESC cells to 2 × 12-well plates containing confluent mitomycin C-treated END2 cells.

(i) Refresh medium on days 5, 9, and 12.

(j) Score beating areas by microscopic examination 12 days after plating. Beating generally starts on days 5–7 and is maximal on day 12. Immunostaining with antibodies against α-actinin (dilution 1:800) and tropomyosin (dilution 1:400) indicates the number of cardiac cells in the beating clumps. This is usually 20–25% of the cells. Alternatively, quantification may be carried out by Western blotting (e.g., using troponin I antibodies; dilution 1:100).

Notes on hESC Differentiation to Cardiomyocytes

(1) The efficiency of differentiation toward cardiomyocytes in mouse and human ES cells may be variable. Important factors are the initial number of cells per embryoid body and the FBS batch, if the differentiation is not in serum-free medium. It is advisable to test different batches of FBS for differentiation efficiency and choose the batch that performs best for all subsequent differentiation assays.

(2) Mouse ES cells also respond to END2 coculture. Coculture of mESC with END-2 cells starts with *single-cell suspensions*: Cells start to aggregate, and after about 3 days aggregates attach to END2 cells and grow out and differentiate. Beating areas appear after about 8 days [Mummery et al., 2002]. Coculture with mESCs is usually in serum-containing medium, which improves survival.

(3) Some hESC lines respond best in END2 cocultures; others respond better when grown as aggregates in END2 conditioned medium, as described previously for pluripotent P19 embryonal carcinoma cells [van den Eijnden-vanRaaij et al., 1991]. hESC medium is conditioned for 4 days by a confluent END2 cell monolayer.

Protocol 4.3. Dissociation of hESC-Derived Cardiomyocytes

Reagents and Materials

Sterile

- ❑ Coculture dishes from Protocol 4.2
- ❑ Standard hESC medium (*see* Section 4.3.2.1)
- ❑ Dissociation buffers (*see* Section 4.3.3 and Table 4.1):
  - Buffer 1, low Ca^{2+}
  - Buffer 2, enzyme buffer
  - Buffer 3, KB
- ❑ Petri dishes, 3.5 cm
- ❑ Gelatin-coated, 0.1%, coverslips or dishes
- ❑ Blue pipettor tips (200–1000 μL)
- ❑ Fine scissors (e.g., iridectomy scissors)

Nonsterile

- ❑ Parafilm
- ❑ Pipettor, 1 mL

Procedure

(a) Isolate beating areas from coculture plates by cutting them out with scissors and collect excised tissue in standard hESC medium with FBS.

(b) Three buffers are required for dissociation (*see* Section 4.3.3). To start dissociation, transfer excised tissue pieces to a dish with buffer 1, using a 1 mL pipettor with blue tip, and leave for 30 min at room temperature.

(c) Transfer cell clumps from buffer 1 into buffer 2 and incubate at 37°C for ~45 min (cover dish with Parafilm before transferring to the CO_2 incubator).

(d) Transfer cell clumps from buffer 2 into buffer 3. Shake gently in buffer 3 at room temperature for 1 h at 100 rpm on nonpivoting shaker.

(e) Transfer cell clumps from buffer 3 into standard hESC culture medium with 20% FBS to promote attachment and survival. Break up the cell clumps by pipetting up and down (2–4 times) against the bottom of the dish with a 1-mL pipettor. The degree of dissociation required depends on the particular experiments that will be done with the cardiomyocytes:

  (i) For transplantation into animals or immunofluorescent staining, it is sufficient to obtain a mixture of cell clumps and single cells.

(ii) For electrophysiology single cells are required.

Note: Cardiomyocytes are very fragile! If pipetting is too rigorous, cells will fail to recover either in culture or in vivo.

(f) For electrophysiology and immunofluorescent staining, seed dissociated beating areas in standard hESC medium (*see* Section 4.3.2.1) on gelatin-coated coverslips or culture dishes at 37°C, as required.

(g) Allow cells on coverslips to recover for at least 2 days and up to a week for electrophysiology experiments.

(h) For transplantation into mouse heart, keep dissociated cells in suspension in hESC medium at 4°C on ice. Inject 1×10^5–1×10^6 cells into the left ventricular wall in a maximum volume of 15 μL of medium. Larger volumes cause scarring of the cardiac tissue.

Protocol 4.4. Derivation of Mouse Embryos Feeder (MEF) Cells

Reagents and Materials

Sterile

- ❑ Pregnant mice dated by detection of vaginal plug (= day 0)
- ❑ MEF medium (*see* Section 4.3.1)
- ❑ Freezing medium: MEF medium with 20% DMSO
- ❑ PBSA: D-PBS without Ca^{2+} and Mg^{2+}
- ❑ Trypsin/EDTA: 0.05% trypsin, 0.5 mM EDTA in Hanks' BSS without Ca^{2+} and Mg^{2+}
- ❑ Centrifuge tubes, 15 mL
- ❑ Freezing vials (cryotubes)
- ❑ Dissection instruments
- ❑ Syringe and 18G and 21G needles

Procedure

(a) Euthanize female mice on E13.5 of pregnancy.

(b) Isolate embryos and wash once in PBSA:

(i) Remove uterus from mouse and dissect out individual embryos [Freshney, 2005].

(ii) Remove the head and visceral organs (liver, heart, lungs, etc).

(iii) Wash the carcasses twice in PBSA.

(c) Transfer the carcasses to 3-cm-diameter bacteriological dishes, with two in each dish.

(d) Mince two embryo carcasses together into fine pieces, using 2 crossed scalpels and working in a very small volume of PBSA.

(e) Add 1 mL of trypsin/EDTA per embryo

(f) Leave for 10 min at room temperature while gently swirling the dish. Alternatively, leave embryos for 18 h at 4°C and then incubate at 37°C for 10–20 min; this has been described as improving yield and viability (*see* Freshney [2005], p. 187).
(g) Use a syringe first with a pink 18G needle and subsequently with a green 21G needle to dissociate the digested tissue into a single-cell suspension. Transfer the cell suspension to a tube containing 5 mL of MEF medium (*see* Section 4.3.1) per embryo.
(h) Allow the large pieces of tissue debris to settle and transfer the supernate into a clean centrifuge tube. Centrifuge at 250 *g* for 5 min.
(i) Resuspend the cell pellet in MEF medium and plate the suspension in 175-cm^2 tissue culture flasks with approximately 1 embryo per flask.
(j) Culture the cells for 24–48 h and trypsinize the flasks.
(k) Resuspend the cells in MEF medium.
(l) Dilute 1:1 with freezing medium and dispense into freezing vials (cryotubes) at 2 vials per original embryo.
(m) Freeze with standard procedures (*see* Freshney [2005], Chapter 20).

Notes on MEFs

The mouse strain used for MEF isolation may critically determine self-renewal of some hESC lines. In our hands hES2 and hES3 grow preferentially on 129Sv strain MEFs, HUES1 and 7 on CD1 or 129Sv strain MEFs. Both grow on human foreskin fibroblasts.

Protocol 4.5. Mitomycin C Treatment of MEFs for hESC Coculture

Reagents and Materials

Sterile or aseptically prepared

- ❑ Cryotube, 1.5 mL, containing MEF cells (*see* Protocol 4.4)
- ❑ MEF medium (*see* Section 4.3.1)
- ❑ Standard hESC medium (*see* Section 4.3.2) (for refreshment after 24 and 48 h)
- ❑ PBSA
- ❑ Mitomycin C stock: dissolve 2-mg vial in 1 mL of PBSA and filter sterilize
- ❑ Trypsin/EDTA: 0.05% trypsin, 0.5 mM EDTA in Hanks' BSS without Ca^{2+} and Mg^{2+}
- ❑ Falcon 35–3037 center well organ culture dish (0.1% gelatin coated).
- ❑ Syringe needles, 18G
- ❑ Syringe filters, 1 × 0.22 μm
- ❑ Syringe, 2.5 mL

Nonsterile

- ❑ Mitomycin C, 2-mg vial

Procedure

(a) Grow MEFs to confluence in 75-cm^2 tissue culture flask.
(b) Treat with mitomycin C (5 μL/mL stock; final concentration 10 μg/mL) for at least 23/3 h, 3 h maximum.
(c) Wash 1× with large excess of MEF medium.
(d) Wash 2× with PBSA.
(e) Trypsinize (trypsin/EDTA) max 2 min.
(f) Replate at 1.75 × 10^5 per organ culture dish in 1 mL of medium.

Protocol 4.6. Mitomycin C Treatment of END2 Cells for hESC Coculture

Reagents and Materials

Sterile or aseptically prepared

❑ END2 cells can be obtained from Christine Mummery at the Hubrecht Laboratory after completion of a Material Transfer Agreement (www.niob.knaw.nl), e-mail: christin@niob.knaw.nl
❑ END2 culture medium (*see* Section 4.3.1.2)
❑ Mitomycin C stock, 2 mg/mL
❑ PBSA
❑ Trypsin/EDTA
❑ Tissue culture flask, 25 cm^2, coated with 0.1% gelatin (*see* Section 4.3.4).
❑ Tissue culture flask, 175 cm^2, coated with 0.1% gelatin (*see* Section 4.3.4).
❑ Multiwell plates, 12-well, coated with 0.1% gelatin (*see* Section 4.3.4).
❑ Coverslips treated with 0.1% gelatin, in a 12-well plate (*see* Section 4.3.4).

Procedure

(a) On day 1 (preferably Monday), seed a 25-cm^2 tissue culture flask coated with 0.1% gelatin with END2 cells in END2 culture medium (*see* Section 4.3.1.2). END2 cells should be split 1:8 from a confluent flask.
(b) On day 5, seed a 175-cm^2 flask coated with 0.1% gelatin with END2 cells, using all the cells from the previous 25 cm^2 flask
(c) On day 8, the 175-cm^2 flask should be 100% confluent and is ready for mitomycin C treatment, as described for MEFs (*see above*).
(d) Add mitomycin C, 5 μL/mL medium from 2 mg/mL stock solution to the culture medium to give a final concentration of 10 μg/mL.
(e) Incubate flasks at 37°C for at least 2$\frac{3}{4}$ h, 3 h maximum.
(f) Aspirate medium; wash wells once with END2 culture medium, followed by two washes with PBSA

Caution: the waste containing mitomycin C is highly toxic

(g) Trypsinize and count the cells.
(h) Resuspend the cells in END2 culture medium.

(i) Plate at a concentration of 1.75×10^5 cells/mL in 12-well plates coated with 0.1% gelatin, or in 1.5-cm-diameter gelatin-coated coverslips, as required for further experimentation.

4.5. SOURCES OF MATERIALS

Item	Catalog number	Supplier
Antibodies:		
α-Actinin, mouse monoclonal	A7811	Sigma
Tropomyosin rabbit polyclonal	AB1627	Sigma
Troponin I	AB1627	Chemicon International
Collagenase A	11088793001	Roche
Dispase	17105-041	Invitrogen
DMEM (high glucose)	11960-044	Invitrogen
DMEM/F12 (1:1)	31331-028	Invitrogen
DMSO	D-2650	Sigma
D-PBS	14040-91	Invitrogen
D-PBS without Ca^{2+} and Mg^{2+} (PBSA)	14190-094	Invitrogen
Fetal bovine serum (FBS)	SH30070.03	Hyclone
	Lot APA20428	Perbio
	14-506F	Cambrex
Filter unit, Stericup-GV	SC GVU05RE	Millipore
Gelatin	G1890	Sigma
L-Glutamine 200 mM, 100× (5 mL)	25030-024	Invitrogen
GMEM (BHK-21), 1× (500 mL)	21710-025	Invitrogen
Insulin, transferrin, selenium (ITS)	41400-045	Invitrogen
2-Mercaptoethanol	31350-010	Invitrogen
Mitomycin C (5×2 mg)	M0503	Sigma
Nonessential amino acids, 100×	11140-035	Invitrogen
Organ culture dish, center well (0.1% gelatin coated)	Falcon 35–3037	B-D Biosciences
Penicillin-streptomycin, 100× (5,000 U/mL penicillin, 5 mg/mL streptomycin)	15070-063	Invitrogen
Sodium pyruvate, 100 mM (5 mL)	11360-039	Invitrogen
Sterilizing filter, Stericup 0.22 μm	SCGV05RE	Millipore
Trypsin/EDTA (100 mL)	25300-054	Invitrogen

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CHAPTER 5

CULTURE OF THE HUMAN GERM CELL LINEAGE

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Culture of Human Stem Cells, Edited by R.I. Freshney, G.N. Stacey and J.M. Auerbach

5.1. INTRODUCTION

5.1.1. The Objectives of Human Germ Cell Culture

This chapter focuses on the embryonic germ cell (EGC). The goal is to provide a set of protocols with the hope of generating a clear view of how the EGC relates to other human stem cells.

Earlier chapters describe embryonic stem cells (ESCs), to which, in many respects, EGCs are comparable. While ESCs arise from the cells of the inner cell mass of the preimplantation embryo, that is, before gastrulation, EGCs are derived from the primordial germ cells (PGCs) of the postgastrulation fetus (Fig. 5.1; Plate 11). Both the precursor cells and their derived cell types are diploid and express genes such as OCT4 and NANOG [Turnpenny et al., 2006]. In vivo, PGCs complete meiosis after sexual maturity to give rise to the haploid gametes, ova in females and spermatozoa in males, which fuse to generate the fertilized zygote and from which all of the body's 200 or so cell types arise. In laboratory culture, PGCs can delay or avoid meiosis and may acquire a state of rapid mitotic self-renewal—a criterion that marks the derivation of EGCs. This process is very well established for mouse (m)EGCs [Matsui et al., 1992; Resnick et al., 1992]. The protocols described in this chapter are aimed at the derivation of human (h)EGCs.

Beyond therapeutic potential, the value of researching hEGCs extends into at least three other areas. As a potential human pluripotent stem cell, the hEGC offers a cell model with which to research aspects of human development that are inaccessible by other means. In the UK, under the Human Fertilisation & Embryology Act of 1990,

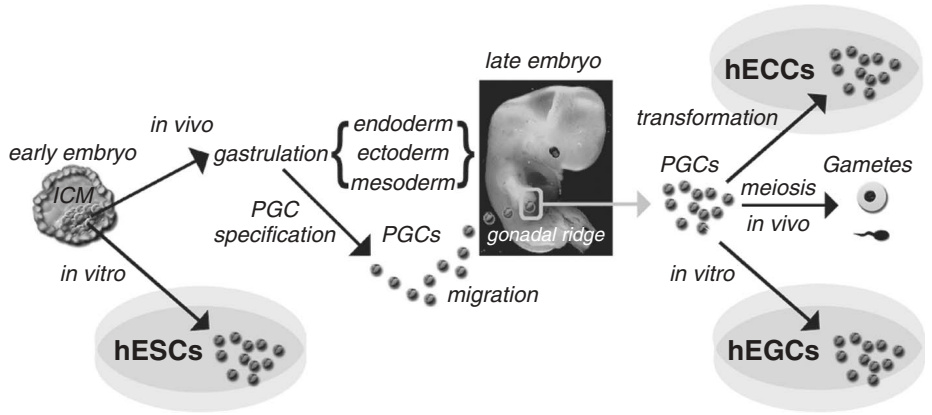

FIGURE 5.1. Schematic of human pluripotent stem cell sources and their respective cells of origin. (*See also* Plate 11.)

it is forbidden to culture human embryos beyond 14 days (*see* Chapter 2). From this stage until the receipt of material from social (also called voluntary) termination of pregnancy at approximately 7–12 weeks of gestation, we know remarkably little of normal human development, for instance, the process of gastrulation, which gives rise to ectoderm, endoderm, and mesoderm. hEGCs, like hESCs, offer a laboratory model with which to mimic these events, albeit in a compromised physiological environment.

Culturing hEGCs may also provide a comparator for the more widely studied hESC. In the quest to understand pluripotency more completely, defining similarities and differences between two cell types with similar theoretical properties may help to distinguish those features that are merely coincidental from those that act to determine pluripotent status.

A third reason for researching the human PGC-EGC lineage is to provide insight into human gonadal tumor biology. Human testicular tumors, which affect between 3 and 9 caucasian males per 100,000, are most frequently of germ cell origin. One particular type, teratocarcinoma, arises from a third human pluripotent stem cell, the malignant human embryonal carcinoma cell (hECC) (*see* Fig. 5.1 and Plate 11; *see* Chapter 6). Studying the PGC and EGC, as the untransformed counterparts of the ECC, provides a range of cell types in which to characterize the germ cell lineage from diploid normality, through either stem cell derivation, or to aneuploid transformed tumor cell.

5.1.2. Alternative Strategies

To date, only a handful of groups have reported culturing the hPGC lineage toward hEGCs [Aflatoonian and Moore, 2005; Park et al., 2004; Shamblott et al., 1998; Turnpenny et al., 2003; Liu et al., 2004]. To put these efforts into context, it is first necessary to understand a little more of native PGC biology, a significant amount of which has been inferred from investigations using mouse embryos [Molyneaux et al., 2001]. mPGCs are first discerned during gastrulation as a complex choice of cell fate among the daughter cells of the proximal epiblast. From this position, the PGCs migrate through the gut mesentery into the developing gonadal ridge during the period when gonadal sex (either testis or ovary) is determined. In the human male, the expression of a number of key

transcription factors causes somatic Sertoli cell differentiation during the seventh week of development [Hanley et al., 1999, 2000]. These cells cluster as testicular cords and induce the mitotic arrest of germ cells, with which they are intimately associated. In the absence of these events, an ovary develops. Ultimately, the ovarian germ cells enter the first phase of meiosis. However, this occurs only after a more protracted period of PGC proliferation, such that from 8 to 12 weeks of development, germ cell number is greater in the developing ovary.

Landmark experiments by the independent groups of Peter Donovan and Brigid Hogan derived mEGCs from PGCs in 1992 [Matsui et al., 1992; Resnick et al., 1992]. This came 11 years after the initial achievements of the Kaufman/Evans and Martin groups in deriving mESCs [Evans and Kaufman, 1981; Martin, 1981]. By itself, this time lapse suggests that although the culture and derivation processes were similar, precise definition of the necessary additives was arduous. These complexities are reviewed in greater depth elsewhere [Turnpenny et al., 2006; Donovan and de Miguel, 2003]. Nevertheless, these studies have remained the foundation for attempts at hEGC derivation. It is of particular note that the derivation of EGCs from mPGCs has proven to be more successful from earlier stages of development [McLaren and Durcova-Hills, 2001]. This includes the culture of germ cells before their arrival at the gonad. Comparable human material predates that accessible from first-trimester termination of pregnancy. This means that the approach of all groups reporting hEGC derivation from PGCs has centered on isolating the gonadal ridge during the late embryonic (until 56 days postconception) or early fetal (thereafter) periods. An interesting alternative has been proposed by Harry Moore and colleagues at the University of Sheffield, who have used hESCs as the starting point [H. Moore, personal communication]. By devising conditions that generate the germ cell lineage from these cells, it becomes possible, at least theoretically, to take differentiated cells through to EGC derivation. This approach offers potential access to "earlier" stages of PGC development; however, it invokes complex laboratory mimicry of the normal in vivo differentiation of the germ line.

5.1.3. The Current Status of Human EGC Research

The groups reporting hEGC derivation from first-trimester human material have all described very similar approaches, based on experiences with mEGC derivation, which itself drew on mESC culture methodology [Turnpenny et al., 2006]. Therefore, the details described here claim no significant advantage over others. More importantly, most groups have experienced significant difficulty in maintaining undifferentiated hEGC cultures over prolonged passaging. To date, no lines are banked in public repositories. Indeed, the starting material is heterogeneous, making the existence of clonal hEGC "lines" highly debatable. In some ways these difficulties, compared to the relative ease of hESC and hECC culture, are predictable. Unlike the spontaneous tendency for teratoma formation in the 129/sv strain of mice [Donovan and de Miguel, 2003; Stevens, 1981], the predominant human testicular germ cell tumor is the seminoma, which lacks a stem cell component. In addition, it took 11 years of intense effort for the success of mESC derivation to translate into a comparable achievement for mEGCs. Once attained, the pluripotency of mEGCs has been irrefutable. Therefore, it seems plausible that the current efforts of hEGC research lie somewhere along this time line, with critical factors that will assist long-term culture maintenance awaiting discovery. This chapter and its methodology aim to facilitate their identification.

5.2. DETAILS OF PREPARATION OF MEDIA AND REAGENTS

5.2.1. Gonadal Cell Dissociation Mix (CDM)

(i) Hanks' balanced salt solution (HBSS)
(ii) Collagenase IV, 2.5 mg/mL
(iii) DNase I, 20 U/mL
(iv) Heat-inactivated newborn calf serum, 2% v/v
(v) Calcium chloride ($CaCl_2$), 0.54 mM (60 μg/mL)

5.2.2. Feeder Cell Culture Medium (FCM)

(i) Dulbecco's modified Eagle's medium (DMEM)
(ii) Fetal bovine serum (FBS), 10% v/v
(iii) Penicillin, 100 U/mL
(iv) Streptomycin, 100 μg/mL

5.2.3. Germ Cell Culture Medium (GCM)

Knockout DMEM with:

(i) Knockout serum replacement (KO-SR), or ESC-tested fetal bovine serum (ESC-FBS) — 15% v/v
(ii) L-Glutamine — 1 mM
(iii) 2-Mercaptoethanol — 0.1 mM
(iv) Nonessential amino acids, 100× — 1% v/v
(v) Penicillin — 100 U/mL
(vi) Streptomycin — 100 μg/mL
(vii) Other additives (subject to definitive proof of requirement):
  a. Forskolin — 10 μM
  b. Human recombinant FGF-2 — 4 ng/mL
  c. Human recombinant LIF — 1000 U/mL

5.2.4. Feeder Freeze Down Medium (FFDM)

(i) Dulbecco's modified Eagle's medium (DMEM) — 40% v/v
(ii) Fetal bovine serum (FBS) — 50% v/v
(iii) Dimethyl sulfoxide (DMSO) (tissue culture grade) — 10% v/v

5.2.5. Germ Cell Freeze Down Medium (GFDM)

(i) Knockout DMEM — 40% v/v
(ii) Knockout serum replacement (KO-SR) or ESC-FBS — 50% v/v
(iii) DMSO — 10% v/v

5.2.6. Karyotyping

Hypotonic solution. 1:1 mixture of 54 mM (4 g/L) potassium chloride (KCl) and 27 mM (8 g/L) sodium citrate or 75 mM (5.6 g/L) KCl (keep stock for no longer than 3 weeks at 4°C).

5.2.7. Alkaline Phosphatase Detection Buffer

(i) Tris-HCl (pH 9.5)—100 mM
(ii) Magnesium chloride ($MgCl_2$)—50 mM
(iii) Sodium chloride (NaCl)—100 mM

5.3. CELL CULTURE METHODOLOGY

All cultures are maintained in 5% CO_2 and 95% humidity at 37°C and handled in a laminar flow hood (Class II microbiological safety cabinet). We use 75% ethanol for cleaning surfaces, tubes, and utensils. All cell culture plasticware is sterile.

5.3.1. Culture of Feeder Cells

Various mouse and human embryonic cell types have been trialed for their capacity to support the growth of PGCs and promote the derivation of hEGCs. However, we have yet to find a feeder cell line that improves significantly on our experience with STO fibroblasts, a finding shared by others [Shamblott et al., 1998, 2004] and to which the following procedures apply. STO is an immortalized line of SIM (Sandoz Inbred Mice) embryonic fibroblasts that expresses murine membrane-bound stem cell factor, a critical factor in the biology of germ cells in vivo and in vitro [Turnpenny et al., 2006]. Nevertheless, as with any immortalized cell line, the properties of STO can fluctuate between sublines in prolonged culture. Therefore, for consistency, it is advisable to prepare stocks of growth-arrested STO cells in batches, after limited passaging from a frozen stock. Such source vials are available from commercial suppliers, such as the ATCC-LGC Promochem partnership or the European Collection of Cell Cultures (ECACC). If a decline in efficiency for supporting the growth of PGCs is apparent, the batch should be discarded and fresh cells prepared.

Protocol 5.1. Preparation and Culture of Feeder Cells

Reagents and Materials

Sterile or aseptically prepared

- ❑ STO fibroblasts (ATCC CRL-1503; ECACC)
- ❑ Feeder cell medium (FCM; see Section 5.2.2)
- ❑ Feeder freeze down medium (FFDM; see Section 5.2.4)
- ❑ PBSA

- ❑ Trypsin, 0.25%, EDTA, 1 mM, in PBSA
- ❑ Culture flasks, 75 cm^2 or 150 cm^2
- ❑ Sterile conical tubes, 15 mL and 50 mL

Procedure

(a) Recover a vial of STO fibroblasts from liquid nitrogen storage and promote rapid thawing by partial immersion in a 37°C water bath.
(b) When cells are approximately 90% thawed, transfer the vial to a cell culture hood and clean the exterior of the vial with 75% ethanol.
(c) Recover the cells by adding prewarmed FCM to the vial and transfer to 6 mL of prewarmed FCM in a 15-mL conical tube.
(d) Centrifuge at 250 *g* for 3 min.
(e) Discard supernate, resuspend cell pellet in prewarmed FCM and transfer to a cell culture flask (culture volume: 10 mL per 75-cm^2 flask or 20 mL per 150-cm^2 flask). Place in the feeder cell culture incubator.
(f) Replace medium regularly (every 2 days).
(g) When cells are at or approaching confluence, aspirate medium and wash gently with prewarmed sterile PBSA.
(h) To passage, detach cells from the flask with prewarmed trypsin-EDTA (3 mL per 75-cm^2 flask; 6 mL per 150-cm^2 flask), ensuring coverage of the entire culture surface. After approximately 3 min with occasional tapping of the flask, the cell layer will be seen detaching from the flask (it is not necessary to return the flask to the incubator).
(i) Inactivate the trypsin by adding prewarmed FCM (3 mL FCM per 1 mL trypsin). Dislodge the remaining adherent cells by repeated gentle pipetting.
(j) Transfer resuspended cells to sterile 15- or 50-mL conical tubes and pellet by centrifugation at 250 *g* for 3 min.
(k) Resuspend pellets in 10 mL of warm FCM and replate at ~1:5 dilution.
(l) Repeating the passaging procedure once should generate the equivalent of approximately twenty to thirty 75-cm^2 flasks of confluent STO fibroblasts.

5.3.2. Arresting the Growth of Feeder Cells

Cells can be growth arrested by treatment with mitomycin C or by exposure to ionizing radiation. We have greater experience with the use of γ-irradiation. Our approach is laborious, requiring prior planning to coordinate access to appropriate equipment. However, multiple vials of ready-to-use cells are prepared for frozen storage (1 vial per confluent 75-cm^2 flask), ensuring uniform cell monolayers without recourse to continuous preparation and cell counting (as is necessary with the use of mitomycin C). The numbers in Protocol 5.2 purposefully overestimate needs to allow for an inevitable degree of cell death during the irradiation/freeze-thaw procedure. Overall, in our experience this approach is economical on time and consumables and provides consistent monolayers of STO fibroblast feeder cells.

Protocol 5.2. Growth Arresting Feeder Cells

Reagents and Materials

Sterile

- ❑ Growing STO fibroblasts (*see* Protocol 5.1)
- ❑ Feeder cell medium (FCM; *see* Section 5.2.2)
- ❑ Feeder freeze down medium (FFDM; *see* Section 5.2.4)
- ❑ PBSA
- ❑ Trypsin, 0.25%, EDTA, 1 mM, in PBSA
- ❑ Culture flasks, 75 cm^2 or 150 cm^2
- ❑ Conical tubes, 50 mL sterile
- ❑ Cryovials
- ❑ Source of γ-irradiation (e.g., ^{137}Cs)
- ❑ Controlled-rate freezing vessel containing 250 mL isopropanol

Procedure

(a) Cells should be irradiated at confluence (approximately 8×10^6 cells per 75-cm^2 flask). Our experience is that it is best to discard cells proceeding beyond this point.

(b) Replace culture medium the evening before irradiation.

(c) Aspirate medium and wash cells gently in prewarmed sterile PBSA.

(d) Detach cells from the flask with prewarmed trypsin-EDTA (3 mL per 75-cm^2 flask; 6 mL per 150-cm^2 flask), ensuring coverage of the entire surface. After approximately 3 min with occasional tapping of the flask, the cell layer will be seen to detach from the flask (it is not necessary to return the flask to the incubator).

(e) Inactivate the trypsin by adding prewarmed FCM (3 mL FCM per 1 mL trypsin). Dislodge the remaining adherent cells by repeated pipetting.

(f) Transfer resuspended cells to sterile 50-mL conical tubes (handling is easiest with volumes no greater than 30 mL per tube) and pellet by centrifugation at 250 *g* for 3 min.

(g) Resuspend pellets in 10 mL of warm FCM and seal the tubes.

(h) Mitotically inactivate resuspended STO cells by exposure to 50 Gy γ-radiation (our practice uses a 37-MBq ^{137}Cs source for 23 min to achieve this dose). This dose is optimal for this quantity and density of STO cells; different cell lines or different numbers of cells may require titration of the exposure time.

(i) Pellet cells by centrifuging at 250 *g* for 3 min.

(j) Thoroughly resuspend cells in prewarmed FFDM in a volume of 1 mL per 75-cm^2 flask.

(k) Maintaining uniform suspension, transfer 1-mL aliquots to individual cryovials and place at $-80^\circ C$ overnight in a controlled-rate freezing vessel.

(l) The following morning, transfer vials to liquid nitrogen storage.

5.3.3. Plating Feeder Cells in Monolayer

The predictability of acquiring human clinical material for laboratory research in the necessary quantity and at a specific time is always less than that for animal models. Growth-arrested monolayers of fibroblast feeder cells are ideally plated the day before commencing a new human germ cell culture. However, feeders can still be used if plated a day earlier. In the event of unforeseen material collection, we have found that feeder cells can adhere successfully to the culture surface in under 5 hours. In part, this reflects the difference of culture medium for plating feeders from that for germ cells: FCM contains serum, which includes factors that promote attachment of cells to cell culture plastic. However, the knockout serum replacement (KO-SR; *see* Section 5.7) in GCM lacks these "attachment factors."

The establishment of germ cell cultures is inherently variable (*see* Section 5.3.4), and it may be over a week before growth becomes evident. The feeder monolayer is viable, on average, for about 10 days; hence, this is a reason for not commencing germ cell cultures on feeders plated more than 2 days previously. Also, by switching from FCM to GCM on the morning of tissue collection, time is allowed for conditioning of the GCM by factors released by the feeder cells.

From Protocol 5.2, recall that irradiated feeders were frozen down at a ratio of one vial per confluent 75-cm^2 flask, which has a surface plating area approximately equivalent to a 10-cm-diameter culture plate. Thus one vial contains sufficient cells to form a monolayer on a 10-cm-diameter cell culture plate, or by scaling down, three to four 6.0-cm-diameter dishes, eight 3.5-cm-diameter dishes or a 6-well plate, one and three-quarters 12-well plates, and a little over one and a half 24-well plates. The principle also extends to 96-well plates or fibronectin-coated glass slides (Protocol 5.4).

Protocol 5.3. Plating Growth-Arrested Fibroblast Feeder Cells in Monolayer

Reagents and Materials

Sterile or aseptically prepared

- ❑ Mitotically inactivated STO fibroblasts (*see* Protocol 5.2)
- ❑ Feeder cell medium (FCM; *see* Section 5.2.2)
- ❑ Germ cell medium (GCM; *see* Section 5.2.3)
- ❑ PBSA
- ❑ Trypsin, 0.25%, 1 mM EDTA, in PBSA
- ❑ Cell culture vessels (6- or 3.5-cm-diameter dishes or 24-,12-, or 6-well plates)
- ❑ Conical tubes, 15 mL

Procedure

(a) One day before initiating a germ cell culture, recover a frozen vial of irradiated STO fibroblasts from liquid nitrogen storage and promote rapid thawing by partial immersion in a 37°C water bath.

(b) When the vial contents are approximately 90% thawed, transfer the vial to a cell culture hood and clean the exterior of the vial with 75% ethanol.

(c) Transfer cells to a 15-mL conical tube containing 6 mL of prewarmed FCM, mix briefly by gentle pipetting, and centrifuge at 250 *g* for 3 min.
(d) Resuspend cells in FCM to half the intended total culture volume; dispense the other half volume to the culture plates or wells; proportion the resuspended feeders evenly across the plate(s) or wells. Carefully place the culture vessels in the cell culture incubator.
(e) The following morning (i.e., the day of tissue collection), aspirate the FCM and gently wash the cells with sterile PBSA.
(f) Remove the PBSA and gently add prewarmed GCM to half of the intended total gonad cell culture volume (*see* Protocol 5.5).
(g) Return culture vessels to the incubator until the time of plating the gonad-derived cells.

In parallel with plating irradiated fibroblast feeder cells, and subsequently human gonadal cells, on tissue culture plastic, it is also useful to prepare glass slides for cell culture. Chambered glass culture slides are available for this purpose and allow fluorescence immunocytochemistry as part of the later analysis of cell type (Protocol 5.8). For fibroblasts to adhere efficiently, the glass slides need to be coated beforehand with some mimic of the extracellular matrix. We have found fibronectin to be the cheapest and most reliable factor for this purpose, although the principle is no different for agents such as laminin or Matrigel. Similarly, we also use gelatin-coated plates for EGC differentiation studies.

Protocol 5.4. Coating Chambered Glass Slides or Tissue Culture Plates

Reagents and Materials

Sterile

- ❑ Gelatin, 2% sterile solution
- ❑ Fibronectin (bovine; 1 mg/mL)
- ❑ Chambered glass slides

Procedure

(a) Remove chambered slides from sterile packaging within cell culture hood.
(b) Pipette fibronectin into the center of each well to provide 5 μg/cm^2. By expelling air from the pipette, form a bubble by which to drag the fibronectin and cover the entire well surface.
(c) Replace chambered slide cover and leave to dry in the tissue culture hood. In our experience, prepared slides can be stored at 4°C for at least 1 week.
(d) For coating tissue culture dishes with gelatin, use a working solution of 0.1% gelatin in sterile water and cover the surface area of the dish. Leave to stand for 1 hour and then aspirate the fluid. Leave the lid ajar and allow dishes to dry in the tissue culture hood. Gelatin-coated dishes can be stored for at least 1 month at 4°C.

5.3.4. Initiating Cultures from the Human Fetal Gonad

In the UK, the collection of human embryonic and fetal material is carried out according to code of practice guidelines issued by the Polkinghorne committee in 1989 [Polkinghorne, 1989]. In addition, projects require current ethical approval, and material is only collected after written informed consent is obtained from women undergoing social (or voluntary) termination of pregnancy.

Our experience has been to collect the gonadal ridge from approximately 7 weeks postconception (wpc) to 11 wpc for identification and dissection under stereomicroscopy using sterilized equipment and vessels. This practice can minimize the risk of subsequent infection; however, it is important to realize that material obtained *per vagina* is not sterile. It is therefore our practice to isolate primary human germ cell cultures from all other cultures by using a dedicated incubator.

The dissected gonadal ridge is placed in sterile HBSS and transferred to the culture hood for cell dissociation. We describe two dissociation methods, both performing variably, probably reflecting a number of uncontrollable factors that include: the developmental stage of the material, the time between collection and processing, and the number of samples being processed. The resulting suspension will consist of a mixture of single cells, small aggregates, and undigested larger clumps. The clumps sink, allowing recovery of the single cells and smaller aggregates by pipette for plating. Whereas the first method probably yields a greater number of cells, the use of proteolytic enzymes lyses a proportion of the cells and potentially damages important surface proteins. Conversely, the second method probably releases fewer cells but avoids protracted washing and resuspension and minimizes potential damage. We find both methods effective in initiating hEGC cultures; however, at present we are unable to conclude whether one is more productive. Similarly, we have identified no difference in cultures that retain or remove the mesonephros from the gonadal ridge (Fig. 5.2).

Our germ cell culture medium is listed in Section 5.2.3. In our opinion, it remains unclear whether the addition of forskolin, human recombinant fibroblast growth factor 2 (FGF-2), and human recombinant leukemia inhibitory factor (LIF) makes clear differences in establishing and maintaining germ cell cultures. Published reports of hEGC derivation, including our own, all include these factors [Shamblott et al., 1998; Turnpenny et al., 2003; Liu et al., 2004; Park et al., 2004].

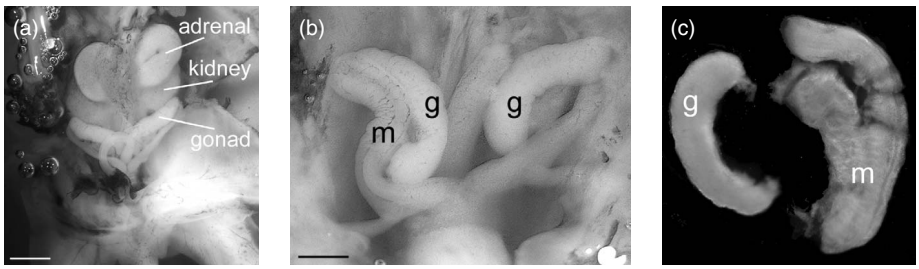

FIGURE 5.2. Identification and dissection of the human gonad and mesonephros. (**a**) Abdomen shown from fetus at 8 weeks postconception. (**b**) Gonad (g) and mesonephros (m) shown in situ. (**c**) The gonad and mesonephros have been removed and separated. Size bar = 2 mm (**a**) and 1 mm (**b**).

Protocol 5.5. Dissection and Disaggregation of Human Fetal Gonads and Plating Cells

Reagents and Materials

Sterile or aseptically prepared

- ❑ Pre-plated growth-arrested STO fibroblasts (*see* Protocol 5.3)
- ❑ Germ cell medium (GCM; see Section 5.2.3)
- ❑ Hanks' balanced salt solution (HBSS)
- ❑ EDTA, disodium salt, 0.3 mM (0.01%) in PBSA
- ❑ Cell dissociation mix (CDM; *see* Section 5.2.1)
- ❑ Conical tubes, 15 mL
- ❑ Scalpels
- ❑ Needles, 18 gauge
- ❑ Glass plates or glass slides for dissection
- ❑ Nylon gauze, 100-μm mesh, cut according to the size of the culture vessel and sterilized by autoclaving.

Nonsterile

- ❑ Shaking incubator, set to 37°C and approximately 200 rpm

Procedure

(a) Dissect gonads (with or without mesenephros) and place in sterile HBSS (*see* Fig. 5.2).

(b) Prepare cells for plating by either of the following methods:

Version 1:

i) Immerse tissue in EDTA for 10 min and then return to HBSS.

ii) Mechanically disaggregate the tissue with a sterile scalpel and forceps in a small volume of HBSS in a sterile glass plate (or on a glass slide). Do not allow to dry.

iii) Transfer the material to a 2-mL aliquot of cell dissociation mix (CDM) in a 15-mL conical tube and place in a shaking incubator at 37°C, for 1–2 h, with occasional trituration by syringe with an 18-gauge needle.

iv) Centrifuge at 250 *g* for 3 min.

v) Carefully remove and discard the supernate. Washing is optional. If excluded, the pellet from Step (iv) can be resuspended directly in Step (vii).

vi) Add 3 mL of HBSS, gently resuspend the cell pellet by pipetting, and then centrifuge again at 250 *g* for 3 min. Carefully remove and discard the supernate.

vii) Resuspend the cell pellet in prewarmed GCM [the remaining half of the total culture volume; refer back to Protocol 5.3, Step (f)]. Allow cells to stand for 3–5 min.

Version 2:

i) Immerse the tissue in EDTA for 20 min, and then return to HBSS.
ii) Place the tissue in a small volume of prewarmed GCM in a sterile glass plate.
iii) Under a dissecting microscope, repeatedly puncture and squeeze the intact organ with sterile needles and forceps, releasing cells directly into the medium.
iv) Transfer the medium to a 15-mL conical tube containing prewarmed GCM [the remaining half of the total culture volume; refer back to Protocol 5.3, Step (f)]. Allow cells to stand for 3–5 min.

(c) Recover the supernatant single and/or small aggregates of cells by pipette for plating. Alternatively, sieve the suspension through sterile 100-μm nylon gauze (wet the gauze by dipping the underside into medium, and apply cell suspension to the top side).
(d) Gently distribute cells across selected dishes or multiwell plates containing preplated feeders by pipetting directly into the medium, taking care to avoid damage to the feeder layer.
(e) Carefully place the culture vessel in a dedicated CO_2 incubator.

5.3.5. Maintenance of Gonad-Derived Cell Cultures

It is our practice to include antibiotics in all our gonad-derived primary cultures, at least in part relating to the nonsterile source of the starting material. We have not found it necessary to use antifungal agents. However, the ever-present risk of mycoplasma infection necessitates the initiation and maintenance of early cultures in a dedicated incubator. Once a culture is established, routine testing for mycoplasma should be carried out; for instance, concurrent with characterization by karyogram (*see* Protocol 5.9). We have used a variety of the commercially available methods and have developed no particular preference.

Cultures are left undisturbed for the first 2 days to allow gonadal cells, including the germ cell lineage, to attach to feeders. As with ESC culture, no specific methodology is mandatory. Our rationale for minimal intervention is to allow cells to adjust to a radically altered environment without further insult. After 2 days, the cultures may contain a lot of cell debris that can be removed by complete medium exchange. Alternatively, where cultures appear to contain fewer dead cells, half the medium can be exchanged to preserve more conditioning from the feeder cells. Thereafter, cultures should be briefly monitored daily, concurrent with medium exchange, to minimize time out of the incubator. As well as observing proliferation of the culture, this opportunity allows evaluation of the feeder cell integrity. These criteria determine the timing of the first passage, carried out during the second week, usually between days 9 and 11 of culture. Analysis of culture samples is carried out thereafter, as appropriate (*see* Section 5.4).

Protocol 5.6. Maintenance and Passaging of Germ Cell Cultures

Reagents and Materials

Sterile

- ❑ Germ cell medium (GCM; *see* Section 5.2.3)
- ❑ Sterile PBSA
- ❑ Trypsin, 0.25%, EDTA, 1 mM, in PBSA
- ❑ Pre-plated mitotically inactivated STO fibroblasts (*see* Protocol 5.3)
- ❑ Feeder cell medium (FCM; see Section 5.2.3)
- ❑ Cell culture vessels (6- or 3-cm-diameter dishes or 24-,12-, or 6-well plates)
- ❑ Conical tubes, 15 mL

Procedure

(a) On day 3, remove cultures from the incubator and evaluate the turbidity of the culture medium. Proceed according to one of the following:
  (i) If the culture has succumbed to a rare infection, discard it.
  (ii) If the medium is turbid because of the presence of copious debris, perform complete medium change.
  (iii) If the culture medium is reasonably clear, exchange half of the medium volume.

(b) Carefully aspirate the medium (complete or partial) and gently replace with fresh, prewarmed GCM.

(c) Thereafter, repeat (a) and (b) daily.

(d) During the second week, evaluate the culture (*see* Section 5.4) and perform the first passage on selected cultures, as follows:

(e) Carefully aspirate medium and wash cells gently with prewarmed sterile PBSA.

(f) Add the minimum volume of prewarmed trypsin-EDTA that covers the cell surface (e.g., 1 mL for a 6-cm-diameter dish). Return the culture vessel to the incubator.

(g) After approximately 3 min, observe plate under microscope for detaching cells. Do not prolong exposure to trypsin once detachment has occurred.

(h) Inactivate trypsin by adding 5 mL of prewarmed GCM to the culture vessel. Dislodge the remaining adherent cells by gentle pipetting; if necessary, more adherent colonies can be detached by gentle nudging with a pipette tip or a sterile cell scraper. Alternatively, if only a small volume of trypsin has been used, it can be inactivated satisfactorily by adding half of the subsequent total volume of GCM rather than the 5 mL listed above, in which case proceed to Step (l).

(i) Transfer (and potentially pool) cells to a 15-mL conical tube.

(j) Centrifuge at 250 *g* for 3 min and discard the supernate.

(k) Resuspend the cell pellet gently in half of the subsequent total culture volume.

(l) Remove preplated feeders from incubator and gently pipette cells into the medium across selected plates or dishes (dropwise addition onto the medium

can potentially damage the cells), taking care to avoid damaging the feeder layer. Return the culture vessels to the incubator.

(m) Resume from Step (a) at next passage.

5.4. CULTURE EVALUATION AND CHARACTERIZATION

5.4.1. Distinguishing the Status of Germ Cell Cultures

One of the major barriers to human germ cell culture, in our experience and that of others [Shamblott et al., 2004], has been the difficulty of reliably assessing culture status by cell colony morphology under light microscopy (Fig. 5.3; Plate 5). Although colonies are visible, not all lie on top of feeder cells. Some appear to nestle in spaces between the fibroblasts, feasibly on extracellular matrix deposited by irradiated cells. In either case, it is only after fixing and testing that one can reliably determine whether the composite cells of the colonies are alkaline phosphatase (AP)-positive germ cells. This contrasts with our experience of culturing hESCs or hECCs, where colony morphology is more predictive (*see* Fig. 5.3). To counter this, our cultures are usually initiated and passaged across multiple wells. This permits the occasional sacrificing of wells to identify germ cells and assess their growth, while leaving the bulk of the culture undisturbed. Similarly, samples of resuspended cells at the time of passaging can be taken for additional characterization (*see* Section 5.4.2). These steps have enabled timely prediction of those cultures to which effort and resources should be applied. Within the first 2 weeks (usually at or around the time of first passage), we assess cultures for growth characteristics and morphology and, critically, the presence of cells with AP activity (*see* Protocol 5.7). According to these criteria, cultures are assigned to one of two categories: "PP" (poorly proliferating); or "VP" (vigorously proliferating). We have taken "VP" cells as indicative of hEGC status and have previously reported a derivation efficiency for this step of approximately 15% of starting cultures [Turnpenny et al., 2003].

5.4.1.1. Poorly Proliferating Cultures — "PP". These cultures demonstrate limited germ cell growth. The AP staining only tends to detect solitary or small groups of cells, with minimal colony formation (Fig. 5.4). Similar findings are encountered by immunocytochemistry for other pluripotent markers on fixed cells. Interestingly, these cells may survive passaging for beyond 50 days or so; however, conversion to proliferative growth is very rarely attained. This is despite the continued presence of factors such as FGF-2 and trialling different combinations of medium formulation and culture surface.

5.4.1.2. Vigorously Proliferating Cultures — "VP". These cultures form multiple distinct colonies of varying morphology and/or networks of proliferating "migratory-like" cells, growing on and among the arrested feeder cells; a high proportion (>90%) of cells within colonies and networks stain heavily for AP (*see* Fig. 5.4). Colony growth and network proliferation persists through passaging, with maintenance of AP expression and immunoreactivity for other markers characteristic of pluripotent cells (*see* Protocols 5.7 and 5.8). In contrast to discrete colonies, the networks of cells in VP cultures are striking, potentially mimicking the proliferative capacity of migratory PGCs in vivo.

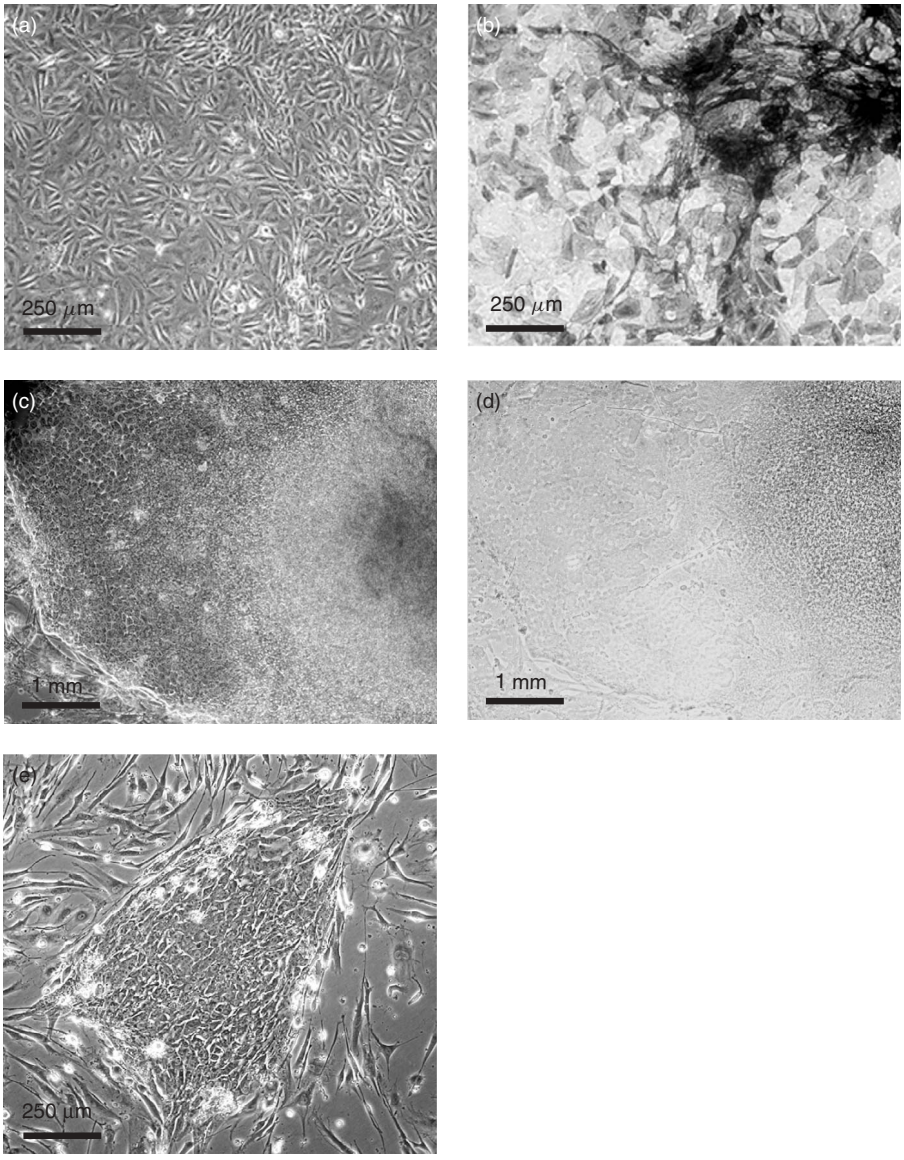

FIGURE 5.3. Colony morphology. This does not necessarily predict staining pattern for alkaline phosphatase (AP). (**a**) Bright field image in which it is difficult to discern a discrete colony. (**b**) Subsequent AP staining of the cells in (**a**) reveals the presence of germ cells. In contrast, the colony easily visible in (**c**) lacks appreciable AP staining (**d**). (**e**) Characteristic colony morphology of hESCs (such colonies always stain for AP activity). (*See also* Plate 5.)

If VP status indicates transition of the PGC to EGC, conversely, the PP culture state suggests failure to undergo this step. As PP cells persist, these findings suggest that cell survival, and its supportive culture conditions, contribute little to the ill-defined "conversion" or "derivation" of EGC cells. At present, the factors that determine hEGC derivation remain very poorly understood.

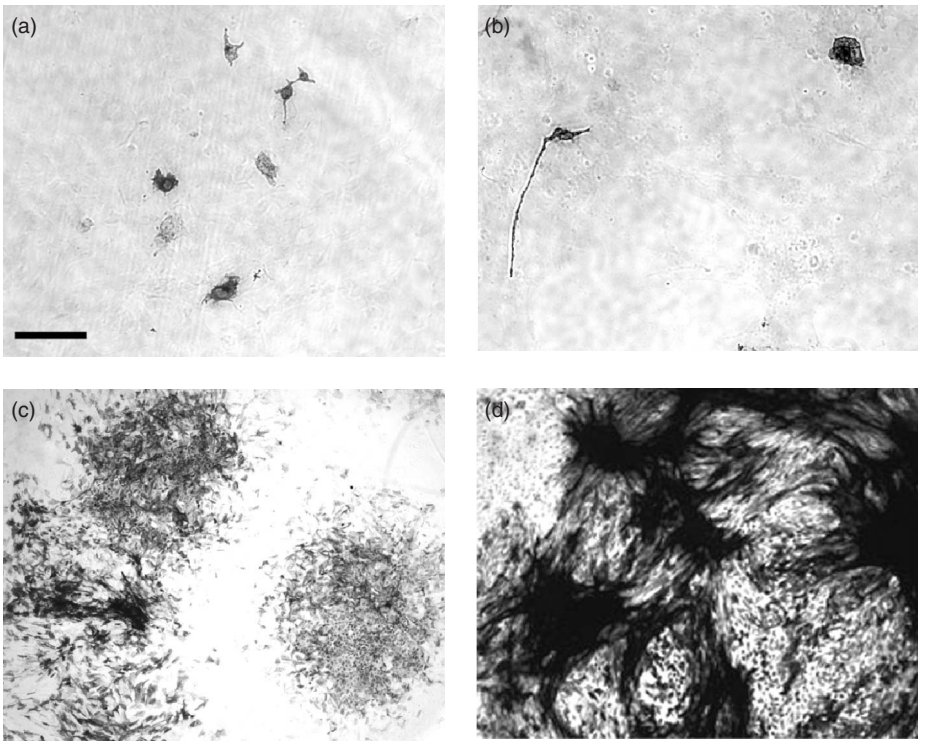

FIGURE 5.4. Poorly proliferative and vigorously proliferative human germ cell cultures. (**a** and **b**) Examples of poorly proliferative cultures with germ cells displaying morphology previously described as "stationary" and "migratory" [Shamblott et al., 1998]. (**c** and **d**) Examples of vigorously proliferative cultures, some of which resemble loosely assembled colonies (**c**), whereas others appear more like networks of cells (**d**). Size bar = 100 μm (**a, b**) and 1 mm (**c, d**).

5.4.2. Characterization of Human Germ Cell Cultures

Characterization studies of human germ cells in culture can be compared to control material, either fixed tissue sections or RNA extracted from the gonadal starting material, or parallel cultures of hESCs or hECCs. We have documented self-renewal of hEGCs by retention of markers such as OCT4, SSEAs, and hTERT within VP cultures. Similarly, loss of this gene expression profile coincides with the onset of differentiation marked by the acquisition of transcripts and proteins characteristic of ectodermal, endodermal, and mesodermal cell lineages. This satisfies in vitro criteria for pluripotency and has been achieved by all groups that have reported hEGC derivation [Shamblott et al., 1998; Turnpenny et al., 2003; Liu et al., 2004; Park et al., 2004].

5.4.2.1. Staining for Alkaline Phosphatase Activity. The detection of alkaline phosphatase activity serves as an accessible, easy, and rapid means of monitoring the germ cell lineage in human gonadal cultures. Different commercial reagents are available, in addition to those specified in Protocol 5.7.

Protocol 5.7. Fixation and Alkaline Phosphatase Staining of Germ Cell Cultures

Reagents and Materials

Nonsterile

- ❑ PBSA
- ❑ Paraformaldehyde (PFA), 4% w/v
- ❑ Ethanol: 50%, 70%, and 100%, v/v
- ❑ Detection buffer (*see* Section 5.2.7)
- ❑ Nitro-blue tetrazolium chloride (NBT), 100 mg/mL
- ❑ 5-Bromo-4-chloro-3′-indolyphosphate *p*-toluidine salt (BCIP), 50 mg/mL
- ❑ Aquamount
- ❑ Distilled water

Procedure

(a) Aspirate the medium from the culture vessel and wash the attached cells gently in PBSA.

(b) Aspirate PBSA and replace with 4% PFA in PBSA for 3 min.

(c) Aspirate fixative and repeat the wash in PBSA. If specimens are to be stored, proceed with Steps (d) and (e). If continuing immediately, brief equilibration in detection buffer is needed before proceeding directly to Step (g).

(d) Remove PBSA and dehydrate the cells through increasing ethanol concentrations: 50%, 70%, and 100%, sequentially for 2 min each.

(e) Allow culture vessels and the fixed cells to air dry at room temperature and store at 4°C.

(f) Rehydrate cells by covering the culture surface in detection buffer for 2 min.

(g) Replace detection buffer with a further aliquot that covers the cell surface and contains 4.5 μL/mL of the color substrates NBT and BCIP.

(h) Leave the culture vessel for 2 h at room temperature in the dark.

(i) If positive, the color reaction should be visible to the naked eye. Wash cells in distilled water for 5 min and mount with glass coverslips, using an aqueous mounting solution (e.g., Aquamount).

(j) Visualize cells by microscopy.

5.4.2.2. Immunocytochemistry. In addition to detecting AP activity, immunocytochemistry (ICC) allows assessment of pluripotent stem cell markers. A range of biotechnology companies sell robust antibodies for stem cell research. Some of the primary antibodies that we currently use are listed in Table 5.1. Detection can either use fluorescence or precipitated color reactions visible by bright field microscopy. Fluorescence ICC requires observation through glass rather than tissue culture plastic. This is easily achieved by establishing cultures on fibronectin-coated glass slides (Protocol 5.4).

Protocol 5.8 can be applied to cultures directly at their termination where cells are still wet or can be performed on specimens rehydrated in PBSA after dehydrated storage.

Protocol 5.8. Immunocytochemistry of Fixed Germ Cell Cultures

Reagents and Materials

Nonsterile

- ❑ PBSA
- ❑ Fixative, e.g., 4% PFA in PBSA or 40% (v/v) methanol/40% (v/v) acetone in water
- ❑ Trypsin, 0.25%, 1 mM EDTA, in PBSA
- ❑ Primary antibodies (*see* Table 5.1)
- ❑ Biotinylated or fluorescently conjugated secondary antibodies raised against the primary antibodies
- ❑ Sodium citrate (pH 6.0), 10 mM or 1% (v/v) Triton X-100
- ❑ Toluidine Blue
- ❑ DAPI

Procedure

(a) Aspirate the medium and wash cells gently in PBSA.

(b) Add fixative (choice determined by experience with each primary antibody) for 3 min.

(c) Wash cells gently in PBSA. As in Protocol 5.7, cells can be dehydrated through ethanol for storage or proceed to ICC [Step (d)]. If dehydrated, rehydration for 5 min in PBSA will be necessary after Step (d).

(d) Fluid movement can be restricted if desirable by drawing around colonies or regions of interest with paraffin wax. We have also found commercially available pens or nail varnish to be useful.

(e) Permeabilization depends on primary antibody-dependent need and tissue culture surface. Immersion in boiling sodium citrate is possible for cells on glass slides but impractical with tissue culture plastic, where 20 min exposure to 1% Triton X-100 is preferable. In our experience, where possible, the

TABLE 5.1 Primary Antibodies for Characterizing Pluripotent Stem Cell Markers

Name	Species	Company	Catalog Number	Working Dilution
NANOG	Goat polyclonal	R&D Systems	AF1997	1:20
OCT4	Goat polyclonal	Santa Cruz Biotechnology Inc.	sc-8629	1:150
SSEA1	Mouse monoclonal	DSHB	MC-480	1:20
SSEA3	Rat monoclonal	DSHB	MC-631	1:20
SSEA4	Mouse monoclonal	DSHB	MC-813-70	1:20
PGC surface marker	Mouse monoclonal	DSHB	EMA-1	1:20
hTERT	Mouse monoclonal	Novocastra	NCL-L-hTERT	1:50
TRA-1-60	Mouse monoclonal	Chemicon Ltd	MAB4360	1:50
TRA-1-81	Mouse monoclonal	Chemicon Ltd	MAB4381	1:50

transcription factor antibodies listed in Table 5.1 benefit from exposure to boiling sodium citrate for approximately 5 min. The other antibodies have needed no specific ''unmasking.''

(f) Aspirate the permeabilization solution and wash the cells in PBSA.

(g) Remove PBSA and apply standard ICC protocols or those provided by the supplier of the primary antibody.

(h) Depending on whether brightfield or fluorescence ICC has been performed cells can be counterstained with Toluidine Blue or DAPI to aid visualization of cell nuclei. Similarly, choice of mounting solution will depend on the use of glass slide or tissue culture plastic.

5.4.2.3. Reverse Transcription PCR Analysis of Cultures. RT-PCR is a useful adjunct to immunocytochemistry in the analysis of human germ cell cultures. We routinely store cell samples in Qiagen's "RNA Later" and extract total RNA for cDNA synthesis and PCR using commonly available commercial reagents.

5.4.2.4. Assessing the Karyotype of Human Germ Cell Cultures. Human stem cell research is largely driven by the exciting potential for future therapies. This makes the chromosomal stability of stem cells very important. Studying the chromosomes at metaphase resolves chromosome structure to a few megabases. If coupled with Giemsa staining, characteristic "G-banding" patterns are visible that, to experienced cytogeneticists, add much more information on chromosomal integrity. hESC cultures, which have been maintained to far higher passage numbers than hEGCs, have developed recurrent patterns of chromosome disruption, highlighting regions of instability [Draper et al., 2004]. To date, no such developments have been reported in hEGC cultures, although this quite probably reflects the difficulty in maintaining these cultures through freeze-thaw cycles and to high enough passage number.

Protocol 5.9. Preparing Metaphases from Germ Cell Cultures

Reagents and Materials

Sterile

- ❑ Colcemid (KaryoMAX)
- ❑ Trypsin, 0.25%, EDTA, 1 mM, in PBSA

Nonsterile

- ❑ Hypotonic solution (*see* Section 5.2.6)
- ❑ Fixative: 3:1 v/v of methanol:glacial acetic acid, prepared fresh
- ❑ DAPI: 4′, 6-diamidino-2 phenylindole, 5 μg/mL
- ❑ Glass slides

Specialized Equipment

- ❑ Fluorescence microscope

Procedure

(a) Grow a VP culture to occupy one 75-cm^2 flask or equivalent. Renew the medium the day before Step (b).

(b) Add 100 μL Colcemid (per 10 mL medium) and incubate for 15–20 min.

(c) Detach cells with trypsin and pellet by centrifugation as in Protocol 5.6.

(d) Resuspend the cell pellet carefully in a few drops of hypotonic solution. Add 1 mL gently with plastic Pasteur pipette, continue gentle resuspension, ''top up'' volume to ~8 mL, gently remixing the cells, and incubate at 37°C for 15–20 min.

(e) Slowly, add 0.5 mL of fixative dropwise, allowing drops to run down the wall of the tube to avoid damaging the delicate cells. Pipette up and down approximately 20 times, again using the wall of the tube. Add more fixative to a total volume of 10 mL and pipette repeatedly to cause frothing.

(f) Centrifuge at 250 *g* for 5 min.

(g) Resuspend the cells in 5 mL of fixative and leave at 4°C for at least 30 min. Cells can be left at this stage for several days if desired.

(h) Centrifuge at 250 *g* for 5 min and resuspend cells in 2 mL of fixative.

(i) Immerse untreated, uncoated glass slides in fixative for 30 min. Wipe the slides dry with paper towel.

(j) Pre-wet the slides with fixative. Using a glass pipette, mix the cell/fixative suspension and apply a few drops onto a prepared slide. Tilt the slide to drain liquid downwards. Allow the slide to air dry. Store the remaining cell suspension at −20°C.

(k) Place one drop of mounting fluid with DAPI stain onto the slide and apply a glass coverslip. The edge of the coverslip can be sealed with nail varnish. View by fluorescence microscopy. DAPI emits at 460 nm when bound to DNA.

(l) Stained slides can be stored in the dark at 4°C. Dried unstained slides from Step (j) can be stored at room temperature for approximately 2 weeks. Thereafter, slides can be stored at −20°C in a sealed box that contains desiccant. However, if the experiment is delayed, it is better to produce new slides from the suspended cells of Step (j) rather than rely on slides in prolonged storage at −20°C.

5.5. DIFFERENTIATION OF HUMAN GERM CELL CULTURES

5.5.1. In Vitro Assessment of Pluripotency

In our experience, the VP germ cells/hEGCs have proven particularly prone to spontaneous differentiation in culture, despite strict maintenance of the conditions that effect their derivation. These difficulties have been alluded to by most of the groups that have reported on human germ cell culture [Turnpenny et al., 2003; Liu et al., 2004; Onyango et al., 2002]. However, during their early proliferation, when positive for undifferentiated markers, VP cultures provide material for the in vitro demonstration of pluripotency. Our practice has been to encourage cells to aggregate, either through maintained growth beyond confluence or in suspension culture. The concomitant withdrawal of LIF, FGF-2, and forskolin and the loss of feeder cell influence has also been reported to promote differentiation, resulting in structures that share some similarity to

the embryoid bodies (EBs) gained from hESC cultures (*see* Chapter 2). Consistent with our previous interpretation of culture morphology, this process is exceptionally limited in PP suspension cultures, whereas VP germ cell cultures readily yield multiple, distinct EB-like structures ($_{gc}$EBs, "gc" denoting germ cell origin). Unlike ESC differentiation, however, no spontaneous contractions have been observed, consistent with a lesser tendency for mesodermal differentiation toward cardiomyocytes. However, we and others have observed a propensity for neuronal differentiation as evidenced by the outgrowth of neuronal projections [Turnpenny et al., 2005].

Once formed, $_{gc}$EBs can be cultured independently under differing conditions [Shamblott et al., 2001], for instance, in the presence of different growth factors in 96-well plates. On flat-bottomed tissue culture plastic, $_{gc}$EBs will tend to adhere and cells grow out. In untreated rounded-bottom 96-well plates, the detached $_{gc}$EB structure is maintained and amenable to characterization by immunohistochemistry and RT-PCR. Evidence for the gradual decline in pluripotency is demonstrable through the progressive and permanent loss of AP activity: AP staining in early culture $_{gc}$EBs (<2 days) confirms aggregation of the undifferentiated cell type, the presence of which declines as $_{gc}$EBs "mature."

Protocol 5.10. Facilitating In Vitro Differentiation in Embryonic Germ Cells

Reagents and Materials

Sterile

- ❑ Trypsin, 0.25%, 1 mM EDTA, in PBSA
- ❑ PBSA
- ❑ Calcium chloride ($CaCl_2$), 1 M in sterile ultrapure water
- ❑ Gelatin-coated culture vessels (*see* Protocol 5.4)
- ❑ Petri dishes, non-tissue culture treated
- ❑ Cell culture vessels (6- or 3.5-cm-diameter dishes or 24-,12-, or 6-well plates)

Procedure

(a) Resuspend cells from VP cultures, as for passaging (*see* Protocol 5.6), but in GCM without LIF, FGF-2, and forskolin.

(b) Aggregation of cells can be promoted by either of the following methods:

Version 1:

(i) Add $CaCl_2$ to final concentration of 4.5 mM.

(ii) Transfer suspension to an untreated sterile Petri dish.

(iii) Allow cells to aggregate for several days; partially replace the medium every 2 days.

Version 2:

(i) Transfer suspension to gelatin-coated tissue culture vessels (Protocol 5.4).

(ii) Allow cultures to grow beyond confluence; replace the medium every 2 days.

(c) Collect the developing $_{gc}$EBs for ongoing individual culture in untreated round-bottom 96-well plates.

(d) Replace the medium every 2 days. We have maintained $_{gc}$EB cultures for periods ranging from 2 to 21 days.

$_{gc}$EBs are difficult to handle because of their small size (approximately 1- to 3-mm diameter). Therefore, to ease their processing for characterization by immunohistochemistry, $_{gc}$EBs can be embedded in drops of agarose for paraffin embedding, sectioning, and mounting on glass slides for standard immunohistochemical investigation.

Protocol 5.11. Processing of Germ Cell Embryoid Body Structures

Reagents and Materials

Nonsterile

- ❑ Low-melting point (LMP) agarose
- ❑ PBSA
- ❑ Fixative: PFA, 4% (w/v), or methanol:acetone, 3:1
- ❑ Heating block
- ❑ Ethanol, 70%, 80%, 90%, and 100%, v/v, in deionized water
- ❑ Chloroform
- ❑ Paraffin wax
- ❑ Vacuum oven

Procedure

(a) Dissolve LMP agarose in a tube of PBSA at 1.5% w/v and place the tube in a 37°C water bath.
(b) Arrange small drops of molten agarose on a Petri dish placed on a heating block at 50°C (to prevent the agarose from solidifying).
(c) Remove individual $_{gc}$EBs from culture and rinse in warm PBSA.
(d) Carefully insert $_{gc}$EBs into agarose drops by pipette and allow cooling by removing the Petri dish from the heating block.
(e) Immerse the solidified agarose-$_{gc}$EBs in either fixative for no longer than 2 h.
(f) Dehydrate through increasing concentrations of ethanol: 70%, 80%, 90%, and 100%, sequentially for 2 h each.
(g) Displace ethanol by immersion in chloroform for 2 hours.
(h) Transfer to molten paraffin wax for standard protocols of embedding.

5.5.2. In Vivo Assessment of Pluripotency

The definitive test of pluripotency is the ability of a stem cell to contribute to chimeric embryos after injection into the blastocyst. In humans, this is not permitted for ethical reasons. In its place, in vivo assessment comes from the formation of teratomas in immunocompromised mice (covered in Chapter 7). These tumors are considered benign proliferations of a stem cell component accompanied by the differentiation of daughter

cells to derivatives of all three germ layers. Although two groups who have reported hEGC derivation have attempted this, no reports have been published of teratoma formation from hEGCs. Perhaps this is in keeping with the difficulty of maintaining undifferentiated cells in vitro, such that in vivo there is an inadequate stock of stem cells from which to form the differentiated structures of the teratoma. These findings are in contrast to those for the inner cell mass, ESCs and ECCs of mouse or human origin. mEGCs, and the more recently isolated mouse multipotent germline cells (mMGS), also give rise to teratomas on engraftment, whereas hEGCs have behaved more akin to mouse PGCs, which are nullipotent in this setting [Donovan and de Miguel, 2003]. The potential reasons for this discrepancy are severalfold and alluded to in the introduction to this chapter. For instance, it may associate with relatively refractory nature of human testis to form teratomas compared to other germ cell tumors, such as seminomas, a characteristic that contrasts with several strains of mice.

5.6. CRYOPRESERVATION

In line with the difficulty of maintaining undifferentiated status in hEGC cultures, our experience has been that cryopreservation is accompanied by a further diminution of cells positive for pluripotent markers. The reagents and protocol that we use are comparable to those used for hESC culture (*see* Chapter 2).

5.7. SOURCES OF MATERIALS

Item	Supplier
Antibodies	*See* Table 5.1 for listing of primary antibodies*
Aquamount	BDH (Merck)
BCIP	Roche Molecular Biochemicals
Chambered glass culture slides	Shandon, Life Sciences International (Europe) Ltd
Collagenase IV	Sigma
Conical centrifuge tubes, 15 mL/50 mL	Falcon
Cryo 1°C Freezing Container	Nalge Nunc
Cryovials	Corning International
DAPI	Vector
Dimethyl sulfoxide (DMSO)	Sigma
DMEM	Invitrogen/PAA
DNase I	Sigma
ESC-tested fetal bovine serum	PAA Laboratories
FGF-2	PeproTech
Fibronectin (bovine; 1 mg/mL)	Sigma
Forskolin	Sigma
Gelatin, 2% sterile solution	Sigma
Hanks' balanced salt solution (HBSS)	Sigma
Heat-inactivated newborn calf serum	Invitrogen
Human recombinant fibroblast growth factor 2 (FGF-2)	Cell Sciences

Item	Supplier
Human recombinant leukemia inhibitory factor (LIF)	Chemicon
KaryoMAX Colcemid	Invitrogen
Knockout DMEM (KO-DMEM)	Invitrogen
Knockout serum replacement (KO-SR)	Invitrogen
L-Glutamine	Sigma
LMP agarose	Sigma
Matrigel	BD Biosciences
2-Mercaptoethanol	Sigma
NBT	Roche Molecular Biochemicals
Nonessential amino acids	Invitrogen
Penicillin/streptomycin	Invitrogen
RNA Later	Qiagen
STO cells (CRL-1503)	ATCC or ECACC
Tissue culture flasks/plates	Iwaki
TRI Reagent	Sigma

All reagents applied to living cells are "tissue culture grade." *Original stocks of TRA antibodies, now commercially available, were a gift from Prof Peter Andrews (University of Sheffield).

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CHAPTER 6

DERIVATION AND CULTURE OF HUMAN EMBRYONAL CARCINOMA STEM CELL LINES

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Culture of Human Stem Cells, Edited by R.I. Freshney, G.N. Stacey and J.M. Auerbach

6.1. BACKGROUND

6.1.1. Introduction

Teratocarcinomas are structurally diverse tumors generally consisting of an array of differentiated cell types, forming what is known as a teratoma, and a population of stem cells, known as embryonal carcinoma (EC) cells. Embryonal carcinoma cells give rise to the differentiated derivatives within the tumor, and this process is thought to represent a caricature of embryogenesis. Teratocarcinomas most frequently develop in the gonads and belong to a class of neoplasms known as germ cell tumors (GCT). Oocytes can become parthenogenetically activated and undergo disorganized embryonic development, resulting in the formation of an ovarian dermoid cyst. This is the most common manifestation of a GCT and is most often benign. In the testis, GCT can arise from the transformation of primitive germ cells before meiosis. Such tumors are usually malignant and may consist of various embryonic tissues (teratoma) and extraembryonic structures of the trophoblast (choriocarcinoma) or yolk sac (yolk sac carcinoma).

For many years it has widely been accepted that the formation of teratocarcinomas is closely related to the processes of embryonic development, and in some ways EC cells resemble the undifferentiated stem cells from the early-stage embryo [*see* for review Andrews et al., 2001]. The experimental study of teratocarcinomas in the laboratory mouse was pioneered by Stevens and Little [1954], who first reported that about one in 100 male mice of Strain 129 spontaneously develop testicular teratomas. Such tumors can also be produced in other strains from ectopically transplanted embryos [Damjanov and Solter, 1974]. Working with mice, Kliensmith and Pierce [1964] demonstrated that a single EC cell was capable of extensive differentiation subsequent to grafting, indicating the pluripotency of such cells. Such potency was further established by experiments showing that EC cells injected into the blastocyst subsequently participate in normal embryogenesis and give rise to differentiated tissues in the resulting chimeric animal [Illmensee and Mintz 1975; Papaioannou et al., 1975]. It is broadly accepted from studies in the laboratory mouse that EC cells most closely resemble the primitive ectoderm during the early stages of embryogenesis. More recent experimental evidence shows this also to be the case in humans, where there are many examples of the similarities between early human embryonic cells, and their malignant counterparts, human EC cells [*see* for review Andrews et al., 2001]. Correspondingly, the study of cultured human EC cell

differentiation provides an opportunity to explore the molecular processes that control cell differentiation in the early human embryo.

Despite their resemblance to cells within the early-stage embryo, it is always important to remember that EC cells are derived from tumors. As a consequence, EC cells are most often aneuploid and possess an abnormal karyotype. Furthermore, there is a strong selective pressure for the growth of EC cells in tumors that have reduced potential for differentiation since cell differentiation itself leads to cells with restricted proliferative ability. As a consequence, many EC cell lines possess a reduced capacity for cell differentiation compared to their normal embryonic counterparts. In some ways, however, this can be an advantage depending on the context in which the experimental model is used. For example, there are many cases in which cultured murine and human EC cells are used to study the process of neural differentiation [Andrews, 1984; Jones-Villeneuve et al., 1982; Macpherson et al., 1995; McBurney et al., 1988; Przyborski et al., 2000; Stewart et al., 2003; *see also* Chapter 3]. The more restricted developmental potential of certain EC cell lines can offer an alternative to primary cells, where consistency and quantity of material are variable, and embryonic stem cells, which are technically more challenging to grow and have the tendency to spontaneously differentiate and form progeny representative of all three germ layers. Research using human EC cells as models of early embryogenesis remains a useful, viable alternative and serves as a simple and robust experimental system to investigate, for example, cell fate determination in the embryonic ectoderm [*see* for review Przyborski et al., 2004].

6.1.2. Culture of Human Embryonal Carcinoma Cell Lines

In general, it is possible to isolate and establish EC cell lines from teratocarcinomas whatever their origin providing there is a resident population of proliferating stem cells. Another generalization is the morphology of such cells, in that all murine and human EC lineages derived so far share a characteristic structure of a sparse ring of cytoplasm surrounding a rounded nucleus with one or two prominent nucleoli. Moreover, such morphology closely resembles that of embryonic stem cells. EC cells generally grow as tightly packed colonies, with cells adhering strongly to one another, and often the boundaries between cells are indistinct.

Many of the widely used EC cell lines have adapted to grow independent of feeder cells. Thus it is technically less challenging to grow EC cells routinely, and this allows the generation of large quantities of homogeneous cellular material. In general, the requirements for culture of human EC cells are modest compared to human embryonic stem cells, in that specialized (and often expensive) media supplements and feeder cells are not needed. Dulbecco's modified Eagle's medium (high-glucose formulation supplemented with 2 mM L-glutamine) is most commonly used as a base medium to grow EC cells, although other investigators have preferred the α-modification of Eagle's minimal essential medium (α-MEM). The medium is usually supplemented by 10% heat-inactivated fetal bovine serum (FBS). It is recommended that different batches of FBS from alternative suppliers are screened to select a batch that is optimal for growth and differentiation by examining several for EC cell growth and ability to differentiate before selection of the batch. We look at the expression of cell surface stem cell markers (SSEA-3, TRA-1-60) and differentiation markers (VINIS, A2B5, after 7-day retinoic acid exposure) by flow cytometry (*see* Protocol 6.5). Standard tissue culture plastic flasks and multiwelled plates from any of the major suppliers of consumables are generally satisfactory for

the culturing of human EC cells. EC cells can also be grown on glass surfaces (e.g., coverslips, glass chamber slides) providing the glass has been coated, for example, with poly-D-lysine (*see* Section 6.2.8) before use. Antibiotics can be included in the growth medium, and this is recommended during the early stages of explanting tumor tissues into culture (see Sections 6.2.1, 6.2.2). Routine use of antibiotics should be avoided for the growth and maintenance of established EC cell lines since they are not required and can conceal poor cell culture technique. Expansion of human EC stem cell populations and induction of cell differentiation are carried out in a standard cell culture suite; all cells are maintained at 37°C in an atmosphere of 5% CO_2.

Embryonal carcinoma cells grow robustly in culture providing they are maintained under optimal conditions. It appears that cell density is the single most significant variable that affects the maintenance of the EC stem cell phenotype. High cell densities ($>5 \times 10^4$ cells/cm^2) should be maintained (*see* Section 6.3.2) in routine growth and passage of undifferentiated human EC cell lines.

The following methods are generic for the growth and maintenance of human EC cell lines. The majority have been developed for the culture of sublineages of the well-established EC cell line TERA2, originally derived by Fogh and Trempe [1975]. These procedures are adaptable and can be adjusted for the particular needs of specific cell lines or adjusted to come into line with local practices of individual laboratories. Particular sublines of the TERA2 lineage, NTERA2.cl.D1 [Andrews et al., 1984] and TERA2.cl.SP12 [Przyborski, 2001], have proved useful as models to investigate the molecular mechanisms that control cell fate in the ectoderm and the formation of terminally differentiated neurons [*see* for review Przyborski et al., 2004]. Examples of methods for the differentiation of these cells are provided and are generally applicable to alternative human EC cell lines, although their developmental potential may differ.

6.2. PREPARATION OF REAGENTS

6.2.1. Growth Medium

(i) Dulbecco's modified Eagle's medium [DMEM; high glucose (4500 mg/L); pyridoxine HCl; $NaHCO_3$; without L-glutamine] — 90%

(ii) Fetal bovine serum (FBS), batch tested, heat inactivated at 56°C for 30 min in a shaking water bath — 10%

(iii) L-Glutamine — 2 mM

(iv) Antibiotics (optional):

a. Penicillin — 2.5 U/mL

b. Streptomycin — 2.5 U/mL

6.2.2. Collection Medium

Growth medium supplemented with antibiotics (penicillin 250 U/mL; streptomycin 250 μg/mL; kanamycin 10 μg/mL; amphotericin B 2.5 μg/mL). Store up to 2 weeks at 4°C.

6.2.3. Freezing Medium

(i) Growth medium — 50%

(ii) FBS — 40%

(iii) DMSO — 10%

6.2.4. Glass Beads

Glass beads (3-mm diameter) require acid washing before use:

(i) Incubate beads in concentrated HCl overnight in fume cupboard.
(ii) Remove acid and wash beads well in tap water.
(iii) Wash beads 3× with ultrapure water (UPW) and drain before autoclaving.
(iv) Dry in oven (~150°C) 1–2 days.
(v) Aliquot ~15 beads per capped glass test tube and autoclave beads in tubes. Beads are now ready to use in cell culture.

6.2.5. Retinoic Acid

All-*trans* retinoic acid (purchased sterile in ampoules), 10 mM stock solution in sterile DMSO. Do not filter. Aliquot 500 μL per vial. Store at −70°C in the dark (light sensitive).

6.2.6. Hexamethylene *bis*-acetamide

For 0.3 M stock solution of hexamethylene *bis*-acetamide (HMBA), dissolve 0.6 g in 10 mL UPW. Filter sterilize and store at 4°C.

6.2.7. Mitotic Inhibitors

(i) Cytosine arabinoside, 1 mM stock in UPW. Filter sterilize and store at 4°C for up to 2 weeks. Use at 1:1000 dilution (final concentration, 1 μM).
(ii) Fluorodeoxyuridine, 1 mM stock in UPW. Filter sterilize and store at −70°C for up to 2 weeks. Use at 1:100 dilution (final concentration, 10 μM).
(iii) Uridine, 1 mM stock in UPW. Filter sterilize and store at −70°C for up to 2 weeks. Use at 1:100 dilution (final concentration, 10 μM).

6.2.8. Substrate Coatings

The following substrate modifiers can be used to coat either plastic or glass surfaces to enhance the adherence or growth of differentiating cells. Stocks (100× concentrated) of each substrate can be filter sterilized, aliquoted, and stored at −20°C. Surfaces are immersed in aqueous solution for 12–24 h, washed, and stored at 4°C or used immediately.

(i) Poly-D-lysine, final concentration 10 μg/mL in UPW
(ii) Poly-L-ornithine, final concentration 10 μg/mL in UPW
(iii) Human placental laminin, final concentration 10 μg/mL in calcium- and magnesium-free phosphate-buffered saline (PBSA)

6.2.9. Trypsin/EDTA

Trypsin, 0.25% w/v, EDTA, 1 mM, in PBSA.

6.2.10. Wash Buffer (WB)

PBSA with FBS, 5%, and sodium azide, 15 mM (0.1% w/v).

6.2.11. Fixatives

(i) Bouin's solution: saturated picric acid, 70% v/v, formaldehyde (37–40%), 25% v/v, glacial acetic acid, 5% v/v

(ii) Paraformaldehyde, 4% w/v, in PBSA

6.3. PROTOCOLS

6.3.1. Derivation of Clonal EC Cell Lines

Cell lines from human teratocarcinomas were first derived and maintained as xenografts [Pierce et al., 1957]. It was not until the 1970s that several human EC stem cell lines were established, including TERA2 [Fogh and Trempe, 1975]. Early reports describing the isolation and cloning of human EC cells from primary explant cultures have recognized that the parent material consists of multiple cell types and suggest that pluripotent EC stem cells represent a fraction of the total parent cell population [Andrews et al., 1984, 1985; Thompson et al., 1984; Przyborski, 2001]. For example, clonal cell lines established directly from parent explant cultures of tumor tissues showed variability, rarely expressed markers indicative of pluripotent stem cells, and often displayed limited capacity for cell differentiation [Andrews et al., 1984, 1985]. Isolation of the EC component and derivation of purified human EC stem cell populations were first achieved by passage of the explanted tumor cell line TERA2 as a xenograft through an immune-deficient host. Subsequent dilution cloning of individual cells explanted from the TERA2 xenograft gave rise to the well-known subclone NTERA2.cl.D1 [Andrews et al., 1984]. Human NTERA2.cl.D1 EC stem cells express high levels of the cell surface antigens SSEA-3 and SSEA-4, form complex teratomas when transplanted into an appropriate host, and maintain the ability to differentiate in vitro [Andrews et al., 1984]. Subsequently, a more direct approach to isolate and derive human EC stem cell lines has been developed that does not require the passage of the explanted tumor line through an animal [Przyborski, 2001]. This method can be applied to an established explant culture or directly to freshly dissected and dissociated tumor tissue as follows. The primary explant technique is used to establish a cell line representative of the original teratocarcinoma. There are ethical and safety issues concerned with collection of human biopsy material (*see* Chapter 2).

Protocol 6.1. Explant Cultures of Human EC Cells

Reagents and Materials

Sterile

- ❑ Collection medium (*see* Section 6.2.2)
- ❑ Growth medium (*see* Section 6.2.1)
- ❑ PBSA
- ❑ Trypsin/EDTA (*see* Section 6.2.9)
- ❑ Plastic culture flasks, 25 cm^2
- ❑ Petri dish, 9 cm

- ❑ Centrifuge tube, 30 or 50 mL
- ❑ Razor blade

Procedure

(a) Working in collaboration with the surgical team (*see* Chapter 2), provide a sterile container of collection medium for immediate storage and transfer of dissected tissues. Ensure that the specimen tube is clearly labeled with information describing the contents of the vessel, date/time of surgery, and necessary contact details.

(b) Transfer the sample immediately to the tissue culture laboratory. Tissues will remain viable for 1–2 days if stored at 4°C in collection medium, although prolonged storage should be avoided.

(c) Wash tissue twice with PBSA and transfer specimen to the 9-cm Petri dish.

(d) Dissect away nontumor tissues (e.g., fat) and remove any obvious necrotic material. Ensure that the specimen remains moist, using small volumes of PBSA.

(e) Finely chop the specimen into 1-mm cubes with a sterile razor blade.

(f) Transfer material in 10 mL of PBSA to a centrifuge tube with a pipette.

(g) Wash the tissues with PBSA 2–3 times, allowing the samples to settle without centrifugation between the washes.

(h) Transfer the pieces to a 25-cm^2 culture flask (approximately 25 pieces per flask).

(i) Remove the PBSA by pipetting from the corner of the tilted flask and add 1 mL of growth medium including antibiotics.

(j) Spread the pieces evenly over the growth surface by tilting the flask.

(k) Incubate the flask for 24 h at 37°C, being careful not the move the flask during this period. If the pieces have adhered to the surface, carefully increase the growth medium volume to 10 mL over the next few days.

(l) Change the growth medium once or twice per week to encourage cell outgrowth. Cell migration and proliferation from the tissue pieces should be evident during this time.

(m) Continue to maintain the culture, frequently changing the growth medium until monolayers of cells become well established around the explanted tissues.

(n) Enzymatically dissociate the culture with 1 mL of trypsin/EDTA solution for 5 min at 37°C.

(o) Dislodge the remaining cells with a sharp lateral motion delivered by tapping the side of the flask with the palm of the hand.

(p) Add 9 mL of growth medium and flush over entire culture. Collect cells by pipette and gently pipette up and down a couple of times to break up any clumps.

(q) Centrifuge cells at 450 *g* and wash in 10 mL of growth medium.

(r) Resuspend the final pellet in 10 mL of growth medium and split the culture equally into two fresh 25-cm^2 flasks.

(s) Maintain cultures at 37°C, changing the growth medium every 2–3 days.

(t) Repeat enzymatic passaging of cultures as described above to establish sufficient quantities of cells to maintain the explant line and cell samples for cryopreservation (*see* Section 6.5).

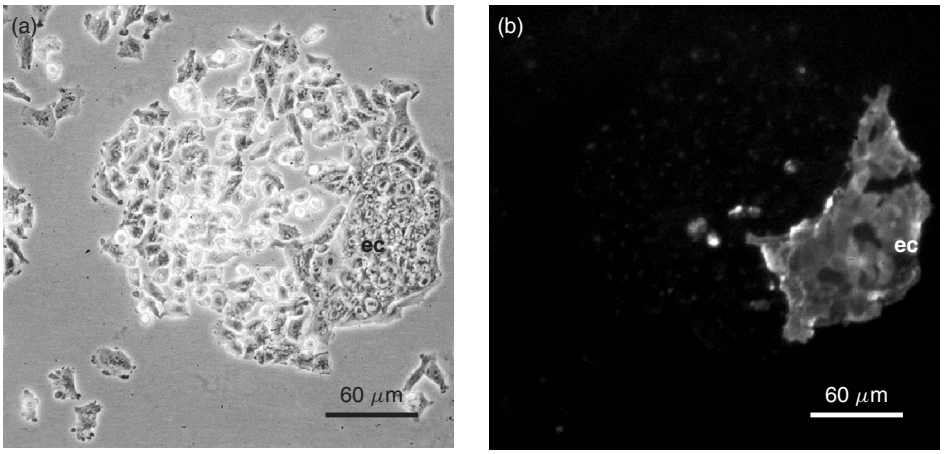

FIGURE 6.1. Phase image (**a**) and corresponding immunofluorescence localization of SSEA-3 (**b**) in an established explant culture from a human germ cell tumor. Note that only a small proportion of the total cell population is positive for SSEA-3 staining, indicating EC stem cells (ec) within this culture. Scale bar: 60 μm.

Notes: It is essential that the appropriate regulations and approvals for the use, collection, and handling of human tissues are in place and followed (*see* Chapter 2). Antibiotics should continue to be included in the growth medium until the explant culture is well established and cryopreserved samples can be retrieved from storage free from infection. Initially the explant culture will appear very heterogeneous and will include a multitude of cell types. Over time, the tumor cells will become the primary cell type. However, even these cultures tend to be heterogeneous and often only consist of 1–2% EC stem cells (Fig. 6.1). It is important to cryopreserve samples as early as possible and at a low passage number to capture the cells in a state most closely related to the parent tumor.

Protocol 6.2. Establishment of Clonal EC Cell Lines

Sterile or aseptically prepared

- ❑ Irradiated STO mouse feeder cells [Martin and Evans, 1975], ~70% confluent when irradiated (*see also* Protocol 11.2)
- ❑ PBSA
- ❑ PBSA/5FB: PBSA containing 5% FBS
- ❑ Trypsin/EDTA (*see* Section 6.2.9)
- ❑ Stage-specific embryonic antigen-3 (SSEA-3), a cell surface antigen marker of pluripotent stem cells (*see* Fig. 6.1)
- ❑ Polyscience BioMag particles, anti-mouse IgM (IgM isotype used to recognize SSEA-3)
- ❑ Polypropylene centrifuge tube, 15 mL
- ❑ Multiwell plates, 6-well and 12-well

- ❑ Petri dishes, 3.5 and 9 cm
- ❑ Glass Pasteur pipettes, flame drawn to fine tip

Nonsterile

- ❑ Hemocytometer
- ❑ Trypan Blue, 0.4% w/v, or Erythrosin B, 0.15% w/v
- ❑ Inverted microscope, swabbed with 70% alcohol, in a vertical laminar flow hood adapted for use with a microscope. Be aware that the biological safety status of a Class II biosafety cabinet may be compromised by adapting it for use with a microscope; this should be checked before use.

Procedure

(a) Work with a confluent flask of the earliest available passage of explanted cells (see above).

(b) Aspirate growth medium from cell culture and rinse with 5 mL of PBSA.

(c) Add 1 mL of trypsin/EDTA and tip the flask to ensure the monolayer of cells is covered.

(d) Incubate the cells at 37°C until they round up and begin to detach (5 min). Dislodge the remaining cells with a sharp lateral motion delivered by tapping the side of the flask with the palm of the hand.

(e) Add 9 mL of growth medium and flush over entire culture surface for maximum harvest. Gently pipette up and down a couple of times to break up any clumps, to produce a single cell suspension.

(f) Count the cells with a hemocytometer. Generally, cell viability is greater than 95% and this can be checked by a simple dye exclusion assay (for example, staining with 0.4% w/v Trypan Blue or 0.15% w/v Erythrosin B [Freshney, 2005]).

(g) Dilute to 1×10^7 cells/mL in 5 mL of PBSA/5FB.

(h) Incubate cell suspension with SSEA-3, diluted 1:5, for 45 min at 4°C in the 15-mL polypropylene centrifuge tube.

(i) Pellet cells by centrifugation (450 *g*, 3 min) and wash 3 times in PBSA/5FB at 4°C.

(j) Isolate cells immunoreactive for SSEA-3 by direct positive magnetic separation with BioMag anti-mouse IgM particles according to the manufacturer's instructions. In brief:

  i) Incubate magnetic particles with cells for 30 min at 4°C.

  ii) Gently mix contents of centrifuge tube and place a magnet against its wall for 30 s to aggregate cells bound to magnetic particles.

  iii) With the magnet remaining in position, carefully tip off supernate, leaving cells against the tube wall.

  iv) Remove the magnet and resuspend the cells in PBSA/5FB.

  v) Repeat isolation process for increased purity of SSEA-3-positive cells.

(k) Resuspend isolated cells in 5 mL of PBSA/5FB and determine cell number with a hemocytometer. Seed approximately 1000 cells into a 9-cm Petri dish containing 10 mL of PBSA/5FB.

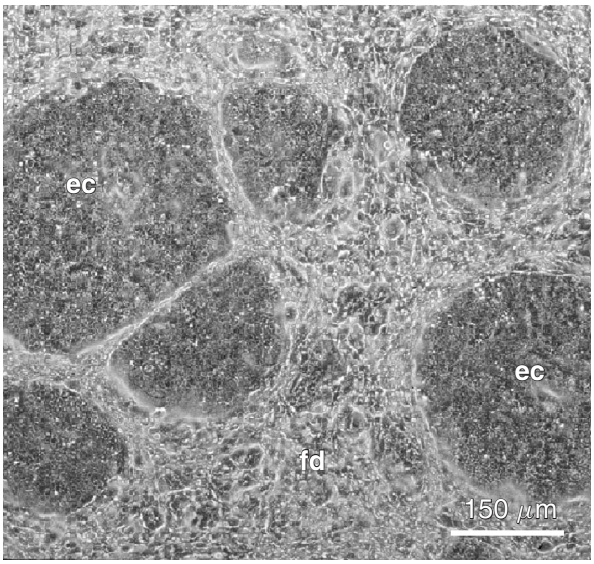

FIGURE 6.2. Colonies of a clonal human EC stem cell lineage (ec) during its initial expansion on STO feeder cells (fd). This phase micrograph was taken at passage 3, when the number of colonies for the same clonal line began to expand and merge. This particular example was not passaged onto fresh feeder cells and was subsequently treated to become feeder independent. Scale bar: 150 μm.

(l) Place an inverted microscope with phase-contrast optics in a sterile environment (for example, an adapted laminar flow hood; swab down microscope stage with ethanol solution to sterilize). Using a flame-drawn fine glass pipette, select individual cells and place into a 100-μL meniscus of PBSA/5FB set up in a separate 3.5-cm Petri dish. Using the microscope's ×40 objective, check that a single cell has been selected.

(m) Transfer each single cell into a separate well of a 12-well plate containing irradiated STO feeder cells in growth medium.

(n) Maintain cocultures for 10 days, changing the growth medium every 2–3 days.

(o) Add 0.3 mL of trypsin/EDTA to each well to remove cocultured cells. Seed entire cell suspension into a 6-well plate containing fresh feeder cells in 3 mL of growth medium. Maintain cocultures as in Step (n). EC cells will become evident among the feeder layer as the stem cell population increases (Fig. 6.2).

(p) When large colonies of EC cells are evident, enzymatically dissociate the culture as described in Steps (b)–(e), and seed into a fresh 6-well plate in growth medium without fresh feeder cells. Existing STO feeders will gradually die away, leaving a clonal population of human EC stem cells that can be maintained independent of feeders (Fig. 6.3).

Note: BioMag magnetic particles are approximately 1 μm in diameter, and because of their nonuniform shape, they provide an increased surface area (>100 m^2/ g), 20–30 times greater than that of uniform spherical particles, allowing for a higher binding

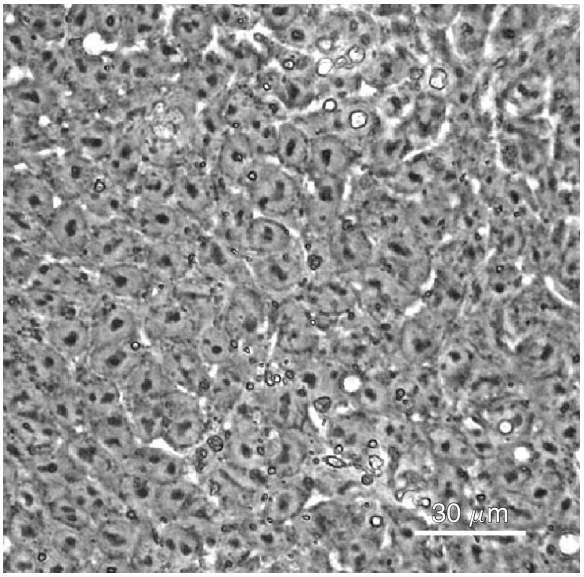

FIGURE 6.3. Clonal lineage of human EC stem cells. The example shown is TERA2.cl.SP12 [Przyborski, 2001]. The cells should be maintained at high confluence and will grow as a homogeneous monolayer under optimal conditions. Scale bar: 30 μm.

capacity while utilizing a lower amount of particle. The magnetic particles detach from the cell membrane spontaneously as the cell surface is turned over during subsequent culturing for up to 48 h. Maintain feeder cell cocultures for at least the first 2–3 passages to enable the newly derived clonal lineages to generate enough numbers to then grow independently. The feeder cells help maintain the EC stem cell phenotype, and without the feeders the EC cells are likely to differentiate spontaneously. Once independent of feeder cells, the clonal EC stem cell lines grow robustly and have a homogeneous appearance. They must be maintained under optimal growth conditions and passaged at high confluence (*see below*); otherwise, they have a tendency to differentiate spontaneously.

6.3.2. Routine Passaging and Harvesting of EC Cells

There are two ways in which this can be achieved. The first method (*see* Protocol 6.3) relies on mechanical disruption, which is quick and simple and avoids complete dissociation of the cell culture. This is important since the maintenance of cell contacts and high confluence is known to limit spontaneous differentiation within the culture. The second method uses enzymatic treatment to dissociate the culture completely into a single-cell suspension. This is necessary when determining cell number with a hemocytometer or for preparing the cells for flow cytometry. The second method (Protocol 6.4) can be used occasionally to passage the cells, but it is not recommended for routine passaging since it may change the nature of the cells over time. In both of these methods, cells from a confluent 75-cm^2 tissue culture flask have been processed.

Protocol 6.3. Subculture of EC Cells by Mechanical Dissociation

Reagents and Materials

Sterile

- ❑ Growth medium (*see* Section 6.2.1)
- ❑ PBSA
- ❑ Acid-washed glass beads (*see* Section 6.2.4)
- ❑ Plastic culture flasks, 75 cm^2

Procedure

(a) Aspirate growth medium from cell culture and rinse with 5 mL of PBSA.
(b) Add ~15 acid-washed 3-mm glass beads and 5 mL of growth medium.
(c) Carefully rock the flask from side to side, backward and forward, to roll the beads over the cells to dislodge them from the surface. Be careful not to tip the contents into the neck of the flask.
(d) Collect the dislodged cells with a 10-mL pipette and gently pipette the suspended cells up and down a couple of times with the tip of the pipette resting in the bottom corner to disrupt the larger aggregates.
(e) Collect the cell suspension with the same pipette and transfer this to a new 75-cm^2 flask containing 50 mL of growth medium.
(f) Ensure that the suspension is completely mixed before splitting the culture equally between three new 75-cm^2 flasks (20 mL per flask) for a 1:3 split.

Protocol 6.4. Subculture and Sampling of EC Cells by Enzymatic Dissociation

Reagents and Materials

Sterile

- ❑ Growth medium (*see* Section 6.2.1)
- ❑ PBSA
- ❑ Trypsin/EDTA (*see* Section 6.2.9)
- ❑ Plastic culture flasks, 75 cm^2

Nonsterile

- ❑ Hemocytometer
- ❑ Trypan Blue, 0.4% w/v, or Erythrosin B, 0.15% w/v

Procedure

(a) Aspirate growth medium from cell culture and rinse with 5 mL of PBSA.
(b) Add 1 mL of trypsin/EDTA and tip the flask to ensure that the entire monolayer of cells is covered.

(c) Incubate the cells at 37°C until they round up and begin to detach (3–5 min). Dislodge the remaining cells with a sharp lateral motion delivered by tapping the side of the flask with the palm of the hand.

(d) Add 9 mL of growth medium and flush over entire culture surface for maximum harvest. Gently pipette up and down a couple of times to break up any clumps.

(e) Count the cells with a hemocytometer. Generally, cell viability is greater than 95%, and this can be checked by a simple dye exclusion assay (for example, staining with 0.4% Trypan Blue or 0.15% Erythrosin B).

(f) Reseed the cells into fresh tissue culture plasticware at 5×10^4 cells/cm^2 (equivalent of 3.75×10^6 cells per 75-cm^2 flask) containing the appropriate amount of growth medium (20 mL per 75-cm^2 flask).

Note: EC stem cells adhere and recover rapidly, forming a fully confluent monolayer in 3–4 days when maintained at 37°C under a humidified atmosphere of 5% CO_2 in air (*see* Fig. 6.3). Cells should not require feeding during this time. Passages involving 1:2 to 1:4 split ratios can be used depending on the confluence of the culture before passaging. It is not necessary to wash the cells for passaging after collection using trypsin, provided that sufficient growth medium containing FBS has been added to inactivate trypsin.

6.4. CHARACTERIZATION

6.4.1. Determination of EC Phenotype

One of the most straightforward approaches to determining the phenotype of cultured human EC stem cells is to examine the expression of cell surface antigens with flow cytometry. The method is relatively simple and provides accurate, quantifiable data regarding the status of the culture and the proportions of stem cell and differentiated cell phenotypes. Comparison of the expression of a variety of antigens on a large panel of human cell lines derived from germ cell tumors confirmed that the cell surface antigens stage-specific embryonic antigens-3 (SSEA-3) and -4 (SSEA-4) and TRA-1-60 are particularly characteristic markers of human EC stem cells [Andrews et al., 1996]. In general, cultures of human EC stem cells maintained at high density post-confluence and under optimal growth conditions express high levels of these markers. However, suboptimal culture conditions and differentiation result in the decreased expression of, for example, SSEA-3, and the upregulation of proteins associated with particular pathways of cell differentiation [Andrews, 1982; Przyborski et al., 2000]. Protocol 6.5 provides a general method for the flow cytometric analysis of human EC stem cells.

Protocol 6.5. Flow Cytometric Detection of Cell Surface Markers in EC Cells

Reagents and Materials

Sterile or aseptically prepared

- ❑ Confluent culture of EC cells
- ❑ PBSA

- ❑ Trypsin/EDTA (*see* Section 6.2.9)
- ❑ Wash buffer (WB; *see* Section 6.2.10)
- ❑ Primary antibody: for example, SSEA-3 supernate (Developmental Studies Hybridoma Bank) diluted 1:5 in WB
- ❑ Secondary antibody, fluorescein-conjugated, diluted in WB: for example, rat anti-mouse IgM diluted 1:20 in WB for the detection of mouse monoclonal anti-SSEA-3
- ❑ Microtitration plate, 96-well, round-bottomed, with lid

Nonsterile

- ❑ Viability stain: Trypan Blue, 0.4% w/v, or Erythrosin B, 0.15% w/v
- ❑ Hemocytometer
- ❑ Microtitration plate centrifuge or plate holders for regular centrifuge
- ❑ Flow cytometer

Procedure

(a) Working with a flask of confluent human EC stem cells (*see* Fig. 6.3), aspirate growth medium from cell culture and rinse with 5 mL of PBSA.

(b) Add 1 mL of trypsin/EDTA and tip the flask to ensure that the entire monolayer of cells is covered.

(c) Incubate the cells at 37°C until they round up and begin to detach (~5 min). Dislodge the remaining cells with a sharp lateral motion delivered by tapping the side of the flask with the palm of the hand.

(d) Add 9 mL of growth medium and flush over entire culture surface for maximum harvest. Gently pipette up and down a couple of times to break up any clumps and produce a single-cell suspension.

(e) Count viable cells with a hemocytometer. Generally, cell viability is greater than 95%, and this can be checked by a simple dye exclusion assay (for example, staining with 0.4% Trypan Blue or 0.15% Erythrosin B).

(f) Pellet cells at 450 *g* for 3 min and resuspend at 1×10^7 cells/mL in WB

(g) Set up 96-well round-bottomed plates with the appropriate antibody solutions (50 μL per well) in every other well (avoid using adjacent wells to minimize any overspill and contamination), for example, SSEA-3 supernate diluted 1:5 in WB. Purified monoclonal antibodies will normally have much higher dilutions, for example, 1:100 in WB. (NB: The appropriate dilutions should be chosen by prior titration to give maximal binding in the assay.)

(h) Add 50 μL of cell suspension to each well containing antibody solution (i.e., approximately 5×10^5 cells/well).

(i) Cover plates with lid and agitate gently on an orbital shaker for 30 min at 4°C.

(j) Spin down cells at 460 *g* for 3 min. Check to see cell pellets in base of wells before removing supernate by inverting the plate.

(k) Add 100 μL of WB, spin down cells, remove supernate, and repeat twice. Do not add fresh WB after last wash.

(l) Add 50 μL of the appropriate fluorescein-conjugated secondary antibody diluted in WB, for example, rat IgM diluted 1:20 in WB for the detection of SSEA-3.

(m) Cover plates with lid and agitate gently on an orbital shaker for 30 min at 4°C in the dark.
(n) Wash cells in WB to remove secondary antibody as described above.
(o) Transfer cells to labeled tubes, appropriate for the flow cytometer, and containing 0.2 mL of WB. Maintain cells on ice until passed through the flow cytometer.
(p) Using the flow cytometer, determine levels of fluorescein fluorescence with the appropriate filter sets according to the manufacturer's instructions.

Notes: It is essential that the single-cell suspension is free of cell aggregates to enable the passage of a single-cell stream through the flow cytometer, so the cell suspension should be checked on a microscope before use. A negative control can also be included to enable background thresholds to be set. It is also possible to fix cells with a mild solution of paraformaldehyde (0.2% w/v in PBSA) and keep them at 4°C overnight to preserve samples temporarily should immediate access to the cytometer be restricted.

6.4.2. Assessment of Developmental Potential

6.4.2.1. Growth of Teratomas In Vivo. It is well known that human embryonic stem cells and their malignant counterparts, human EC stem cells, form complex teratomas consisting of a range of differentiated cell types when engrafted into an immune-deficient host [Andrews et al., 2001; Przyborski 2005; Cooke et al., 2006]. This was first demonstrated in by Kleinsmith and Pierce [1964], who showed that the transplantation of a single murine EC cell from one tumor to a new host resulted in the formation of a new teratocarcinoma containing structures similar to those seen in the parent tumor. Indeed, this was the way an EC stem cell lineage could be maintained before the derivation of EC stem cell lines. Today, many scientists use cell transplantation and the growth of teratomas as a routine method to evaluate the developmental potential of newly derived embryonic stem cell and EC stem cell lines [*see* for review Przyborski 2005]. In vitro experiments enable the characterization of cell differentiation in a more controlled manner, but this approach does have limitations. Cell culture does not currently provide an appropriate environment that allows for three-dimensional cell growth, promotion of interactions between adjacent cells and tissues, and the exposure to growth factors and combinations of signaling molecules, etc., and this may be why engrafted stem cells are capable of far greater differentiation. Protocol 6.6 describes a simple method for producing subcutaneous xenograft tumors in immune-deficient mice from human EC stem cells.

Protocol 6.6. Growth of EC Cells as Subcutaneous Xenografts

Reagents and Materials

Sterile or aseptically prepared

- ❑ Human EC cells
- ❑ Collection medium (if resultant tumors to be cultured)

- ❑ Growth medium (if resultant tumors to be cultured)
- ❑ Trypsin/EDTA (*see* Section 6.2.9)
- ❑ PBSA
- ❑ Hamilton syringe and 21-gauge needle

Nonsterile

- ❑ Nude (*nu/nu*) mice
- ❑ Hemocytometer
- ❑ Viability stain: Trypan Blue, 0.4% w/v, or Erythrosin B, 0.15% w/v

Procedure

(a) Harvest human EC stem cells (*see* Fig. 6.3) with 1 mL of trypsin/EDTA solution to produce a single-cell suspension and determine the viable cell number by hemocytometer as described in Protocol 6.5, Step (e).

(b) Wash cells and resuspend in PBSA at a concentration of 1×10^7 cells/mL.

(c) Transplant 5×10^5 cells in 50 μL of PBSA with a 21-gauge needle (a Hamilton syringe can be used) subcutaneously into the flank of a adult male Nude (*nu/nu*) mouse.

(d) Maintain mice as normal for up to 12 weeks and monitor animal welfare and tumor progression regularly. A teratoma is usually first identified as a small contained, palpable mass beneath the skin near the transplantation site. When identified, record location and size of the tumor with measuring calipers and monitor weight of the animal.

(e) When appropriate (for example, the tumor mass has reached 1 cm in diameter), sacrifice the animal (with an approved, regulated procedure) and immediately dissect the tumor mass, being careful to remove any host tissues.

(f) How tissues are to be analyzed will determine how they are processed. For example, samples may be frozen for cryostat sectioning or fixed for paraffin embedding and histological analysis. There are numerous methods for tissue fixation, including Bouin's solution (*see* Section 6.2.11), which preserves tissue morphology well and is useful for general histology (Fig. 6.4; Plate 6), or 4% paraformaldehyde, which also preserves tissue structure but is more amenable to immunohistochemistry after paraffin embedding and sectioning).

(g) Alternatively, tumor tissues can be explanted into culture. To maintain cell viability, place dissected materials into collection medium at 37°C for transfer to the culture facility. Explant tissues into culture, following procedures described above (*see* Protocol 6.1).

Notes: These procedures must adhere to the regulations of the authority that licenses the use of animals for research purposes and must only be carried out by appropriately trained and authorized personnel. It is essential to monitor the welfare of the host at all times. Immune-deficient animals, such as Nude (*nu/nu*) mice, are required as hosts for these xenograft experiments. Transplantation into Nude mice is particularly useful when locating subcutaneous grafts. The animal's weight is a useful indicator of tumor progression, especially if a growing xenograft is not palpable.

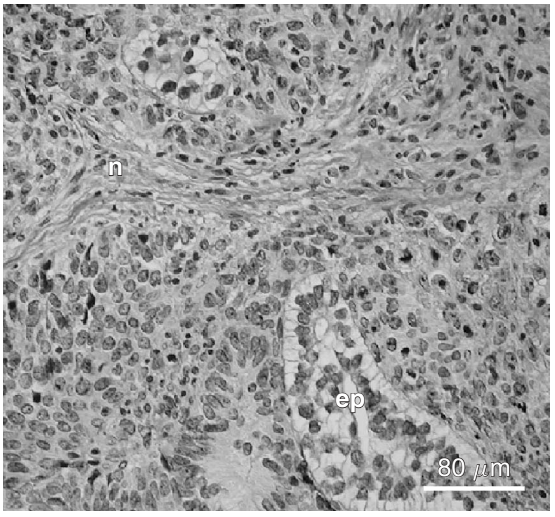

FIGURE 6.4. Formation of teratomas by human EC stem cells can be used to demonstrate their developmental potential. TERA2.cl.SP12 stem cells readily form xenograft tumors when transplanted subcutaneously into immune-deficient hosts. General staining with hematoxylin and eosin shows several diverse structures in the tumor, including neural tissue (n) and primitive epithelia (ep). Scale bar: 80 μm. (*See also* Plate 6.)

6.4.2.2. Differentiation In Vitro in Response to Exogenous Stimuli. Cultured mammalian EC stem cells are well recognized as useful models with which to study the early stages of embryogenesis in vitro. Pioneering work by Jones-Villeneuve et al. [1982] and McBurney et al. [1988], first demonstrated that cultured murine EC stem cells differentiated in response to retinoic acid, forming populations of neurons and glial cells. In a subsequent report, Macpherson and McBurney [1995] published detailed methods for the growth and differentiation of murine P19 EC stem cells for research into neural development. These procedures have since been adapted by other investigators and applied to study of cell differentiation by human EC stem cells [Andrews et al., 1984; Pera et al., 1989; Stewart et al., 2003; Thompson et al., 1984]. Sublines of the TERA2 lineage have proved particularly useful for this purpose [Andrews et al., 1984; Horrocks et al., 2003; Przyborski 2001; Przyborski et al., 2000; *Stewart et al.,* 2003]. The following are typical methods that are used to induce cultured human EC to form neural derivatives. Two protocols are described: Protocol 6.7 describes the growth of cells as adherent monolayers; while in Protocol 6.8, cells are grown in suspension before being plated onto coated surfaces.

Protocol 6.7. Differentiation of EC Stem Cells as Adherent Monolayers

Reagents and Materials

Sterile or aseptically prepared

- ❑ Culture of EC stem cells (*see* Protocol 6.2)
- ❑ Growth medium (*see* Section 6.2.1)

- ❑ Trypsin/EDTA (*see* Section 6.2.9)
- ❑ Retinoic acid (*see* Section 6.2.5)
- ❑ Cytosine arabinoside (*see* Section 6.2.7)
- ❑ Fluorodeoxyuridine (*see* Section 6.2.7)
- ❑ Uridine (*see* Section 6.2.7)
- ❑ Culture flasks, 75 cm^2

Procedure

(a) Harvest EC stem cells (*see* Fig. 6.3) with 1 mL trypsin/EDTA solution to produce a single-cell suspension and determine cell number as described above (*see* Protocol 6.4).

(b) To induce cell differentiation, seed cells at 2×10^4 cells/cm^2 (equivalent to 1.5×10^6 cells per 75-cm^2 flask) in growth medium (20 mL per 75-cm^2 flask) containing 10 μM retinoic acid (Stage 1). Cells will adhere to the flask surface within 24 h and produce a loosely confluent monolayer in 3–4 days. Cell density will continue to increase, but in general the culture will not overgrow as the vast majority of cells begin to differentiate and eventually become postmitotic. Populations of neurons among other. nonneuronal cells will become visible within 2–3 weeks (Fig. 6.5).

(c) Replace growth medium containing retinoic acid every 3–4 days throughout the first period of the differentiation procedure (Stage A1).

(d) After 21 days dissociate culture with trypsin/EDTA solution as described above. Incubation times at 37°C may need to be extended to 7–10 min.

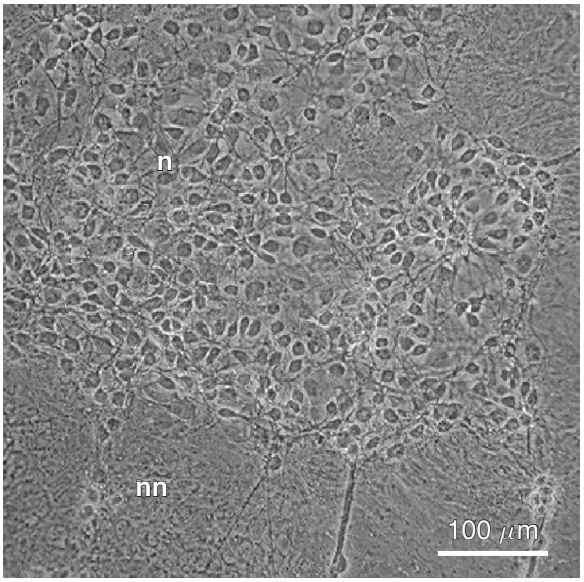

FIGURE 6.5. Generation of neurons from human EC stem cells after 3-week exposure to 10 μM retinoic acid. Phase micrograph showing a heterogeneous culture of differentiating cells including morphologically identifiable neurons (n) and nonneuronal cell types (nn). Scale bar: 100 μm.

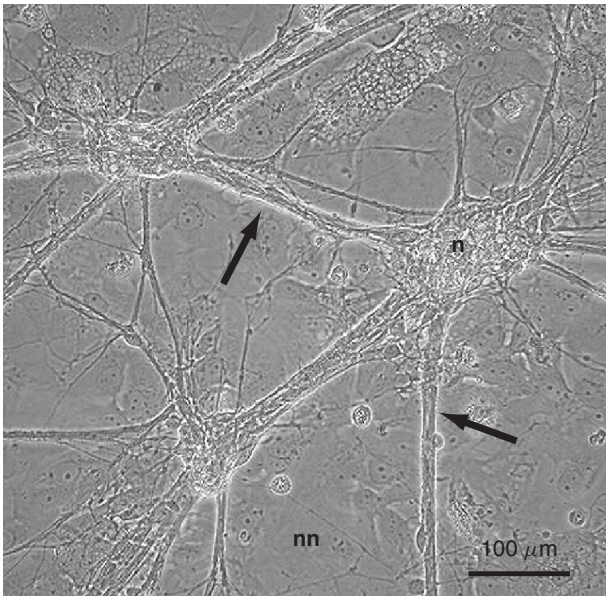

FIGURE 6.6. Dissociation of retinoic acid-induced cells after 21 days of differentiation results in the release of neurons from the monolayer. When the culture is reestablished after passaging, neurons (n) readily grow on top of a background of nonneuronal cells (nn). Neuronal perikarya form aggregations that are linked to one another by bundles of neurites (arrows). Scale bar: 100 μm.

(e) Add 10 mL of growth medium and gently pipette cell suspension up and down to break up any remaining cell clumps and produce a single-cell suspension.

(f) Split cell suspension equally 1:4 between 4 fresh flasks [of the same size used in Step (b) above] and culture in growth medium without retinoic acid for a further 4–6 days (Stage A2). During this time, neurons are readily identified above a background of flat nonneuronal cells (Fig. 6.6).

(g) Remove growth medium from Stage A2 cells, add 1 mL of trypsin/EDTA solution, and incubate for 2 min at room temperature.

(h) Detach cells loosely attached to the surface of the culture by applying a lateral motion provided by five short, sharp blows with the palm of the hand to the side of the horizontal tissue culture flask.

(i) Collect cells displaced by enzymatic/mechanical disruption in 10 mL of growth medium.

(j) Combine cell suspensions from more than one flask, determine cell number, and wash and resuspend pellet in growth medium.

(k) Seed cell suspensions at 2×10^5 cells/cm^2 (Stage A3) and culture in growth medium containing either: (i) 1 μM cytosine arabinoside (for the first 10 days only), 10 μM fluorodeoxyuridine, 10 μM uridine, to enhance the purity of neurons and reduce the proliferation of nonneuronal cell types (Fig. 6.7) or (ii) 0.1 μM cytosine arabinoside (for the first 10 days only), 3 μM fluorodeoxyuridine, 5 μM uridine, to allow limited proliferation of astrocytic glial cells.

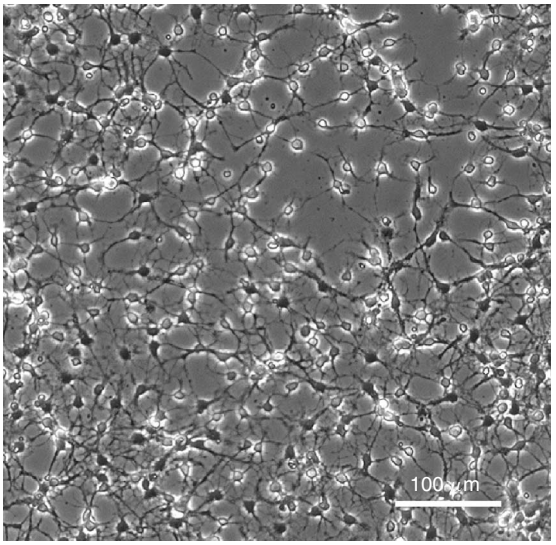

FIGURE 6.7. Purified populations of neurons derived from human EC stem cells. Cells are maintained in medium containing mitotic inhibitors to control the proliferation of any nonneuronal cell types. Scale bar: 100 µm.

Notes: These procedures were developed with sublines of the TERA2 lineage as previously described [Andrews et al., 1984; Pleasure et al., 1992; Przyborski et al., 2000], in particular the clonal line TERA2.cl.SP12 [Przyborski 2001; Stewart et al., 2003] and can be adapted for use in other EC stem cell lines. After 2–3 weeks of growth (Stage A3), almost pure (>95%) populations of EC stem cell-derived neurons can be obtained on the basis of differences in cell adhesion. Astrocytic glial cells adhere well to the tissue culture surface, whereas neurons are more loosely attached. After Stage A3, Steps (g)–(k) may be repeated to increase the purity of neuronal cultures from >95% to >99%. Astrocytic glia can be cultured directly on tissue culture plastic or substrates coated with poly-D-lysine (10 µg/mL). To enhance neuronal development, particularly neurite outgrowth, seed neurons onto substrates coated with poly-L-ornithine and laminin. Human EC stem cells are also known to respond to other types of exogenously applied chemical reagents and differentiate in alternative ways. For example, TERA2.cl.SP12 EC cells exposed to 3 µM HMBA for 3–4 weeks form relatively homogeneous cell cultures devoid of neurons but consisting of flat epithelial-like cells [Przyborski et al., 2004]; Fig. 6.8).

Protocol 6.8. Differentiation of EC Stem Cells as Suspended Cell Aggregates

Reagents and Materials

Sterile or aseptically prepared

- ❑ Culture of EC stem cells (*see* Protocol 6.2)
- ❑ Trypsin/EDTA (*see* Section 6.2.9)

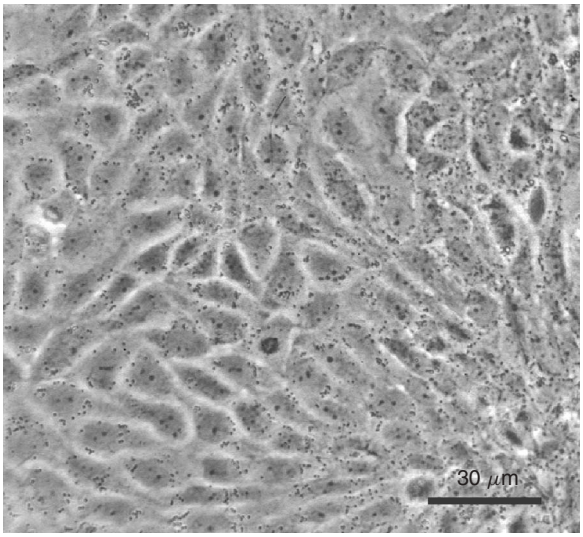

FIGURE 6.8. Human EC stem cells exposed to 3 μM HMBA for 21 days form relatively homogeneous cultures consisting of differentiating cells epithelial-like in nature. There is very little, if any, evidence of neuronal differentiation. Scale bar: 30 μm.

- ❑ Retinoic acid (*see* Section 6.2.5)
- ❑ Cytosine arabinoside (*see* Section 6.2.7)
- ❑ Fluorodeoxyuridine (*see* Section 6.2.7)
- ❑ Uridine (*see* Section 6.2.7)
- ❑ Plastic Petri dishes, bacteriological grade, 9 cm
- ❑ Centrifuge tubes, 15 mL
- ❑ Coated plates or dishes: poly-L-ornithine and laminin (*see* Section 6.2.8)

Procedure

(a) Harvest EC stem cells with 1 mL of trypsin/EDTA solution to produce a single-cell suspension and determine cell number as described above.

(b) To induce cell differentiation, seed cells at 5×10^5 cells per sterile 9-cm Petri dish in 10 mL of growth medium.

(c) Maintain suspension cultures for 24 h before adding retinoic acid to a final concentration of 10 μM.

(d) Incubate cells for a further 2 weeks, changing the growth medium supplemented with retinoic acid every 3–4 days. Suspended aggregations of cells readily form during the first week of culture (Fig. 6.9). It is necessary to pellet aggregates by gentle centrifugation at 20 *g* for 2 min before resuspending cells in fresh growth medium.

(e) Transfer cell suspension to a 15-mL centrifuge tube and gently pellet cell aggregates at 20 *g* for 2 min. Resuspend in fresh growth medium without retinoic acid and seed onto substrates coated in poly-L-ornithine (10 μg/mL) and laminin (10 μg/mL).

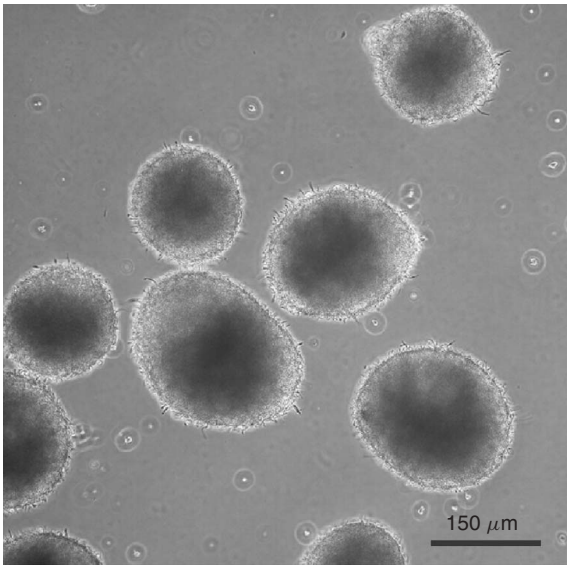

FIGURE 6.9. Growth of dissociated human EC stem cells in suspension and in the presence of 10 μM retinoic acid results in the formation of cell aggregates within 4–7 days. Scale bar: 150 μm.

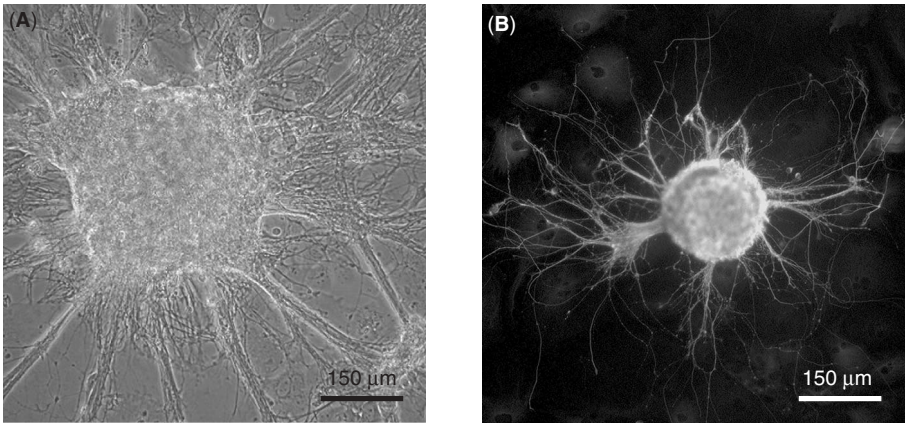

FIGURE 6.10. Suspended aggregates of differentiating human EC cells plated onto to poly-L-ornithine- and laminin-coated surfaces often form elaborate neurite outgrowths as seen by phase microscopy (**a**) and immunofluorescent staining (**b**) for the neural marker TuJ1. Scale bars: 150 μm. (*See also* Plate 7.)

(f) Incubate cells for a further 2–3 weeks in growth medium containing mitotic inhibitors [1 μM cytosine arabinoside (for the first 10 days only), 10 μM fluorodeoxyuridine, 10 μM uridine]. During this time, the cell aggregates will attach to the culture surface and the majority will develop extensive neurite projections (Fig. 6.10; Plate 7).

Notes: It is essential that a fresh bacteriological Petri dish is used each time the growth medium is changed to prevent cells from adhering to the culture surface. Cell aggregates may range from 50 to 750 μm in diameter and can be visible to the naked eye. It is therefore relatively straightforward to select individual aggregates and place them in different culture conditions (for example, adjacent to other aggregates or in coculture with other cell types).

6.5. CRYOPRESERVATION

When a cell line has been derived, and it displays characteristics typical of an EC stem cell and is free from contamination, it can be readily preserved cryogenically to generate frozen stocks. EC stem cells are readily stored in this manner and show excellent recovery from cryopreservation.

Protocol 6.9. Freezing EC Stem Cells

Reagents and Materials

Sterile or aseptically prepared

- ❑ Culture of EC stem cells, 25 cm^2 or 75 cm^2 (*see* Protocol 6.2)
- ❑ Freezing medium (*see* Section 6.2.3)

Nonsterile

- ❑ Insulated box for freezing
- ❑ Ultra deep-freeze at −70°C
- ❑ Liquid nitrogen freezer or equivalent

Procedure

(a) Harvest cells according to procedure described above (*see* Protocol 6.4) and pellet at 450 *g* for 3 min.

(b) Remove supernate and resuspend pellet in freezing medium (3 mL per 75-cm^2 flask; 1 mL per 25-cm^2 flask).

(c) Aliquot 1 mL of suspension per cryogenic vial.

(d) Label vial with name of cell line, passage number, and date.

(e) Slowly freeze samples overnight by placing vials in an insulated box and transferring the box into the −70°C freezer.

(f) Transfer frozen samples next morning into long-term storage facility, −140°C (mechanical freezer) or −196°C (liquid nitrogen).

(g) Ensure that records are updated and accurate.

Protocol 6.10. Thawing EC Stem Cells from Cryostorage

Reagents and Materials

Sterile

- ❑ Growth medium (*see* Section 6.2.1)

Nonsterile

- ❑ Water bath at 37°C

Procedure

(a) Remove cells from storage facility and transfer to portable liquid nitrogen storage canister.

(b) Thaw vial rapidly in a 37°C water bath.

(c) Transfer contents of vial into a 15-mL centrifuge tube containing 10 mL of growth medium at 37°C.

(d) Spin down cells at 450 *g* for 3 min and remove supernate.

(e) Resuspend cells in 20 mL of growth medium and split equally between two 25-cm^2 flasks.

(f) Culture cells overnight and inspect the next day. Change growth medium.

(g) Ensure that records are updated to reflect removal of cells from storage facility.

6.6. SOURCES OF MATERIALS

Item	Catalog No.	Supplier
Acetic acid, glacial	A 6283	Sigma
Amphotericin B	A 2942	Sigma
BioMag particles; anti-mouse IgM magnetic particle (IgM isotype used to recognize SSEA-3)		Polysciences
Bouin's solution	HT-10-1-32	Sigma
Cytosine arabinoside	C 1768	Sigma
DMEM	D 5671	Sigma
DMSO	D 2650	Sigma
Fetal bovine serum	10106	Invitrogen
Fluorodeoxyuridine	F 8791	Sigma
Formaldehyde	F 1635	Sigma
Glass beads, 3-mm diameter	71013	Merck Biosciences
Glutamine	G 7513	Sigma
Hexamethylene *bis*-acetamide (HMBA)	H 6260	Sigma
Kanamycin	K 0129	Sigma

Item	Catalog No.	Supplier
Laminin, human, placental	L 6274	Sigma
Microtitration plates, round-bottomed	163320	Nunc (now Fisher)
Paraformaldehyde	P 6148	Sigma
Penicillin/streptomycin	15140	Invitrogen
Picric acid	925-40	Sigma
Poly-D-lysine	P 7280	Sigma
Poly-L-ornithine	P5666	Sigma
Retinoic acid, all-*trans*	R 2626	Sigma
SSEA-3, antibody to		Developmental Studies Hybridoma Bank
STO transformed mouse embryo cells	86032003	ECACC
Trypsin, 0.25% (1:250 grade), 1 mM EDTA ·4Na	25200	Invitrogen
Uridine	U 3003	Sigma

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CHAPTER 7

CULTURE OF UMBILICAL CORD- AND CORD BLOOD-DERIVED STEM CELLS

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Culture of Human Stem Cells, Edited by R.I. Freshney, G.N. Stacey and J.M. Auerbach

7.1. INTRODUCTION

Bone marrow (BM) transplantation is a well-established classic treatment for patients with hematologic malignancies including leukemia or fatal metabolic diseases. Mononuclear cells (MNCs) in human BM consist of hematopoietic stem cells (HSCs), which are the building blocks of blood and immune systems in the body, and marrow stromal cells or mesenchymal stem cells (MSCs), which are capable of differentiating into various cell lineages under specific microenvironmental conditions.

Umbilical cord blood (UCB) is the blood remaining in the umbilical cord (UC) and placenta after birth. This had been regarded as medical waste and was discarded routinely in the past. However, in recent years, UCB is being widely accepted as a rich alternative source of HSCs and other stem cells with practical and ethical advantages. Since the first UCB transplantation was performed in 1988 for a child with Fanconi anemia, it has become a safe and accepted mode of HSC transplantation for recipients because of the low viral exposure and reduced incidence of the more severe grades of acute graft-versus-host disease (GvHD) when one or two human leukocyte antigen (HLA)-mismatched unrelated donor transplants are performed, whereas BM transplantation requires strict histocompatibility between donors and recipients.

In addition to HSCs, it is known that the UCB also contains other stem cells such as MSCs, which have the potential to differentiate into various other types of cells and can be used to repair damaged cells and tissues in the human body.

The human UC embryologically formed at day 26 of gestation is the lifeline between the fetus and the placenta. The UC normally contains two umbilical arteries and one umbilical vein, a main reservoir of UCB. These are embedded within a loose, proteoglycan-rich matrix known as Wharton's jelly (WJ). First described by Thomas Wharton in 1656 [Wharton, 1996], the jelly has physical properties like a polyurethane pillow, which serves to protect the critical vascular lifeline that connects the placenta and the fetus. Recently, stem cells have been isolated separately from umbilical veins and WJ [McElreavey et al., 1991; Naughton et al., 1997; Purchio et al., 1999; Romanov et al., 2003].

7.1.1. Umbilical Cord-Derived Stem Cells

UC-derived stem cells have been isolated and cultured mainly from umbilical vein or WJ. Romanov et al. [2003] suggested that MSC-like cells are present in the subendothelial layer of the human umbilical vein and could be successfully isolated, cultured, and expanded with routine technical approaches. The results of morphological studies and immunophenotyping of cultured MSC-like cells from human umbilical vein have shown that these cells closely resemble cultured MSCs obtained from bone marrow and other sources [Campagnoli et al., 2001; Deans and Moseley, 2000; Erices et al., 2000; Minguell et al., 2000; Zuk et al., 2001]. Sarugaser et al. [2005] also reported that human umbilical cord perivascular (HUCPV) cells, which were either discarded or not specifically isolated, should contain a subpopulation of cells that would be capable of exhibiting a functional mesenchymal phenotype.

Another potential alternative source of mesenchymal stem cells is the Wharton's jelly, as reported by McElreavey et al. [1991]. Thereafter, Naughton et al. [1997] and Purchio et al. [1999] isolated "prechondrocytes," derived from UC-WJ, and Mitchell et al. [2003], using a similar approach, isolated fibroblast-like cells from WJ, which could be induced to differentiate into "neural-like" cells.

7.1.2. Umbilical Cord Blood-Derived Stem Cells

In addition to HSCs in UCB, potential alternative stem cells such as MSCs, unrestricted somatic stem cells (USSCs), cord blood-derived embryonic-like stem cells (CBEs), and cord blood multipotent progenitor cells (CB-MPCs) have been isolated and characterized by their different growth conditions. Because UCB-derived cells have been regarded as

more primitive than BM, UCB-derived stem cells would be more popular sources for cellular therapies, regenerative medicine, and tissue engineering.

7.1.2.1. Hematopoietic Stem Cells (HSCs). It has been proved that UCB is an important source of HSCs, and cumulative results of transplantation for more than a decade support its usefulness as an alternative to BM. HSCs in cord blood are a rare, heterogeneous population of immature hematopoietic precursor cells, occurring at a frequency of approximately 1 in 10^4 to 1 in 10^5 cells postnatally, and are multipotent, with the ability to commit to one of 10 or 11 functional hematopoietic lineages. These HSCs, through their multipotent and long-term repopulating ability, are able to populate the whole hematopoietic system within an individual's life span [Martin-Rendon and Watt, 2003a and b]. UCB has a higher content of primitive HSCs than BM and mobilized peripheral blood and has a higher proliferative potential associated with an extended life span and longer telomeres [Migliaccio et al., 1986; Szilvassy et al., 2001; Vaziri et al., 1994; Zanjani et al., 1993].

7.1.2.2. Mesenchymal Stem Cells (MSCs). The marrow stromal cells derived from BM retain a subpopulation of cells that have been shown to be capable of differentiation into cells of different tissue lineages such as bone, cartilage, tendon, muscle, fat, and stromal connective tissue, which supports hematopoietic cell differentiation. Many studies have defined conditions for isolation, expansion, and in vitro and in vivo differentiation of the stromal cells. These cells are referred to as marrow stromal cells or mesenchymal stem cells (MSCs), since they are known to have the capacity to proliferate and differentiate into the mesenchymal lineage. Isolation of MSCs is primarily based on plastic adherence and growth under specific culture conditions related to culture media and growth factors. To date, the most popular source of MSCs has been the BM, but aspiration of BM from the patient is an invasive procedure and, in addition, differentiation potential of BM-MSCs decreases with age. Therefore, the search for alternative sources of MSCs has become a matter of concern. Recently, MSCs have been isolated from various sources, including UCB [Bieback et al., 2004; Erices et al., 2000; Goodwin et al., 2001; Javajon et al., 2004; Lee et al., 2004a; Lee et al., 2004b; Rosada et al., 2003; Yang et al., 2004]. Although some investigators have failed to isolate MSCs from UCB cell populations, many recent studies have successfully isolated MSCs from UCB that had a capacity for multidifferentiation into osteoblasts, chondrocytes, adipocytes, myogenic cells, and neuronal cells.

7.1.2.3. Unrestricted Somatic Stem Cells (USSCs). Recently, Kögler et al. [2004] identified rare CD45 and HLA class II-negative stem cell candidates in UCB, termed unrestricted somatic stem cells (USSCs), displaying robust in vitro proliferative capacity without spontaneous differentiation but with intrinsic and directable potential to differentiate into various spectra of cell lineages including mesodermal, endodermal, and ectodermal cell fates. In contrast to MSCs from BM [Pittenger et al., 1999], the USSCs have a wider differentiation potential and differ in immunophenotype [Deans and Moseley, 2000] and in their mRNA expression profile.

7.1.2.4. Cord Blood-Derived Embryonic-Like Stem Cells (CBEs). McGuckin et al. reported reproducible production of untransformed adherent human stem cell populations, with an embryonic stem cell phenotype, from UCB, termed cord blood-derived

embryonic-like stem cells (CBEs) [Forraz et al., 2004; McGuckin et al., 2004]. The CBEs had formed embryoid body-like colonies, which were immunoreactive for primitive human embryonic stem cell-specific genes [Gerrard et al., 2005; Martin et al., 2004]. Thus McGuckin et al. [2004] suggested that CBEs have the capacity to differentiate into neuronal, hepatic, and pancreatic cells, bone, fat, skeletal muscle, and blood vessels.

7.1.2.5. Cord Blood-Derived Multipotent Progenitor Cells (CB-MPCs). Our group has studied whether other stem cells such as MSCs are present in fresh or cryopreserved UCB. We have also isolated a novel cell line from a population of stem cells found in the human UCB, which had characteristics different from those of HSCs and MSCs. Seeded UCB-MNCs formed adherent colonies of cells in optimized culture conditions. Over a 3- to 4-week culture period, the colonies gradually developed into adherent monolayer cells, which exhibited homogeneous fibroblast-like morphology and immunophenotypes and were highly proliferative; these will be referred to here as cord blood-derived multipotent progenitor cells (CB-MPCs). CB-MPCs had the capacity to differentiate into various spectra of cell types, including osteoblast, endothelial, hepatic, and neuronal cells. Neuronal-differentiated cells expressed cell type-specific markers, such as tyrosine hydroxylase (dopaminergic neurons), acetylcholinesterase (cholinergic neurons), and glutamate decarboxylase (GABAergic neurons), suggesting that CB-MPCs differentiate into functionally specific neurons [unpublished results].

7.2. PREPARATION OF MEDIA AND REAGENTS

7.2.1. Media

7.2.1.1. Transport Medium for Umbilical Cord. Eagle's basal medium (EBM) supplemented with 300 U/mL penicillin, 300 μg/mL streptomycin, 150 μg/mL gentamicin, and 1 μg/mL fungizone

7.2.1.2. DMEM for Mesenchymal Stem Cells. Low-glucose Dulbecco's modified Eagle's medium (LG-DMEM) with 2 mM L-glutamine and supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin

7.2.1.3. IMDM for Hematopoietic Stem Cells. Iscove's modified Dulbecco's medium (IMDM) with 2 mM L-glutamine and supplemented with 1% bovine serum albumin, 10 μg/mL insulin, 0.1 mM 2-mercaptoethanol, 50 U/mL penicillin, and 50 μg/mL streptomycin

7.2.1.4. IMDM for Coculture of Hematopoietic Stem Cells and Mesenchymal Stem Cells. Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin

7.2.1.5. DMEM for Cord Blood-Derived Multipotent Progenitor Cells. High-glucose Dulbecco's modified Eagle's medium (HG-DMEM) with 2 mM L-glutamine and supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 100 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF)

7.2.1.6. IMDM for Cord Blood-Derived Embryonic-Like Stem Cells. Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and TPOFLK cytokine mix: thrombopoietin (10 ng/mL), Flt-3 ligand (50 ng/mL), and c-kit ligand (20 ng/mL)

7.2.1.7. Starting Culture Medium for Unrestricted Somatic Stem Cells. LG-DMEM with 2 mM Ultraglutamine and supplemented with 30% heat-inactivated FBS, 0.1 μM dexamethasone, 100 U/mL penicillin, and 100 μg/mL streptomycin

7.2.1.8. Expansion Culture Medium for Unrestricted Somatic Stem Cells. LG-DMEM with 2 mM Ultraglutamine and supplemented with 30% heat-inactivated FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin

7.2.2. Reagents

7.2.2.1. Buffers.

PBSA: Dulbecco's phosphate-buffered saline solution A (without Ca^{2+} and Mg^{2+})

Column buffer: PBSA pH 7.2 supplemented with 0.5% bovine serum albumin and 2 mM EDTA or 0.6% acid citrate dextrose formula-A (ACD-A; *see* Section 7.5). De-gas buffer by applying vacuum.

ACD-A: acid citrate dextrose formula-A (0.6% ACD-A) and bovine serum albumin, 0.5%, fraction V (*see* Section 7.5)

7.2.2.2. Enzymes.

Collagenase A, 0.1% in PBSA

Trypsin, 0.05% in PBSA with 0.53 mM EDTA and trypsin, 2.5% in PBSA

7.2.2.3. Anticoagulant. Citrate-phosphate-dextrose-adenine (CPDA-1): 122 mM (26 g/L) sodium citrate, 0.142 M (25.5 g/L) dextrose, 15.6 mM (3.0 g/L) citric acid, and 18.3 mM (2.2 g/L) monobasic sodium phosphate

7.3. STEP-BY-STEP PROTOCOLS

7.3.1. Preparation of Umbilical Cord (UC) and Cord Blood (UCB)

In placental mammals, the umbilical cord is a tube that connects a developing embryo or fetus to its placenta. The umbilical cord varies from no cord (achordia) to a length of 300 cm, with diameters up to 3 cm. Umbilical cords are helical in nature, with as many as 380 helices. Six percent of cords are shorter than 35 cm, and 94% of cords are longer than 80 cm. It contains major arteries and veins (notably two umbilical arteries and an umbilical vein, buried within Wharton's jelly) for the exchange of nutrient- and oxygen-rich blood between the embryo and the placenta. The umbilical stub on the newborn's belly dries and comes off after a few days. It leaves only a small scar (the umbilicus) behind [Robinson, 2000].

Human umbilical cord and cord blood must be collected after informed consent of the mothers and according to the guidelines approved by the Institutional Review Board

or Independent Ethics Committee. After vaginal or cesarean section delivery, cord blood should be collected as soon as possible, ideally within 10 minutes of birth. To maximize volume, it is recommended that cord blood be collected in utero (the placenta is still inside the uterus) rather than ex utero (after the placenta is delivered, following the baby's delivery). In utero collection is also preferred for uncomplicated cesarean deliveries. Collection of cord blood should be performed in a manner that will not (1) alter the delivery of the infant, (2) increase the likelihood of any adverse reaction in the infant or mother, or (3) preclude appropriate medical management of the infant or mother, including collection of cord blood for diagnostic specimens [Galanakis et al., 2003].

Protocol 7.1. Preparation of Umbilical Cord

Reagents and Materials

Sterile

- ❑ Transport medium: *see* Section 7.2.1.8.
- ❑ Clamps
- ❑ Scissors

Procedure

(a) After birth, close the umbilical cord with two clamps on the end adjacent to the infant and one on the end adjacent to the placenta.

(b) Snip between the two clamps near the infant and cut off the placenta from the other end.

(c) Carry the umbilical cord to the laboratory in transport medium and process within 6–12 h.

Protocol 7.2. Preparation of Umbilical Cord Blood: the Bag or Syringe Method

Reagents and Materials

Sterile

- ❑ Anticoagulant: CPDA-1(*see* Section 7.2.2.3)
- ❑ Betadine or 70% alcohol
- ❑ Clamps
- ❑ Syringe needle, 18 gauge
- ❑ Cord blood collection bag, 175 mL, containing 24.5 mL of CPDA-1
- ❑ Syringe, 50 mL, containing 200 I.U. of heparin

Procedure

(a) Clamp and cut the cord as close as possible to the infant.

(b) Swab the needle insertion site at the fetal end of the cord with Betadine or 70% alcohol.
(c) To maximize collection volume, minimize manipulation of the cord.
(d) Insert the needle with the attached collection bag or 50-mL syringes at the insertion site of the umbilical vein prepared in Step (b).
(e) Keep the bag at a lower level than the insertion site so that cord blood is allowed to fill the container by gravity or slowly aspirate the blood from the umbilical vein with 50-mL syringes. Allow as much blood to collect as possible. The range of volumes obtained will be 60 to 150 mL.
(f) If the vein collapses, reinsert the needle farther up the cord after swabbing with Betadine or 70% alcohol.
(g) After the blood flow has stopped, activate the needle safety cover by pushing it into locked position. ***Note:*** The cord blood should remain at room temperature. Do not refrigerate.
(h) For the bag method, clamp the tubing with the attached clasp and tie two secure knots in the tubing as close to the blood bag as possible to prevent leakage, then cut off the needle and discard it in a sharps container.
(i) Gently invert the bag or syringe several times to thoroughly mix the cord blood and anticoagulant.

7.3.2. Preparation of Stem Cells from Umbilical Cord

Romanov et al. [2003] isolated and cultured MSC-like cells from the subendothelial layer of umbilical vein containing no endothelium- or leukocyte-specific antigens but expressing α-smooth muscle actin and several mesenchymal cell markers. WJ-derived stem cells, one potential alternative source of mesenchymal cells, have been isolated and cultured by the explant method [Naughton et al., 1997; Purchio et al., 1999] or the enzymatic digestion method [Mitchell et al., 2003].

Protocol 7.3. Isolation of Stem Cells from Umbilical Vein

Reagents and Materials

Sterile

- ❑ Culture medium: complete LG-DMEM (*see* Section 7.2.1.2)
- ❑ PBSA
- ❑ Trypsin, 0.05% with EDTA (*see* Section 7.2.2.2)
- ❑ Collagenase (*see* Section 7.2.2.2)
- ❑ Culture flasks
- ❑ Catheter

Procedure

(a) Collect and process umbilical cord within 6–12 h after normal delivery.
(b) Catheterize umbilical vein and wash twice internally with PBSA.

(c) Clamp the distal end.
(d) Fill the vein with 0.1% collagenase solution.
(e) Clamp the proximal end.
(f) Incubate the umbilical cord at 37°C for 20 min.
(g) Massage the cord gently, collect the suspension of endothelial and subendothelial cells, and centrifuge at 600 *g* for 10 min.
(h) Resuspend the cell pellet in culture medium.
(i) After counting, seed cell suspension in 75-cm^2 culture flasks with a density of approximately 1×10^3 cells/cm^2.
(j) Remove nonadherent cells after 3 days by changing the medium and keep adherent cells in culture, feeding with fresh medium every 3 days until the outgrowth of fibroblastoid cells about 2 weeks later.
(k) At that time, harvest cells with 0.05% trypsin/EDTA and passage into a new flask for further expansion.

Protocol 7.4. Isolation of Stem Cells from Wharton's Jelly: the Explant Method

Reagents and Materials

Sterile

- ❑ Culture medium: complete LG-DMEM (*see* Section 7.2.1.2)
- ❑ PBSA
- ❑ Scissors
- ❑ Forceps

Procedure

(a) Obtain umbilical cord after normal delivery and store in PBSA for 1–24 h before tissue processing.
(b) Remove blood vessels and dice the Wharton's jelly into small fragments.
(c) Transfer the explants onto 6-well plates containing DMEM/FBS.
(d) Leave them undisturbed for 5–7 days to allow migration of cells from the explants (Fig. 7.1; Plate 8), at which point replace the medium.

Protocol 7.5. Isolation of Stem Cells from Wharton's Jelly: the Enzymatic Digestion Method

Reagents and Materials

Sterile

- ❑ Culture medium: complete LG-DMEM (*see* Section 7.2.1.2)
- ❑ PBSA
- ❑ Collagenase (*see* Section 7.2.2.2)

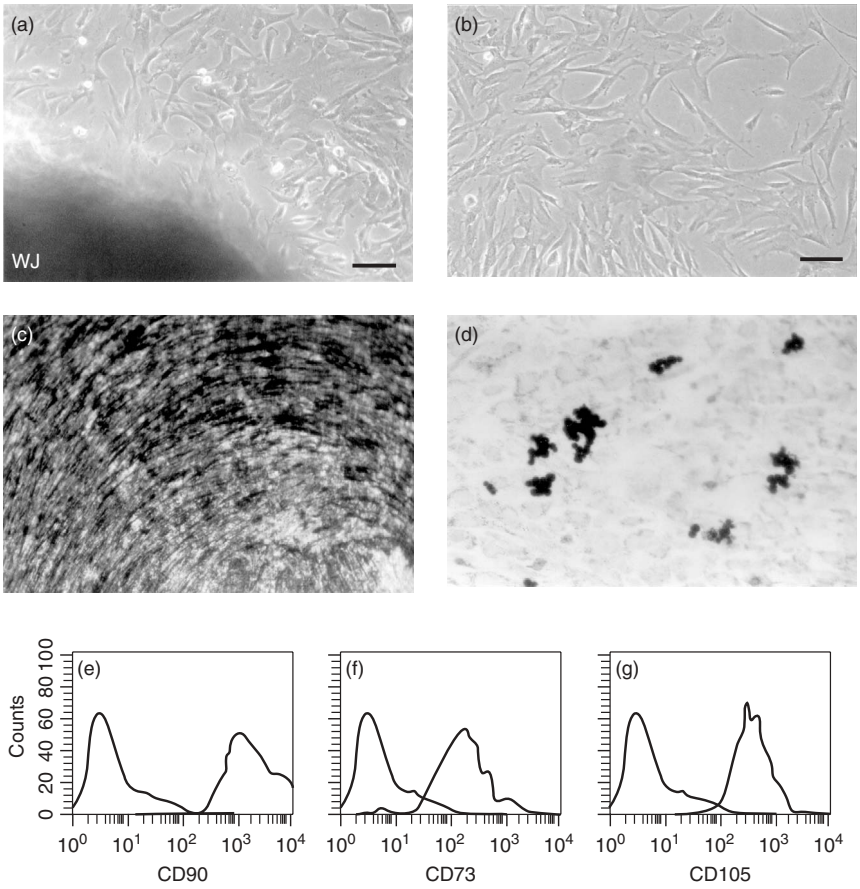

FIGURE 7.1. Photomicrographs and cell surface markers of stem cells isolated from Wharton's jelly (WJ) by the explant method. (**a**) Photomicrographs of primary WJ-derived stem cells and (**b**) subcultured cells at second passage. Bar is 100 μm. (**c**) Osteogenic differentiation evaluated by von Kossa and (**d**) adipogenic differentiation evaluated by Oil-red O staining. (*See also* Plate 8.) (**e–g**) FACS analysis of cell surface markers of primary WJ-derived stem cells. Isotype controls are indicated as thin graph lines on plots.

- ❑ Trypsin, 2.5% (*see* Section 7.2.2.2)
- ❑ Culture flasks
- ❑ Conical centrifuge tubes, 50 mL
- ❑ Scissors
- ❑ Forceps

Procedure

(a) After obtaining umbilical cord, store it in PBSA for 1–24 h before tissue processing.

(b) Remove blood vessels, and dice the Wharton's jelly into ~0.5-cm cubes.

(c) Transfer the chopped tissue to a 50-mL conical centrifuge tube, wash the tissue with serum-free DMEM, and centrifuge at 250 *g* for 5 min at room temperature.

(d) Discard the supernate, disperse the pellet, immerse in 0.1% collagenase (about 3-fold the volume of pellet), and digest for 16–18 h at 37°C.
(e) Add an appropriate volume of PBSA (double the volume of digest) and centrifuge at 250 *g* for 5 min at room temperature.
(f) Remove the supernate and treat the pellet with 2.5% trypsin (about double the volume of pellet) at 37°C for 30 min with agitation.
(g) Add appropriate FBS (10% of volume of digest) to neutralize the excess trypsin.
(h) Wash the cells with culture medium.
(i) Resuspend isolated cells in culture medium and seed in culture flasks at a density of approximately 1×10^3 cells/cm^2.

7.3.3. Preparation of Stem Cells from Umbilical Cord Blood

Umbilical cord blood (UCB) is well known to be a rich source of hematopoietic stem cells (HSCs) with practical and ethical advantages. HSCs have been defined as primitive, undifferentiated cells that are capable of both self-renewal and differentiation into all blood cell types. The majority of HSCs express the CD34 antigen, an integral membrane glycoprotein of 90–120 kDa that functions as a regulator of hematopoietic cell adhesion to stromal cells of the hematopoietic microenvironment. Thus HSCs have been isolated mostly by using reactivity with anti-CD34 antibody. Several types of stem cells, which contain CB-MSCs, USSCs, CBEs, and CB-MPCs, have been isolated from fresh or cryopreserved UCB, under different growth conditions.

Protocol 7.6. Isolation of Mononuclear Cells (MNCs) by Density Separation

Reagents and Materials

Sterile

❑ Appropriate culture medium (*see* Sections 7.2.1.2–7.2.1.6) or freezing medium (*see* Protocol 7.7)
❑ PBSA
❑ Ficoll-Hypaque (density 1.077 g/L)
❑ Pasteur pipettes
❑ Conical centrifuge tubes, 50 mL

Procedure

(a) Dilute the cord blood sample 1:1 with PBSA.
(b) Pipette 15 mL of Ficoll-Hypaque into a 50-mL conical centrifuge tube.
(c) Slowly layer 30 mL of the mixture of PBSA and sample over the Ficoll-Hypaque. Do not disturb the Ficoll-Hypaque/sample interface.
(d) Centrifuge for 20–30 min at 450 *g* at room temperature.
(e) After centrifugation, a layer of mononuclear cells should be visible on top of the Ficoll-Hypaque phase, as they have a lower density than the Ficoll-Hypaque solution (*see* Fig. 9.1 and Plate 13).

(f) Using a Pasteur pipette, transfer the interface layer containing the mononuclear cells to a centrifuge tube.
(g) Wash the cells with PBSA and recover the cells by centrifugation for 10 min at 200 *g* and room temperature.
(h) Discard the supernate, resuspend the cell pellet in PBSA, and repeat the washing procedure, Step (g).
(i) Finally, resuspend the cells in appropriate medium (freezing or culture medium).

Protocol 7.7. Cryopreservation of CB-MNCs

Reagents and Materials

Sterile

- ❑ FBS
- ❑ Dimethyl sulfoxide (DMSO)
- ❑ Cryovials
- ❑ Slow-freezing container (e.g., Mr. Frosty) or controlled-rate freezer
- ❑ LN_2-resistant storage box

Procedure

(a) Prepare the freezing medium: 90% FBS + 10% DMSO; chill on ice or place in 4°C refrigerator for at least 30 min.
(b) Count cell numbers of the CB-MNCs.
(c) Resuspend CB-MNCs in cold freezing medium and adjust the cell concentration to 5–10 $\times$ 10^6 viable cells/mL.
(d) Dispense 1 mL into cryovials.
(e) Immediately place the cryovials in a slow-freezing container and place the container in a -70°C freezer for 4–24 h. Alternatively, place the cryovials into the freezing chamber of a controlled-rate LN_2 freezer.
(f) After 4–24 h in a -70°C freezer or controlled-rate freezer, transfer the cryovials into a LN_2-resistant storage box and place the box into the vapor phase (approximately -135°C) or liquid phase (-196°C) of a liquid nitrogen freezer.

Protocol 7.8. Thawing CB-MNCs

Reagents and Materials

Sterile

- ❑ Appropriate medium (*see* Sections 7.2.1.2–7.2.1.6) supplemented with 10% FBS
- ❑ PBSA

- ❑ Alcohol, 70%
- ❑ Water bath

Procedure

(a) Thaw cryovials containing CB-MNCs in a 37°C water bath.
(b) Dry off the outside of the cryovials and, before opening, wipe the vials with 70% alcohol to prevent contamination.
(c) Quickly transfer the thawed cell suspension (about 1 mL) to a 15-mL conical centrifuge tube containing 10 mL of chilled medium supplemented with 10% FBS.
(d) Centrifuge at room temperature at 200 *g* for 10 min.
(e) Remove the supernate without disturbing the cell pellet.
(f) Wash once with 10 mL of PBSA and centrifuge at room temperature at 200 *g* for 10 min.
(g) Gently resuspend the CB-MNCs in medium appropriate for the experiment to be performed.

Protocol 7.9. Isolation of $CD34^+$ Cells from CB-MNCs

Reagents and Materials

Sterile

- ❑ Culture medium
- ❑ Column buffer (*see* Sections 7.2.2.1 and Section 7.5)
- ❑ FcR blocking reagent (*see* Section 7.5)
- ❑ CD34 Microbeads (*see* Section 7.5)
- ❑ Column (MS+/RS+ or LS+/VS+) (*see* Section 7.5)
- ❑ Magnetic cell separator (e.g., MiniMACS; *see* Section 7.5)
- ❑ Nylon mesh, 30 μm

Procedure

(a) Prepare the column buffer and de-gas by applying vacuum.
(b) Resuspend cells in a final volume of 300 μL of buffer per 10^8 total CB-MNCs.
(c) Add 100 μL of FcR blocking reagent per 10^8 total CB-MNC suspension to inhibit nonspecific or Fc-receptor-mediated binding of CD34 microbeads to nontarget cells.
(d) Label cells by adding 100 μL of CD34 microbeads per 10^8 total CB-MNCs, mix well, and keep for 30 min in the refrigerator at 6–12°C.
(e) Wash the cells by adding PBSA, centrifuge for 10 min at 200 *g* and room temperature, and resuspend in the appropriate amount of buffer.
(f) Choose a column type (MS+/RS+ or LS+/VS+) according to the number of total CB-MNCs and place it in the magnetic field of the MACS separator. Fill and rinse with buffer (MS+/RS+: 500 μL; LS+/VS: 3 mL).

(g) Pass the cells through 30-μm nylon mesh to remove clumps. Wet the column with buffer before use.
(h) Apply cells to the column; allow unbound cells to pass through the column.
(i) Wash out unbound cells with buffer (MS+/RS+: 3 × 500 μL; LS+/VS: 3 × 3 mL).
(j) Elute bound cells:
  (i) Remove column from separator.
  (ii) Place on a suitable tube.
  (iii) Pipette buffer on to top of column (MS+/RS+: 1 mL; LS+/VS: 5 mL).
  (iv) Firmly flush out retained cells with pressure, using the plunger supplied with the column.
(k) Wash the selected CD34+ cells by adding PBSA, centrifuge for 10 min at 200 *g* and room temperature, and resuspend in appropriate medium.

Protocol 7.10. Ex Vivo Expansion of $CD34^+$ Cells

Reagents and Materials

Sterile

- ❑ Culture medium; complete IMDM (*see* Section 7.2.1.3)
- ❑ Cytokines (Flt-3 ligand, stem cell factor, thrombopoietin, and interleukin-6)
- ❑ Culture flask

Procedure

(a) Resuspend in culture medium with cytokines (50 ng/mL Flt-3 ligand, 50 ng/mL stem cell factor, 20 ng/mL thrombopoietin, and 10 ng/mL interleukin-6).
(b) Seed 2×10^4 $CD34^+$ cells/mL in 25-cm^2 tissue culture flasks.
(c) Replace half the medium and replenish the cytokines twice weekly.

Protocol 7.11. Ex Vivo Expansion of $CD34^+$ Cells by Coculture with Feeder Cells

This procedure is taken from the method of Jang et al. [2005].

Reagents and Materials

Sterile

- ❑ MSC culture medium (*see* Section 7.2.1.2)
- ❑ Multiwell culture plate, 6-well
- ❑ Mitomycin C, 100 μg/mL
- ❑ Coculture medium (*see* Section 7.2.1.4)
- ❑ Cytokines (stem cell factor, interleukin-6, Flt-3 ligand, and thrombopoietin)

Procedure

(a) To use as feeder cells, resuspend cord blood-derived mesenchymal stem cells (CB-MSCs) at 5×10^4 cells/mL in MSC culture medium (*see* Protocol 7.13 to obtain cord blood-derived mesenchymal stem cells).
(b) Seed CB-MSCs into the 6-well culture plate.
(c) Replace half of the medium twice weekly.
(d) When CB-MSCs reach more than 90% confluence, treat with 10 μg/mL mitomycin C for 2.5 h at 37°C.
(e) Wash CB-MSCs twice with serum-free IMDM.
(f) Resuspend 1×10^4/mL $CD34^+$ cells in coculture medium with cytokines (100 ng/mL stem cell factor, 100 ng/mL interleukin-6, 50 ng/mL Flt-3 ligand, 10 ng/mL thrombopoietin).
(g) Seed $CD34^+$ cells (*see* Protocol 7.9) onto CB-MSC feeder cells.
(h) Replace a quarter of the medium twice weekly.
(i) After 2 weeks, harvest nonadherent cells.

Note: BM-MSCs [Dexter et al., 1977; Da Silva et al., 2005], human umbilical vein endothelial cells (HUVEC) [Yildrim et al., 2005], and human placenta-derived mesenchymal progenitor cells [Zhang et al., 2004] can also be used as feeder cells.

Protocol 7.12. Ex Vivo Expansion of $CD34^+$ Cells in Three-Dimensional (3D) Matrix

This procedure is taken from the method of Ehring et al. [2003].

Reagents and Materials

Sterile

❑ Culture medium (*see* Section 7.2.1.3)
❑ Cytokines (Flt-3 ligand, stem cell factor, interleukin-3, and interleukin-6)
❑ Trypsin, 0.05% in EDTA (*see* Section 7.2.2.2)
❑ Multiwell culture plate, 48-well
❑ Fibronectin-coated Cytomatrix® scaffold

Procedure

(a) Resuspend in culture medium with cytokines (100 ng/mL Flt-3 ligand, 100 ng/mL stem cell factor, 20 ng/mL interleukin-3, and 20 ng/mL interleukin-6).
(b) Seed 2.5×10^5 $CD34^+$ cells/mL onto fibronectin-coated Cytomatrix® scaffold in a 48-well culture plate.
(c) Replace half the medium twice weekly.
(d) After 2 weeks, harvest cells:
    (i) Nonadherent cells are harvested from 3D Cytomatrix® scaffolds by centrifugation for 10 min at 250 *g*.

(ii) Adherent cells are collected by incubating Cytomatrix® with 0.05% trypsin/EDTA for 30 min at 37°C and then centrifuging at 250 *g* for 10 min.

Protocol 7.13. Isolation of Cord Blood MSCs

Reagents and Materials

Sterile

- ❑ Culture medium (*see* Section 7.2.2.1)
- ❑ Trypsin, 0.05% in EDTA (*see* Section 7.2.2.2)
- ❑ PBSA
- ❑ Culture flasks, 25 cm^2

Procedure

(a) To obtain fresh CB-MNCs, isolate CB-MNCs by following Steps (a)–(i) of Protocol 7.6.

(b) Resuspend the fresh or frozen MNCs in culture medium. Frozen MNCs should be thawed rapidly at 37°C and washed in medium before plating.

(c) Seed the CB-MNCs in a 25-cm^2 culture flask at a density of 3×10^5 cells/cm^2 in culture medium.

(d) Place the cells at 37°C in a humidified 5% CO_2/air incubator.

(e) Replace the culture medium every 7 days until the fibroblast-like cells at the base of the flask reach confluence.

(f) On reaching confluence, resuspend the cells with 0.05% trypsin/EDTA for 5 min and reseed at 1×10^5 cells per flask.

(g) On reaching confluence, replate the cells diluted 1:5 in culture medium.

Protocol 7.14. Isolation of Unrestricted Somatic Stem Cells (USSCs)

This procedure is taken from the method of Kögler et al. [2004].

Reagents and Materials

Sterile

- ❑ Starting culture medium (*see* Section 7.2.1.7)
- ❑ Expansion culture medium (*see* Section 7.2.1.8)
- ❑ Trypsin, 0.05%, with EDTA (*see* Section 7.2.2.2)
- ❑ PBSA
- ❑ Culture flasks, 25 cm^2

Procedure

(a) Prepare and separate cord blood-derived MNCs (*see* Protocol 7.6).

(b) In the case of cryopreserved CB-MNCs, thawed cells can be used for culture with no further separation steps.
(c) Seed the MNCs at a density of 5–7 $\times$ 10^6 cells/mL in 25-cm^2 culture flasks in starting culture medium.
(d) Incubate the cells in a humidified atmosphere at 37°C with 5% CO_2 and change medium and cytokines weekly for 2–4 weeks.
(e) After formation of USSC colonies, expand the cells in the expansion culture medium.
(f) Incubate the cells at 37°C in 5% CO_2 in a humidified atmosphere.
(g) On reaching 80% confluence, detach USSCs with 0.05% trypsin/EDTA and replate cells at 1:3 dilution under similar culture conditions.

Protocol 7.15. Isolation of Cord Blood-Derived Embryonic-Like Stem Cells (CBEs)

This procedure is taken from the method of McGuckin et al. [2004].

Reagents and Materials

Sterile

- ❑ Culture Medium (*see* Section 7.2.1.4)
- ❑ TPOFLK cytokine mixture (10 ng/mL thrombopoietin, 50 ng/mL Flt-3 ligand, and 20 ng/mL c-kit ligand)
- ❑ ACD-A buffer (*see* Section 7.2.2.1)
- ❑ Mouse monoclonal anti-human CD45, CD33, and CD7 antibody, anti-glycophorin-A antibody, and human gamma globulins (HAG, 2% in PBSA)
- ❑ Dynabeads Human IgG4 monoclonal anti-pan mouse IgG
- ❑ Dynal Magnetic Particle Concentrator
- ❑ Trypsin 0.05% in EDTA (*see* Section 7.2.2.2)
- ❑ PBSA
- ❑ Multiwell culture plate, 6-well

Procedure

(a) Separate and prepare cord blood-derived MNCs (*see* Protocol 7.6).
(b) Purify primitive lineage-restricted stem cells by sequential immunomagnetic depletion as follows.
(c) Place MNCs in 2% HAG in PBS at 4°C for 20 min.
(d) Label MNCs with mouse monoclonal anti-human CD45, CD33, CD7 and anti-glycophorin-A antibody at 4°C for 30 min.
(e) Wash the cells with ACD-A buffer and centrifuge the cells (400 *g*, 10 min, 4°C).
(f) Repeat wash with ACD-A buffer.
(g) Label MNCs with Dynabeads Human IgG4 monoclonal anti-pan mouse IgG for 30 min.

(h) Isolate LinNeg cell population with a Dynal Magnetic Particle Concentrator, following the manufacturer's instructions.

(i) Construct the cell separation apparatus and silicone tubing as follows:

(i) Rinse for 10 min in 70% ethanol.

(ii) Wash twice for 5 min each in water.

(iii) Rinse with 5% BSA/PBSA.

(iv) Attach a pinch clamp to the lower end of the silicone tubing and secure the tubing to the magnetic particle concentrator with clear tape.

(j) Before cell separation, fill the silicone tubing of the cell separation apparatus with culture medium and establish a flow rate through the column of <5 mL/min. Add the mixed cell/bead suspension to the tubing just below the meniscus of the liquid with a micropipette.

(k) Using a Pasteur pipette, continually add culture medium to the tubing to prevent the cells on the side of the tube from drying out.

(l) Collect the flow-through fraction (containing cells not bound to beads) in a 15-mL conical tube (LinNeg cells).

(m) Count the number of viable cells by Trypan Blue dye exclusion.

(n) Seed the UCB-derived LinNeg cells on noncoated 6-well culture plates at a density of 2.7×10^4 cells/mL in culture medium with TPOFLK cytokine mixture.

(o) Incubate the cells in a humidified atmosphere at 37°C with 5% CO_2 and change medium and cytokines weekly.

(p) When required, developing adherent CBEs can be dispersed with 0.05% trypsin/ EDTA and subcultured in new flasks with the same liquid culture conditions as above at 1×10^5 cells/mL.

Protocol 7.16. Isolation of Cord Blood-Derived Multipotent Progenitor Cells (CB-MPCs)

Reagents and Materials

Sterile

- ❑ Culture medium (*see* Section 7.2.1.5)
- ❑ Trypsin, 0.05%, with EDTA (*see* Section 7.2.2.2)
- ❑ PBSA
- ❑ Culture flasks, 25 cm^2

Procedure

(a) Separate and prepare cord blood-derived MNCs (*see* Protocol 7.6).

(b) In the case of cryopreserved CB-MNCs, thawed cells can be used for culture with no further separation steps.

(c) Count the number of viable cells by Trypan Blue dye exclusion within 30 min after thawing.

(d) Seed the CB-MNCs on 25-cm^2 culture flasks at a density of 3×10^5 cells/cm^2 in culture medium.
(e) Incubate the cells in a humidified atmosphere at 37°C with 5% CO_2 and change medium every 7 days for 2–4 weeks.
(f) Remove medium and wash the flasks with PBSA twice.
(g) Resuspend adherent cells gently with 0.05% trypsin/EDTA and reseed resuspended cells at 1×10^6 cells per flask.
(h) On reaching confluence, replate the cells at 1: 3 dilution under similar culture conditions.
(i) Assess the total number of cells by Trypan Blue dye exclusion during the culture period.

7.4. CHARACTERIZATION OF UMBILICAL CORD-DERIVED CELLS

7.4.1. Umbilical Cord-Derived Stem Cells

7.4.1.1. Umbilical Vein. Initially, primary cultured cells were represented mostly by clusters of endothelial cells (ECs) with typical endothelial morphology. However, in contrast to parallel cultures growing in standard endothelial conditions (medium 199 with 10% FBS), cells growing in DMEM with 10% FBS did not spread, migrate, or proliferate. As a result, the endothelial islands remained compact. As soon as 1 week after cultivation, numerous fibroblast-like cells could be observed between ECs. Subsequently, they formed colonies and expanded, and by the third week a homogeneous layer of fibroblastoid (MSC-like) cells occupied the whole plastic surface [Romanov et al., 2003].

Immunophenotypically, this cell population is positive for the CD29, CD13, CD44, CD49e, CD54, CD90, and HLA-class I markers and negative for CD45, CD14, glycophorin A, HLA-DR, CD51/61, CD106, and CD49d [Covas et al., 2003]. These cells have the capacity to differentiate into adipocytes and osteoblasts. Adipogenic differentiation was apparent after 1 week of incubation with adipogenic supplementation. By the end of the second week, cells contained numerous Oil-red O-positive lipid droplets. Similarly, most of the MSC-like cells became alkaline phosphatase-positive when the regular culture medium was replaced by osteogenic medium [Romanov et al., 2003].

This potential remained unchanged over 20 passages when the cells were cultured and maintained at low concentrations. When they reached a high level of confluence, the cells lost their replicating potential and presented morphological changes [Covas et al., 2003].

7.4.1.2. Wharton's Jelly. Stem cells from Wharton's jelly demonstrated a fibroblast-like phenotype. Flow cytometric analysis showed that the cells expressed high levels of matrix markers (CD44, CD105), integrin markers (CD29, CD51), and MSC markers (SH2, SH3) but did not express hematopoietic lineage markers (CD34, CD45) [Wang et al., 2004]. The cells proliferated in culture for more than 80 population doublings [Mitchell et al., 2003].

These cells can be induced to differentiate into cardiomyocytes by treating them with 5-azacytidine or by culturing them in cardiomyocyte-conditioned medium. Both sets of conditions resulted in the expression of cardiomyocyte markers, namely N-cadherin and

cardiac troponin I. Furthermore, these cells have multilineage potential and are able to differentiate into cells of the adipogenic, chondrogenic, and osteogenic lineages under appropriate growth conditions [Wang et al., 2004].

7.4.2. Cord Blood-Derived Stem Cells

7.4.2.1. Hematopoietic Stem Cells. HSC activities should be determined in two ways, in vitro and in vivo. In vitro assays include the long-term culture-initiating cell (LTC-IC) assay, the cobblestone area-forming cell (CAFC) assay, the high proliferative potential colony-forming cell (HPP-CFC) assay, and the colony-forming unit-blast (CFU-BL) assays [Heike et al., 2002]. The in vivo method uses the engraftment assay in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice as described below. Six- to eight-week-old mice irradiated with 270 cGy received 5×10^3 expanded cells via tail vein injection. Mice were sacrificed at 10–12 weeks after the transplantation. Femurs and tibiae were collected and aspirated with 5% FBS-containing PBSA to liberate mouse bone marrow. Cell suspensions were filtered through a sterile 40-μm cell strainer to get rid of clumps and debris and were then processed for flow cytometric analysis. Presence of at least 0.1% human $CD45^+$ cells in mouse bone marrow after transplantation was considered proof of human cell engraftment [Leung et al., 2005].

HSCs contain a small population of primitive and pluripotent stem cells that express a $CD34^+$ cell surface marker pattern and are capable of self-renewal and generation of committed progenitors of myeloid and lymphoid compartments. However, the number of HSCs in cord blood is limited. Therefore, cord blood-derived HSCs after ex vivo expansion [Astori et al., 2005; Flores-Guzman et al., 2002] or coinfusion of two or more units can serve as a reliable resource for hematopoietic stem cell transplantation.

7.4.2.2. Cord Blood-Derived MSCs. Frozen UCB-derived MNCs were plated and resulted in adherent heterogeneous cell populations after 4–7 days in culture that consisted of round, spindle-shaped cells. The cells proliferated slowly in the initial passage of the culture and gave rise to confluence in 14–21 days. When subcultured, the heterogeneous cell populations change into a homogeneous population with flat and fibroblast-like shape [Lee et al., 2004a].

After three passages in culture, the cell surface antigen profiles of UCB-derived cells were analyzed and compared with that of the UCB-MNC fraction before culture. The immunophenotypical profile of the MNC fraction greatly changed after the culture period, turning to a typical MSC immunophenotype. The cultured cells were strongly positive for MSC-specific surface markers such as CD105 (SH2), CD73 (SH3, SH4), and CD166 (ALCAM), while being negative for CD14 (monocyte antigen), CD31 (endothelial cell antigen), CD34 (HSC antigen), CD45 (leukocyte common antigen), and CD86 (costimulating molecule). The cell surface antigen profile of UCB-derived cells was essentially the same as that of BM-derived MSCs.

UCB-derived MSCs were highly proliferative until passage 6 and resulted in approximately 1250-fold expansion in cell number, yielding a minimum of 6.8×10^8 cells from one flask with the first seeding. The rapid expansion of the UCB-derived cells during the early passages would allow these cells to produce a sufficient quantity for therapeutic application, and these cells have the capacity for differentiation into neuronal cells, osteoblasts, chondrocytes, and adipocytes.

7.4.2.3. USSCs. USSCs are adherent, spindle-shaped cells and have a size of 20–25 μm. USSCs are negative for CD14, CD33, CD34, CD45, CD49b, CD49c, CD49d, CD49f, CD50, CD62E, CD62L, CD62P, CD106, CD117, glycophorin A, and HLA-DR. USSCs express high levels of CD13, CD29, CD44, CD49e, CD90, CD105, vimentin, and cytokeratin 8 and 18 and express low levels of CD10 and FLK1 (KDR) [Kögler et al., 2004].

USSCs can be cultured for >20 passages, equivalent to >40 population doublings, without any spontaneous differentiation. USSCs express various transcripts for cytokine receptors, transcription factors, and surface markers including epidermal growth factor receptor, platelet-derived growth factor receptor, insulin-like growth factor receptor, Runt-related transcription factor (Runx1), YB1, CD49e, and CD105. The cells are negative for the chondrogenic extracellular protein chondroadherin, the bone-specific markers collagenase X and bone sialoprotein, the liver- and pancreas-specific markers Cyp1A1 and PDX-1, and neural markers such as neurofilament (NF) protein, synaptophysin, tyrosine hydroxylase (TH), and glial fibrillary acid protein (GFAP) [Kögler et al., 2005].

USSCs have the capacity to differentiate into various lineages including neuronal cells, osteoblasts, chondrocytes, adipocytes, hematopoietic cells, cardiomyocytes, Purkinje fibers, and hepatic cells both in vitro and in vivo [Kögler et al., 2004].

7.4.2.4. CBEs. One week after the initial plating of the primary culture, adherent cell clusters form embryoid body-like colonies. These embryoid body-like colonies increase in size and number progressively. The adherent cell colonies can be dissociated at week 6 or 7 and reseeded in second-generation liquid cultures. Second-generation CBEs form embryoid body-like structures with morphology similar to that of their first-generation progenitor colonies. CBEs were grown for up to an additional 6 weeks and demonstrated an exponential cell proliferation pattern. Second-generation CBE populations significantly expanded (168-fold) from the 10^5 cells/mL baseline concentration to yield $1.68 \times 10^7 \pm 8.84 \times 10^5$ cells [McGuckin et al., 2005].

CBEs are negative for hematopoietic lineage markers, such as CD45, glycophorin A, CD38, CD7, CD33, CD56, CD16, CD3, and CD2. The cells are positive for CD34, CD133, and CD164. CBE colonies express embryonic stage-specific antigens SSEA-3 and SSEA-4. The cells are negative for embryonic antigen-1 (SSEA-1). The cell colonies expressed embryonic extracellular matrix components TRA 1-60 and TRA 1-81 and embryonic stem cell transcription factor Oct-4 [Forraz et al., 2004; McGuckin et al., 2004]. CBEs also have a capacity for differentiation into neuronal cells and hepatocytes. The investigators reported that Multi-Lineage Progenitor Cells™ (MLPCs™; from BioE), an improved and commercialized source of cells, have the capacity to differentiate into multiple lineages including bone, fat, skeletal muscle, blood vessels, and liver and pancreatic cells.

7.4.2.5. CB-MPCs. CB-MPCs are proliferative cells with fibroblast-like morphology. At the stable passage stage in culture, cells are negative for CD49a, CD62E, CD73, CD90, and CD104 and express high levels of CD14, CD31, CD44, CD45, and CD54, with variable expression of CD104, CD105, and CD166. Those cells are highly proliferative, with a 28-fold increase in number at 12 weeks. Cell cycle analysis revealed that ≤82% of the cells were in the G_0/G_1 phase, with about 18% actively involved in proliferation.

CB-MPCs have the capacity to differentiate into tissue-specific cell types, including osteoblast, endothelial, hepatic, and neuronal cells, representing mesoderm, endoderm, and

neurectoderm, as verified by reverse transcription-polymerase chain reaction (RT-PCR), immunocytochemistry, Western blot, and in vitro functional analysis (unpublished results).

7.4.3. Summary

We have discussed the characteristics of several stem cells isolated from umbilical cord or cord blood. These cells possess high proliferation potential, potential for multidifferentiation into mesenchymal lineages and/or others, and cell surface antigens known as stem cell-specific markers. Characteristics of these cells are summarized in Table 7.1.

TABLE 7.1 Characteristics of Stem Cells Isolated from Umbilical Cord and Cord Blood

	Proliferation Potential	Lineage Marker	Differentiation Capacity	References
CB-MPCs	>28-Fold at 12 weeks	**Positive:** CD14, CD31, CD44, CD45, and CD54 **Negative:** CD49a, CD62E, CD73, CD90, and CD104	Osteoblast, endothelial, hepatic, and neuronal cells	Unpublished results
CBEs	168-Fold at 2nd generation	**Positive:** CD34, CD133, CD164, SSEA-3, SSEA-4, Tra 1–60 and Tra 1–81, and Oct-4 **Negative:** CD2, CD3, CD7, CD16, CD33, CD38, CD45, CD56, SSEA-1, and glycophorin A	Bone, fat, skeletal muscle, blood vessels, hepatic, and pancreatic and neuronal cells	Forraz et al., 2004; McGuckin et al., 2004, 2005
USSCs	>40 Population doublings	**Positive:** CD13, CD29, CD44, CD49e, CD90, CD105, vimentin, and cytokeratin 8 and 18 and expressed low levels of CD10 and FLK1 (KDR) **Negative:** CD14, CD33, CD34, CD45, CD49b, CD49c, CD49d, CD49f, CD50, CD62E, CD62L, CD62P, CD106, CD117, glycophorin A, and HLA-DR	Osteoblasts, chondrocytes, adipocytes, hematopoietic cells, myocardial cells, Purkinje fibers, and hepatic and neuronal cells	Kögler et al., 2004, 2005

(*continued*)

TABLE 7.1 (*continued*)

	Proliferation Potential	Lineage Marker	Differentiation Capacity	References
CB-MSCs	>10 Passages	**Positive:** CD13, CD29, CD44, CD49e, CD54, CD73, CD90, CD105, CD166, and HLA-ABC **Negative:** CD14, CD31, CD34, CD45, CD49d, CD80, CD86, CD106, and HLA-DR	Osteoblasts, chondrocytes, adipocytes, and myogenic and neuronal cells	Erices et al., 2000; Goodwin et al., 2001; Hou et al., 2003; Lee et al., 2004a; Gang et al., 2004
Cord vein MSCs	>20 Passages	**Positive:** CD13, CD29, CD44, CD49e, CD54, CD73, CD90, CD105, CD166, and HLA-ABC **Negative:** CD14, CD31, CD34, CD45, CD49d, CD51/61, CD106, CD133, cadherin-5, glycophorin A, HLA-DR, and KDR	Osteoblasts, adipocytes, chondrocytes, and cardiomyocytes	Covas et al., 2003; Romanov et al., 2003; Kadivar et al., 2006
UC-WJ MSCs	>80 Population doublings	**Positive:** CD10, CD13, CD29, CD44, CD51, CD73, CD90, and CD105 **Negative:** CD14, CD31, CD33, CD34, CD38, CD40, CD40L, CD45, CD56, CD80, CD86, CD117, and HLA-DR	Osteoblasts, adipocytes, chondrocytes, cardiomyocytes, and neuronal cells	Mitchell et al., 2003; Wang et al., 2004; Sarugaser et al., 2005; Fu et al., 2005; Weiss et al., 2005

7.5. SOURCES OF MATERIALS

Item	Supplier
Antibiotics: penicillin/streptomycin, gentamicin, fungizone	Invitrogen
Antibodies: mouse monoclonal anti-human CD45, CD33, CD7 antibody; anti-glycophorin A antibody;	Autogen Bioclear Dako
Acid citrate dextrose formula-A (ACD-A)	Baxter
Betadine	Mundipharma

(*continued*)

Item	Supplier
	Purdue
Blood collection bag and syringe	Green Cross MS
	Baxter
Bovine serum albumin (BSA)	Sigma
Cell counting chamber (hemocytometer)	Marienfeld GmbH, Germany
Cell counting solution: Trypan Blue stain	GIBCO (Invitrogen)
Centrifuge tubes	Falcon (BD Biosciences)
Collagenase A (from *Clostridium histolyticum*)	Roche
Column: MS+/RS+, LS+/VS+	Miltenyi Biotec
Cryotubes	Nalge Nunc
Cytokine: Flt-3 ligand, stem cell factor, thrombopoietin, interleukin-6	R & D Systems
Cytomatrix®	Cytomatrix
Dexamethasone	Sigma, D4902
Dimethyl sulfoxide (DMSO)	Sigma, D2650
DMEM	Invitrogen
Dynabeads human IgG4, monoclonal anti-pan mouse IgG	Invitrogen
Dynal magnetic particle concentrator	Invitrogen
Eagle's basal medium (EBM)	Invitrogen
FBS	Invitrogen
FcR blocking agent	Miltenyi Biotec
Fibronectin	Boehringer Mannheim (Roche Diagnostics)
Ficoll-Hypaque	Sigma, H8889
Flasks and plates	Nalge Nunc
Granulocyte-macrophage colony-stimulating factor (GM-CSF)	R & D Systems
Insulin	Sigma, I2767
Iscove's modified Dulbecco's medium (IMDM)	Invitrogen
L-Glutamine	Invitrogen
Magnetic cell separator	Miltenyi Biotec
β-Mercaptoethanol	Invitrogen
Microbeads: CD34	Miltenyi Biotec
Nylon mesh	BD Biosciences
PBS (PBSA)	Hyclone
Penicillin, 10^4 U/mL, streptomycin, 10 mg/mL mixture	Invitrogen
TPOFLK cytokine mix	R & D Systems
Trypsin/EDTA	Invitrogen
Ultraglutamine	Cambrex

ACKNOWLEDGMENTS

I gratefully acknowledge my colleagues Young-Woo Eom, Mal Sook Yang, Sun Kyung Kim, In Keun Jang, Young Joon Moon, and Jong Eun Lee in the LifeCord Biomedical

Research Institute for their excellent support. The success of our experimental studies described herein would not have been possible without their efforts. In addition, I also wish to thank Jessica Mu, Dr. Jun ho Jang, and Dr. Swan Choi for their expert review of the manuscript.

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CHAPTER 8

MULTIPOTENT STEM CELLS IN DENTAL PULP

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Culture of Human Stem Cells, Edited by R.I. Freshney, G.N. Stacey and J.M. Auerbach

8.1. INTRODUCTION

The crowns of human teeth consist of enamel, dentin, and dental pulp tissue. During tooth growth and development, ameloblasts form enamel and odontoblasts generate primary dentin. After tooth eruption, ameloblasts disappear from the surface of the enamel; consequently, enamel formation ceases to occur naturally in vivo. In contrast, odontoblasts, along the inner surface of the dentin inside the pulp chamber, continue to deposit dentin matrix to form secondary dentin throughout life [Baume, 1980; Smith et al., 1995]. In addition to secondary dentin, odontoblasts can form tertiary (reactionary/reparative) dentin in response to several stimuli, such as mechanical, chemical, and/or bacterial stimulation [Cox et al., 1992; Kitamura et al., 1999; Smith et al., 1990, 1994]. Even when odontoblasts have been damaged, the reparative dentin can be formed in the dental pulp to protect against further disruption of the pulp tissue. This reparative dentinogenesis has been thought to be mediated by newly generated odontoblasts that seem to arise from dental pulp tissue [Ruch, 1998; Sveen and Hawes, 1968]. These findings have led to the speculation that odontogenic progenitor cells or stem cells may exist in dental pulp tissue [Butler et al., 1997; Ruch, 1998; Sveen et al., 1968]. Several reports have demonstrated that pulp tissue contains proliferating odontoblast-like cells and that these cells are capable of forming mineralized nodules in vitro [Buurma et al., 1999; Couble et al., 2000; Kuo et al., 1992; Shiba et al., 1995; Tsukamoto et al., 1992]. However, in these studies, the cells isolated from dental pulp appear to have a limited capacity to differentiate into odontoblast-like cells and an inability to differentiate into other cell types such as adipocytes or neurons. More recently, Tecles and colleagues confirmed that proliferating odontogenic precursor cells appear to be mobilized from blood vessels to sites of damaged pulp or dentin tissue [Tecles et al., 2005]. Collectively, these studies describe the presence of preodontoblast cells present in dental pulp tissue, leading to speculation of the existence of putative dental stem cell populations.

Mesenchymal stem cells were first isolated from bone marrow (bone marrow mesenchymal stem cells; BM-MSCs); they are a population of multipotent postnatal stem cells [Gronthos et al., 1994, 1996, 2003; Owen et al., 1988; Pittenger et al., 1999; Prockop, 1997; Simmons et al., 1991; Sonoyama et al., 2005]. One of the most important characteristics of BM-MSCs is their capacity to form single-cell-derived colony clusters called colony forming unit-fibroblast (CFU-F) in vitro [Friedenstein, 1976; Friedenstein et al., 1970]. Accumulated knowledge regarding the phenotypic characteristics of BM-MSCs has permitted us to isolate putative stem cell populations from the dental pulp of human third molars (dental pulp stem cells; DPSCs) and deciduous teeth (stem cells from human exfoliated deciduous teeth; SHED), which exhibit properties similar to those of BM-MSCs. Stem cells in dental pulp were found to reside in a specific perivascular microenvironment, where they are quiescent and maintain their basic stem cell characteristics, including a self-renewal capacity and undifferentiated status [Shi and Gronthos, 2003]. This specific microenvironment is called the "stem cell niche" [Bianco and Robey, 2001; Doherty et al., 1998; Fuchs et al., 2004; Moore and Lemischka, 2006]. Thus any isolation of mesenchymal stem cells must give consideration to their niche microenvironment in order to identify the factors that maintain the "stemness" of cultured mesenchymal stem cells, which gradually lose their stem cell-like properties after ex vivo expansion. In this chapter, we provide detailed procedures for the isolation, purified preparation, expansion, and tissue regeneration potential of DPSC and SHED.

8.2. PREPARATION OF MEDIA AND REAGENTS

8.2.1. Culture Media

All media should be sterilized by filtration through a 0.22-μm membrane filter and stored at 4°C.

8.2.1.1. Mesenchymal Stem Cell Medium (MSC Medium). Prepare α-modified minimal essential medium (α-MEM) with 2 mM glutamine and supplemented with 15% fetal bovine serum (FBS), 0.1 mM L-ascorbic acid phosphate, 100 U/ml penicillin, and 100 μg/ml streptomycin (Table 8.1). Selection of a suitable lot of FBS is critical for successful MSC culture. We select FBS on the basis of its colony-forming efficiency. Briefly, primary MSCs are seeded at the same density with several kinds of FBS, and then colonies are counted. Usually, a higher colony number is associated with better proliferation of MSCs.

TABLE 8.1 Preparation of MSC Medium

Reagent	Stock Solution Concentration	Volume of Reagent Needed (mL)	Final Concentration
α-MEM	1×	410	1×
FBS	—	75	15%
Glutamine	200 mM	5	2 mM
L-Ascorbic acid phosphate	10 mM	5	0.1 mM
P/S antibiotics	10,000 U/mL	5	100 U/mL
Final volume		500	

8.2.1.2. Odontogenic Differentiation Medium. Add 0.01 μM dexamethasone sodium phosphate and 1.8 mM monopotassium phosphate (KH_2PO_4) to MSC medium (Table 8.2A).

8.2.1.3. Adipogenic Differentiation Medium. Add 0.5 μM isobutylmethylxanthine, 60 μM indomethacin, 0.5 μM hydrocortisone, and 10 μg/ml insulin to MSC medium (Table 8.2B).

8.2.1.4. Neural Differentiation Medium. Prepare Neurobasal A with B27 supplement, 20 ng/ml epidermal growth factor (EGF), 40 ng/ml basic fibroblast growth factor (bFGF), 100 U/ml penicillin, and 100 μg/ml streptomycin (Table 8.2C).

TABLE 8.2 Preparation of Differentiation Media

Reagent	Stock Solution Concentration	Volume of Reagent Needed (mL)	Final Concentration
A. Preparation of odontogenic differentiation medium			
α-MEM	1×	404.5	1×
FBS	—	75	15%
Glutamate	200 mM	5	2 mM
L-Ascorbic acid phosphate	10 mM	5	0.1 mM
P/S antibiotics	10,000 U/ml	5	100 U/ml
Dexamethasone	10 μM	0.5	0.01 μM
KH_2PO_4	180 mM	5	1.8 mM
Final volume		500	
B. Preparation of adipogenic differentiation medium			
α-MEM	1×	398.25	1×
FBS	—	75	15%
Glutamate	200 mM	5	2 mM
L-Ascorbic acid phosphate	10 mM	5	0.1 mM
P/S antibiotics	10,000 U/ml	5	100 U/ml
Isobutylmethylxanthine	50 mM	5	0.5 mM
Hydrocortisone	0.5 mM	0.5	0.5 μM
Indomethacin	6 mM	5	60 μM
Insulin	4 mg/ml	1.25	10 μg/ml
Final volume		500	
C. Preparation of neural differentiation medium			
Neurobasal A	1×	484.45	1×
B27 supplement	50×	10	1×
EGF	200 μg/ml	0.05	20 ng/ml
bFGF	40 μg/ml	0.5	40 ng/ml
P/S antibiotics	10,000 U/ml	5	100 U/ml
Final volume		500	

8.2.2. Solutions for Tissue Digestion

8.2.2.1. Collagenase Solution. Dissolve 4 mg/ml collagenase (type I) in Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBSA), and sterilize by filtration. Store at $-20^{\circ}C$.

8.2.2.2. Dispase Solution. Dissolve 2 mg/ml dispase in PBSA, and sterilize by filtration. Store at $-20^{\circ}C$.

8.2.3. Freezing Medium

8.2.3.1. Freezing Medium I. FBS, 100%. Store at $4^{\circ}C$.

8.2.3.2. Freezing Medium II. FBS, 80%, and dimethyl sulfoxide (DMSO), 20%. Sterilize by filtration. Store at $4^{\circ}C$.

8.3. DETAILED PROTOCOLS FOR CULTURE

8.3.1. Safety Precautions

The health status of donors may not always be known. Therefore, great care should be taken to avoid transmission of diseases in handling tissue and possibly contaminated instruments and materials.

8.3.2. Tissue Handling

The extracted tooth should be immersed in a sterile, normal saline solution or PBSA immediately after extraction. Addition of double-strength antibiotics (e.g., penicillin and streptomycin) to these solutions is optional but advised. When a baby tooth is shed unexpectedly, it can be kept in sterile solution as described above. In either case, it should be stored at $4^{\circ}C$ to maintain cell vitality. Although it is better to isolate cells from a tooth as soon as possible, cells can be isolated as much as 24 h later if the tooth is stored under appropriate conditions.

8.3.3. Isolation of Dental Pulp Tissue

To maintain cell vitality, it is important to keep the pulp tissue at a low temperature and in a wet condition even during the cutting of the tooth.

The tooth can be contaminated because it is exposed to a huge amount of bacteria in the oral cavity, although dental pulp surrounded by dental hard tissues (enamel, dentin, and cementum) is not exposed to the oral cavity. However, in SHED isolation, dental pulp of the baby tooth is sometimes exposed as a result of resorption of the tooth root. Therefore, it is critical for successful cell isolation to disinfect the teeth on the outside with a disinfectant reagent before isolation of pulp tissue.

Protocol 8.1. Isolation of Dental Pulp Tissue

Reagents and materials

Sterile

- ❑ PBS without Ca^{2+} and Mg^{2+} (PBSA)
- ❑ MSC medium (*see* Section 8.2.1.1.)
- ❑ Petri dishes
- ❑ Fine forceps (small and large)
- ❑ Dental carbide burs
- ❑ Dental excavator
- ❑ High-speed dental hand piece

Nonsterile

- ❑ Equipment to use high-speed dental hand piece (e.g., air compressor)
- ❑ Disinfectant reagent (e.g., povidone-iodine)

Procedure

(a) Clean tooth surface well by washing three times with PBSA.

(b) Disinfect with disinfectant reagent and again wash well with PBSA.

(c) Cut the tooth around the cementum–enamel junction with a dental carbide bur equipped with a high-speed dental hand piece, and thereby reveal the pulp chamber (Fig. 8.1a,b; Plate 12A, B). Soak the tooth in ice-cold PBSA intermittently to avoid heating while cutting the tooth. In SHED isolation, this process is sometimes not required because the pulp is already exposed by root resorption.

(d) Separate the pulp tissue gently from the pulp chamber with small fine forceps or a dental excavator (Fig. 8.1c,d; Plate 12C,D).

(e) Put the pulp tissue into a small amount of MSC medium on a Petri dish. Be careful not to allow the tissue to become dry.

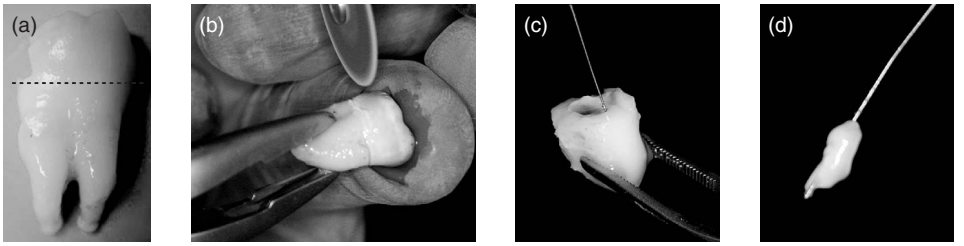

FIGURE 8.1. Isolation of pulp tissue from an extracted third molar. (**a**) Gross view of an extracted tooth. The dotted line shows the cementum–enamel junction. (**b–d**) The tooth is cut along with the cementum–enamel junction, and then pulp tissue is isolated. All these procedures should be carried out aseptically. (*See also* Plate 12.)

8.3.4. Primary Culture of DPSCs/SHED

This isolation protocol is based on the ability of stem cells to adhere to culture dishes and form discrete colony clusters [Friedenstein, 1976; Friedenstein et al., 1970]. To obtain colonies each derived from a single cell (i.e., clones), it is very important to release DPSCs and SHED from their perivascular niche in the pulp tissue [Shi and Gronthos, 2003]. Use a sterile scalpel blade and chop up the pulp into small segments. Enzyme digestion is then required for harvesting putative stem cells from pulp tissue. However, it may be harmful for the stem cells if enzyme digestion lasts for more than 1 h. Each researcher should find optimal conditions for the digestion. Usually, it takes 30–60 min to fully digest well-minced pulp tissue.

It is recommended that the medium be changed 1 day after cell isolation; this minimizes the incidence of contamination. However, when the risk of contamination is high, the medium can be changed 5 h after the isolation.

Cells isolated from dental pulp are usually seeded at $1–10 \times 10^3$ cells/cm^2 for culture. To evaluate their colony formation rate (CFU-F assay), a lower cell density ($0.1–1.0 \times 10^3$ cells/cm^2) is recommended in order to distinguish each single colony cluster.

Purified preparations of dental pulp stem cells can be obtained by immunoselection prior to culture [Shi and Gronthos, 2003] [*see* Protocol 8.2, Step (g)]. Single-cell suspensions of enzyme-digested dental pulp tissue are obtained by passing the cells through a 70-μm cell strainer. The cells are then incubated with primary antibodies reactive with DPSC, using STRO-1 (mouse anti-human MSC; IgM), CC9 (mouse anti-human CD146/MUC-18; IgG_{2a}), or 3G5 (mouse anti-human pericyte; IgM) [Shi and Gronthos, 2003]. After this, the cells are washed with PBSA containing 1% bovine serum albumin (BSA) and then incubated with either sheep anti-mouse IgG-conjugated or rat anti-mouse IgM-conjugated magnetic Dynabeads. Cells bound to beads are removed with the Dynal MPC® -1 magnetic particle concentrator. The STRO-1-, CD146-, or 3G5-positive cells are then seeded at $1–10 \times 10^3$/ cm^2 in growth medium as described below [*see* Protocol 8.2, Step (h)].

Protocol 8.2. Primary Culture of DPSCs or SHED

Reagents and materials

Sterile

- ❑ PBSA
- ❑ MSC medium (*see* Section 8.2.1.1.)
- ❑ Collagenase (*see* Section 8.2.2.1.)
- ❑ Dispase (*see* Section 8.2.2.2.)
- ❑ Reagents for immunomagnetic separation, if used:
  - (i) PBSA containing 1% BSA
  - (ii) Primary antibodies reactive with DPSC using STRO-1 (mouse anti-human MSC; IgM), CC9 (mouse anti-human CD146/MUC-18; IgG_{2a}), or 3G5 (mouse anti-human pericyte; IgM)

(iii) Sheep anti-mouse IgG-conjugated or rat anti-mouse IgM-conjugated magnetic Dynabeads

- ❑ Culture flasks or dishes
- ❑ Surgical blades and their folders
- ❑ Cell strainer, 70 μm

Nonsterile equipment for immunomagnetic separation, if used:

- ❑ Rotary mixer
- ❑ Dynal MPC® -1 magnetic particle concentrator

Procedure

(a) Mince the dental pulp tissue into tiny pieces with a surgical blade.
(b) Immerse the minced tissue into a mixed collagenase/dispase solution (1:1).
(c) Incubate at 37°C for up to 30–60 min and mix well intermittently.
(d) After the digestion, inactivate the enzyme by dilution in sufficient MSC medium.
(e) Pass the cells through a 70-μm cell strainer to remove tissue debris to obtain a single-cell suspension.
(f) Centrifuge at 500 *g* for 6 min.
(g) Remove supernate and resuspend pellet with MSC medium. Immunomagnetic bead selection can be performed at this stage:
  i) Incubate with primary antibodies reactive with DPSC using STRO-1 (mouse anti-human MSC; IgM), CC9 (mouse anti-human CD146/MUC-18; IgG_{2a}), or 3G5 (mouse anti-human pericyte; IgM) for 1 h on ice at a concentration of 20 μg/ml.
  ii) Wash twice with PBSA containing 1% BSA, spinning at 600 *g* for 6 min.
  iii) Incubate with either sheep anti-mouse IgG-conjugated or rat anti-mouse IgM-conjugated magnetic Dynabeads (4 beads per cell) for 40 min on a rotary mixer at 4°C.
  iv) Remove cells bound to beads with the Dynal MPC® -1 magnetic particle concentrator according to the manufacturer's recommended protocol.
(h) Count the cells and seed them into culture flasks or dishes at $1–10 \times 10^3/\text{cm}^2$.
(i) Culture cells in MSC medium at 37°C and 5% CO_2 in the incubator.
(j) Seven days after the cell isolation, wash the culture vessels with PBSA and change the medium. After that, the medium can be changed twice a week until cell confluence is reached.

8.3.5. Subculture

Usually around 1 week after the cell isolation, colonies are easily identified in the culture vessels, where the cells have a typical fibroblast-like spindle shape (Fig. 8.2). Before the cells become 100% confluent (usually after about 2–3 weeks), they should be subcultured as described below.

Each colony is theoretically derived from a single CFU-F [Friedenstein, 1976, 1980; Friedenstein et al., 1970] and can be isolated by use of a cloning cylinder. In addition,

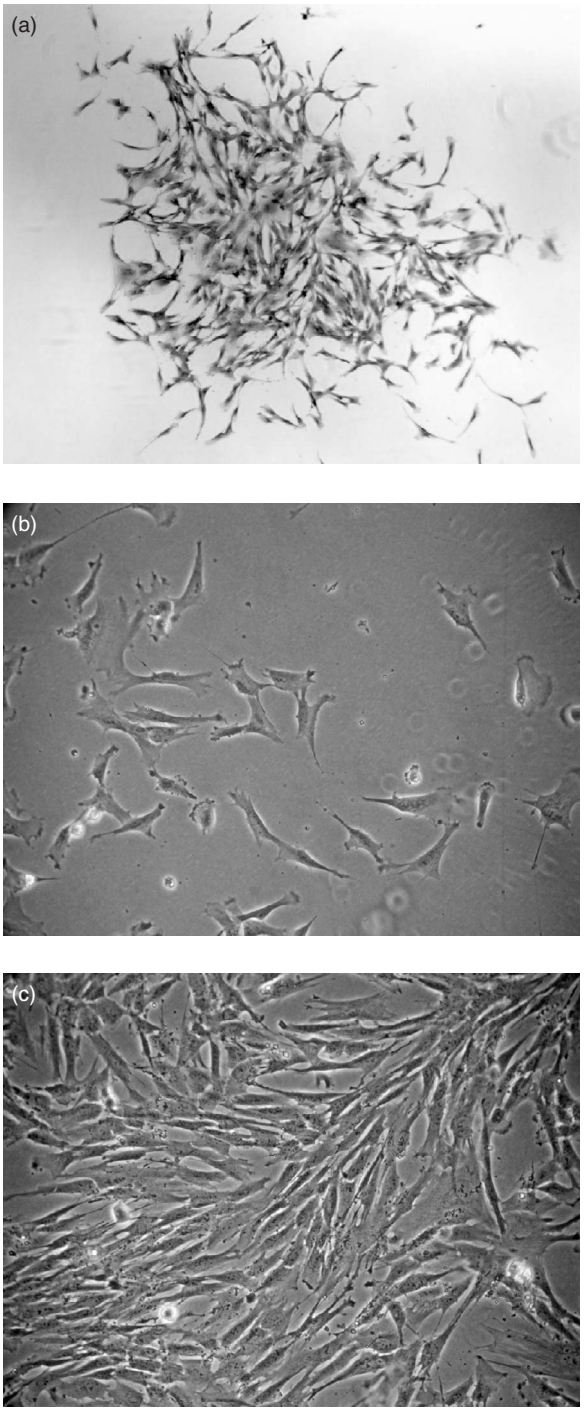

FIGURE 8.2. Morphology of DPSCs. (**a**) Representative colony structure from primary DPSCs (original magnification, ×50). (**b**) Passaged cells at low density (original magnification, ×200). (**c**) Passaged cells at high density (original magnification, ×200).

single colonies can be collected by serial dilution following subculture or by fluorescence activated cell sorting (FACS) using STRO-1 and CD146 antibodies.

If cultures are established with unselected preparations, occasionally colonies of cells with a morphology resembling epithelial cells or endothelial cells can be observed [Huang et al., 2006]. Usually, these contaminating cells disappear in the course of successive cell passages. If the contamination is extensive, the following three procedures can be performed on subculture. The first procedure involves trypsinizing the culture for a shorter time so that only stromal cells are detached, because epithelial- or endothelial-like cells are more strongly attached to the culture flask or dish. The second is changing the medium 4–6 h after subculture, because stromal cells attach to the culture surface earlier than the contaminating cells. The third and most reliable approach to separation of DPSCs from epithelial cells is to use FACS, in which STRO-1 or CD146 can be used to select DPSCs as previously described [Shi and Gronthos, 2003].

Protocol 8.3. Subculture of DPSCs

Reagents and materials

Sterile

- ❑ PBSA
- ❑ MSC medium (*see* Section 8.2.1.1.)
- ❑ Trypsin-EDTA solution: trypsin, 0.05%, EDTA, 0.54 mM (0.2%), in Hanks' balanced salt solution without Ca^{2+} and Mg^{2+}

Procedure

(a) Wash the flasks/dishes with PBSA three times.
(b) Add a sufficient volume of trypsin-EDTA solution and incubate at 37°C.
(c) Confirm that 80% of the attached cells have become detached from the culture surface, then add MSC medium.
(d) Centrifuge at 500 *g* for 6 min.
(e) Remove supernate and resuspend with MSC medium.
(f) Count the cells and seed them at the desired density.
(g) Culture them in MSC medium at 37°C and 5% CO_2 in air.

8.3.6. Cryopreservation and Recovery

Dental pulp stem cells can be cryopreserved and recovered by the usual procedure (see Chapter 2 and Protocol 8.4). The more important points in the procedure are as follows:

(1) Cells harvested near the end of log-phase growth (approximately 80–90% confluent) are best for cryopreservation.
(2) The number of cells should be around $1–2 \times 10^6$/vial containing 1.5 ml of freezing medium. Too low or too high a cell number may decrease the recovery rate.

(3) The cyroprotective agent (DMSO) should be added gradually up to 10% at a low temperature (e.g., on ice).
(4) A high serum concentration (90% FBS and 10% DMSO at final concentration) should be used to assist in cell survival.

Protocol 8.4. Cryopreservation of DPSCs

Reagents and materials

Sterile

- ❑ PBSA
- ❑ Ice-cold Freezing Medium I (*see* Section 8.2.3.1.)
- ❑ Ice-cold Freezing Medium II (*see* Section 8.2.3.2.)
- ❑ Trypsin-EDTA solution: trypsin, 0.05%, EDTA, 0.54 mM (0.2%), in Hanks' balanced salt solution without Ca^{2+} and Mg^{2+}
- ❑ Cryovials, 1.8 mL

Procedure

(a) Wash the flasks/dishes with PBSA three times.
(b) Add a sufficient volume of trypsin-EDTA solution and incubate at 37°C.
(c) Confirm that 80% of the attached cells have become detached from the surface, then add MSC medium.
(d) Centrifuge at 500 *g* for 6 min.
(e) Remove supernate and resuspend cells in ice-cold Freezing Medium I.
(f) Count the cells, then dilute or concentrate them to twice the desired final concentration with Freezing Medium I. Keep the tubes containing the cells on ice.
(g) Add an equal volume of Freezing Medium II little by little on ice while rotating the tube.
(h) Add aliquots to cryopreservation vials.
(i) Place the vials in a programmed freezer. A controlled freezing container and −80°C freezer may also be used to achieve a slow cooling rate. Place the vials in a liquid nitrogen freezer for long-term storage.

Protocol 8.5. Recovery of DPSCs from Frozen Storage

Reagents and materials

Sterile or aseptically prepared

- ❑ Cryovial taken directly from nitrogen freezer
- ❑ MSC medium (*see* Section 8.2.1.1.)

Procedure

(a) Place the vials in a warm water bath (37°C). Quick thawing (1–2 min) is important for the best recovery.
(b) Add sufficient volume of MSC medium and mix well.
(c) Centrifuge at 500 *g* for 6 min.
(d) Remove supernate and resuspend cells in MSC medium.
(e) Count the viable cells with Trypan Blue solution and then seed them at the desired viable cell density.
(f) Culture them in MSC medium at 37°C and 5% CO_2 in air.

8.3.7. Characterization of Undifferentiated DPSCs and SHED

STRO-1 is one of the early cell surface markers for mesenchymal stem cells that can be used to evaluate the undifferentiated status of DPSCs and SHED. Monoclonal antibody for STRO-1 was first described as a potential reagent that reacted with a cell surface molecule highly expressed on human bone marrow CFU-F [Dennis et al., 2002; Gronthos et al., 1994, 1999; Simmons et al., 1994]. STRO-1-positive cells from adult bone marrow contain a CFU-F population and exhibit an ability to differentiate into multiple cell lineages including myelo-supportive stromal cells, osteoblasts, adipocytes, and chondrocytes [Gronthos et al., 2003; Shi and Gronthos, 2003; Simmons et al., 1991]. DPSCs and SHED have been found to contain a STRO-1-positive fraction at around 10–20% [Gronthos et al., 2000; Miura et al., 2003]. CD146 (MUC18), known as a possible marker for BM-MSCs [Filshie et al., 1998], is also expressed in DPSCs and SHED [Gronthos et al., 2000, 2003; Miura et al., 2003; Shi and Gronthos, 2003]. These markers can be used in immunocytochemistry or FACS analysis.

Dentin sialophosphoprotein (DSPP), alkaline phosphatase (AP), bone sialoprotein (BSP), and osteocalcin (OSC) are representative lineage markers of odontoblasts and osteoblasts [Feng et al., 1998; MacDougall et al., 1997]. Core binding factor 1 (Cbfa1)/Runt-related gene factor 2 (Runx2) and osterix are also known as osteoblast-specific transcription factors and are thought to play a pivotal role in tooth development [D'Souza et al., 1999; Ducy et al., 1997; Nakashima et al., 2002; Thesleff and Aberg, 1999]. The genes for these molecules are not expressed, or are markedly less expressed, in undifferentiated DPSCs and SHED as analyzed by the reverse transcription-polymerase chain reaction (RT-PCR) or Western blot analysis [Gronthos et al., 2000, 2002; Miura et al., 2003]. Lineage markers of other cell types, including proliferator-activated receptor-$\gamma 2$ (PPAR$\gamma 2$) and lipoprotein lipase (LPL) for adipocytes or glial fibrillary acidic protein (GFAP) and nestin for neural cells, are also not expressed, or markedly less expressed, by RT-PCR or Western blot analysis before stimulation for cell-specific differentiation [Gronthos et al., 2000, 2002; Miura et al., 2003].

It is important to note that DPSCs and SHED are heterogeneous populations after expansion ex vivo [Gronthos et al., 2002; Kuznetsov et al., 1997; Owen et al., 1988; Pittenger et al., 1999] and begin gradually to lose their stemness (loss of STRO-1 and 3G5 expression) with successive culture passages. For example, DPSCs seem to partially differentiate into an odontoblast lineage after continuous passage without any specific inductive stimulation. The genes for DSPP and OSC become are expressed after passage 4 or 5 (cumulative population doubling is approximately 20) as detected by RT-PCR

[unpublished data]. Therefore, it is very important to use DPSCs and SHED at an early passage if they are to be used as "stem" cells. Further studies are required to establish adequate and ideal culture conditions to maintain the stemness of DPSCs and SHED.

8.4. DIFFERENTIATION CAPACITY AND ASSAY

8.4.1. Odontogenic/Osteogenic Differentiation

DPSCs and SHED can differentiate into odontoblasts and osteoblasts in vitro when they are cultured with a medium containing dexamethasone, inorganic phosphate, and L-ascorbic acid. This differentiation is confirmed by upregulation of odontoblast- and osteoblast-related markers, for example, DSPP, BSP, AP, cbfa1, and osterix, within a few weeks after induction. In the meantime, a downregulation of STRO1 should be observed. After culture for several weeks, mineralized nodules are observed under the microscope as a result of calcium accumulation. This calcium accumulation can be analyzed by Alizarin Red staining (Fig. 8.3a; Plate 9A) or quantified with commercially available kits.

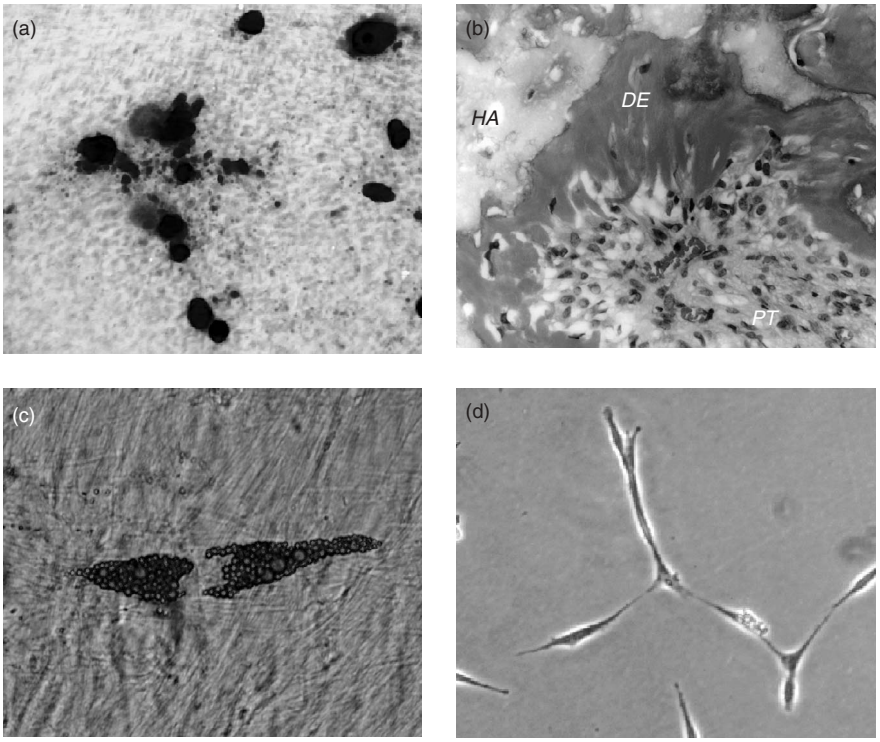

FIGURE 8.3. Differentiation capacity of DPSCs. (**a**) Mineralized nodules formed after 2-week culture under mineralized condition (×100). Nodules are stained by Alizarin Red S. (**b**) Dentin-pulp complex generated by DPSCs after 8 weeks of transplantation (original magnification, ×400). Dentin-like matrix (*DE*), which has a tubular structure, is generated on the surface of carrier (*HA*) with pulplike tissue (*PT*). (**c**) Adipocyte differentiated from DPSC. Lipid clusters are stained by Oil Red O (original magnification, ×400). (**d**) Cells with elongated cytoplasmic processes are observed after 2-week neural stimulation (original magnification, ×400). (*See also* Plate 9.)

Importantly, DPSCs and SHED can regenerate a dentin–pulp complex when transplanted subcutaneously into immunocompromised mice with a hydroxyapatite/tricalcium phosphate (HA/TCP) carrier (Fig. 8.3b; Plate 9B) [Gronthos et al., 2000, 2002; Miura et al., 2003]. The regenerated mineralized matrix has a typical tubular structure along with odontoblastic cells, which is a characteristic of the natural dentin–pulp complex. The origin and characteristics of this matrix are confirmed by immunostaining with antibodies against human-specific mitochondria, DSPP, and BSP [Gronthos et al., 2000; Miura et al., 2003]. Cells that maintain odontoblastic capacity can be recovered from transplanted tissue, and this suggests a self-renewal potential of DPSCs. Interestingly, SHED can be induced to form a bonelike matrix with a lamellar structure by recruiting host cells [Miura et al., 2003]. This capacity for bone generation might be correlated with the nature of the baby tooth, whose root resorption is followed by bone and permanent tooth eruption. Another interesting feature of the bonelike matrix generated by SHED transplants is the lack of bone marrow components. In contrast, the bonelike matrix generated by BM-MSC transplants contains material compatible with bone marrow [Batouli et al., 2003].

Protocol 8.6. Odontogenic and Osteogenic Differentiation of DPSCs In Vitro

Reagents and materials

Sterile

- ❑ MSC medium (*see* Section 8.2.1.1.)
- ❑ Odontogenic differentiation medium (*see* Section 8.2.1.2.)

Procedure

(a) Culture the cells with regular MSC medium until they reach complete confluence.

(b) Switch the medium to odontogenic differentiation medium, and change 2–3 times a week for up to 6 weeks. (After confluence, cells may easily be detached from the culture surface. Therefore, great care should be taken to avoid detachment on changing the medium.)

Protocol 8.7. Odontogenic/Osteogenic Differentiation of DSPCs In Vivo

Reagents and materials

Sterile

- ❑ PBSA
- ❑ MSC medium (*see* Section 8.2.1.1.)
- ❑ Trypsin-EDTA solution: trypsin, 0.05%, EDTA, 0.54 mM (0.2%), in Hanks' balanced salt solution without Ca^{2+} and Mg^{2+}

- ❑ Cryovials, 1.8 mL
- ❑ Appropriate carrier (hydroxyapatite/tricalcium phosphate carrier)

Procedure

(a) Culture the cells with MSC medium until 90% confluent.
(b) Wash the dishes three times with PBSA.
(c) Add sufficient volume of Trypsin-EDTA solution and incubate at 37°C.
(d) Confirm that 80% of cells are detached from the culture surface, then add MSC medium.
(e) Centrifuge at 500 *g* for 6 min.
(f) Remove supernate and resuspend cells in MSC medium.
(g) Count the cells.
(h) Mix 2–4 $\times$ 10^6 cells suspended in MSC medium with carrier in a 1.8-mL cryovial.
(i) Incubate them at 37°C with rotation for 1 h.
(j) Transplant them aseptically and subcutaneously into an immunocompromised mouse. We usually use NIH *bg-nu/nu-xid* mice for transplantation.
(k) Harvest them at an appropriate time, and examine histologically. (In the system with HA/TCP carrier and NIH *bg-nu/nu-xid* mice, a duration of 8 weeks is adequate for the generation of sufficient mineralized matrix by DPSCs and SHED. The time point for harvest depends on the system.)

8.4.2. Adipogenic Differentiation

Although adipocytes have not been observed in dental pulp, DPSCs and SHED can, under appropriate inductive conditions, differentiate into adipocytes along with accumulation of lipid clusters in their cytoplasm [Gronthos et al., 2000, 2002; Miura et al., 2003]. These lipid clusters are easily identified under the microscope and can be examined with Oil Red O staining after several weeks of adipogenic induction (Fig. 8.3c; Plate 9C). Also, upregulation of adipocyte-related genes (PPARγ2 and LPL) are is by RT-PCR.

It is no surprise to find that DPSCs and SHED have a lower capacity to differentiate into adipocytes than BM-MSCs. This might be caused by a difference in the nature of their original tissue (adipose tissue occurs in the bone marrow cavity of long bones in aged humans).

Protocol 8.8. Adipogenic Differentiation of DPSCs In Vitro

Reagents and materials

Sterile

- ❑ MSC medium (*see* Section 8.2.1.1.)
- ❑ Adipogenic differentiation medium (*see* Section 8.2.1.3.)

Procedure

(a) Culture the cells with MSC medium until they reach complete confluence.
(b) Switch the medium to adipogenic differentiation medium, and change 2–3 times a week for up to 6 weeks. (Differentiated adipocytes easily detach from the culture surface. Therefore, great care should be taken to avoid detachment on changing the medium.)

8.4.3. Neural Differentiation

Development of dental pulp is closely associated with neural crest cells [Chai et al., 2000; Nosrat et al., 2001; Thesleff and Aberg, 1992]. Therefore, it is reasonable to assume that DPSCs and SHED might have the capacity to differentiate into neural cells. Indeed, under specified inductive conditions in vitro, DPSCs and SHED can differentiate into neural cells with protruding elongated cytoplasmic processes (Fig. 8.3d) [Gronthos et al., 2000, 2002; Miura et al., 2003]. Neural differentiation is confirmed by morphological appearance and expression of neural-specific molecules, including GFAP, nestin, neurofilament M, neuronal nuclear marker (NeuN), 2′, 3′-cyclic nucleotide−3′-phosphodiesterase (CNPase), βIII tubulin, and glutamic acid decarboxylase (GAD). Some cells are capable of forming spherelike structures, which are observed in neural stem cells. Transplanted SHED, precultured in neural differentiation medium, can survive with neural phenotypes for at least 1 week in mouse brain [Miura et al., 2003].

Protocol 8.9. Neural Differentiation of DPSCs In Vitro

Reagents and materials

Sterile

❑ MSC medium (*see* Section 8.2.1.1.)
❑ Neural differentiation medium (*see* Section 8.2.1.4.)
❑ Gelatin, 0.1%

Procedure

(a) Coat culture plates with gelatin for 30 min and dry.
(b) Seed cells at relatively low density $0.1–1.0 \times 10^3$ cells/cm^2 with neural differentiation medium.
(c) Change medium every day, usually for 4 weeks.

8.5. DISCUSSION

DPSCs and SHED are derived from an easily accessible tissue resource and can be expanded to reach a sufficient number of cells for therapy as a result of their extremely high population-doubling ability. They may be an important source of stem cells for autologous stem cell transplantation. Although DPSCs and SHED are capable of regenerating dentin/pulp and bone tissues in vivo, considerable work is still required to maintain their stemness in vitro, and to achieve optimal tissue regeneration in vivo.

8.6. SOURCES OF MATERIALS

Item	Catalog No.	Supplier
α-Modified minimal essential medium (α-MEM)	22571	Invitrogen (GIBCO)
B27 supplement	17504	Invitrogen (GIBCO)
Basic fibroblast growth factor (bFGF), human recombinant	354060	BD Biosciences
Cell strainer, 70 μm		BD Biosciences
Cryovials	375418	Nunc
Collagenase, type I	CLOSA	Worthington Biochemicals Corp.
Dexamethasone sodium phosphate	D 1159	Sigma
Dimethyl sulfoxide (DMSO)	D 2650	Sigma
Dispase II (neutral protease)		Roche
Dynabeads		Invitrogen (Dynal)
Dynal MPC® -1		Invitrogen (Dynal)
Epidermal growth factor (EGF), human recombinant	GF001	Millipore (Chemicon)
Gelatin	07903	StemCell Technologies Inc.
Glutamine	P300–100	Invitrogen (Biosource)
Hydrocortisone	H 0396	Sigma
Indomethacin	I 7378	Sigma
Insulin	28150	Invitrogen (GIBCO)
Isobutylmethylxanthine	I 7018	Sigma
L-Ascorbic acid phosphate		Wako Chemicals.
Monopotassium phosphate (KH_2PO_4)	P 5655	Sigma
Neurobasal A	10888	Invitrogen (GIBCO)
Penicillin/streptomycin	15140	Invitrogen (Biosource)
Trypsin/EDTA	0249	Invitrogen (GIBCO)

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CHAPTER 9

FUNDAMENTALS OF CULTURE AND CHARACTERIZATION OF MESENCHYMAL STEM/PROGENITOR CELLS (MSCs) FROM BONE MARROW STROMA

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Culture of Human Stem Cells, Edited by R.I. Freshney, G.N. Stacey and J.M. Auerbach

9.1. INTRODUCTION

Human mesenchymal stem cells (hMSCs), also referred to as multipotential stromal cells from bone marrow, are pluripotent, and under the appropriate conditions hMSCs can be expanded while retaining their potential to differentiate into a variety of tissue lineages including osteoblasts, adipocytes, chondrocytes, myoblasts, hepatocytes, and possibly even neural tissue in vitro [Friedenstein et al., 1976; Pereira et al., 1995; Pittenger et al., 1999; Sekiya et al., 2002a; Reyes and Verfaillie, 2001; Jiang et al., 2002a,b, 2003]. In the mid-1970s, Friedenstein and colleagues demonstrated that MSCs could be grown ex vivo and maintain their differentiation capacity in vivo on reimplantation [Friedenstein et al., 1974]. MSCs were later shown to durably engraft in the bone, cartilage, and lungs of mice [Pereira et al., 1998] and to differentiate into chondrocytes, adipocytes, myocytes, cardiomyocytes, bone marrow stromal cells, and thymic stroma on transplantation in sheep [Liechty et al., 2000]. Other recent examples include the repair of kidney [Herrera et al., 2004], lung [Ortiz et al., 2003], and heart [Mangi et al., 2003]. Based on these animal studies, it is not surprising that there has been much interest in their clinical potential for tissue repair and gene therapy [Prockop 1997; Bruder et al., 1994]. To date, human clinical trials have been conducted for the treatment of numerous diseases including osteogenesis imperfecta [Horwitz et al., 1999, 2002], metachromatic leukodystrophy and Hurler syndrome [Koc et al., 2002], myocardial infarction [Katritsis et al., 2005], and stroke [Bang et al., 2005].

Human MSCs are typically isolated from the mononuclear cell layer of the bone marrow after separation by discontinuous density gradient centrifugation [Friedenstein et al., 1976; Pereira et al., 1995; Colter et al., 2000; Sekiya et al., 2002b]. The mononuclear

layer is cultured, and the MSCs adhere to the plastic surface of the tissue culture vessel. Through monolayer culture, the nonadherent hematopoietic cells are gradually washed away, resulting in a fibroblast-like, exclusively adherent, rapidly expanding culture of MSCs. Rodent MSCs are prepared and cultured in a similar manner, with essentially minor variations in the protocol. As the cultures approach high density, the MSCs enter a stationary phase and transform from a spindlelike morphology to a larger, flatter phenotype [Sekiya et al., 2002b]. The MSCs recovered from a 2-mL bone marrow aspirate can be expanded 500-fold over about 3 weeks, resulting in a theoretical yield of $1.25–3.55 \times 10^{10}$ cells, but typically 1×10^8 cells are routinely produced in the laboratory [Sekiya et al., 2002b; Gregory et al., 2005a,b]. Over the expansion period, the cells retain their multipotentiality, but this property can diminish with extensive expansion or suboptimal methodology. The characterization assays described in the latter sections of this chapter are designed to complement the culture process and allow one to appraise the cells during their expansion. This chapter describes the minimum requirements for efficient culture of functional MSCs from bone marrow stroma.

9.2. PREPARATION OF MEDIA AND REAGENTS

9.2.1. Complete Culture Medium (CCM)

α-MEM: α-Minimal essential medium with glutamine but without ribonucleosides or deoxyribonucleosides; supplemented with:

(i) Additional L-glutamine — 2 mM
(Final concentration, including glutamine already in formulation 4 mM)
(ii) FBS hybridoma qualified and not heat inactivated — 20%
(iii) Penicillin — 100 U/mL
(iv) Streptomycin — 100 μg/mL

Filter sterilize. Can be stored at 4°C for up to 2 weeks.

9.2.2. Trypsin/EDTA

Porcine trypsin, 0.25%, EDTA, 1 mM, in PBSA

9.2.3. Crystal Violet Solution

(i) Dissolve Crystal Violet at 3% (w/v) in methanol.
(ii) Filter through a 500-mL vacuum filter unit with a 0.22-μm^2 filtration membrane to remove particulate material.

9.2.4. Bone Differentiation Medium (BDM)

CCM containing 5 mM β-glycerol phosphate, 50 μg/mL ascorbate-2-phosphate, and 1 nM dexamethasone (note the presence of β-methyl cyclodextrin carrier in the dexamethasone when preparing a 1000× stock solution in water). Filter sterilize.

9.2.5. Alizarin Red S (ARS) Solution

1% (w/v) ARS in distilled water adjusted to pH 4.1 with 0.5 N ammonium hydroxide, then sterile filtered

9.2.6. Fat Differentiation Medium (FDM)

(i) Isobutylmethylxanthine (IBMX), 1000× stock, 0.5 mM in methanol
(ii) Indomethacin (IM), 1000× stock, 50 mM in methanol.
(iii) Dexamethasone, 1000× stock, 0.5 mM in water (note the β-methyl cyclodextrin carrier in the dexamethasone).
(iv) FDM: CCM (*see* Section 9.2.1) containing 0.5 μM IBMX, 50 μM IM, and 0.5 μM dexamethasone.

9.2.7. Oil Red O (ORO) Working Solution

(i) ORO 1% (w/v) in isopropyl alcohol _____ 3 parts
(ii) PBSA _____ 2 parts
(iii) Filter through a 70-μm cell strainer.

9.2.8. Stock Solutions (100×) of BMP2 and -6

(i) Dissolve bone morphogenetic proteins (BMPs) at a concentration of 50 μg/mL in sterile PBSA.
(ii) Make 10- to 50-μL aliquots in 0.5-mL microcentrifuge tubes and store at -20°C.

9.2.9. Stock Solution (100×) of TGF-β3

(i) Dissolve TGF-β3 at a concentration of 1 μg/mL in sterile PBSA.
(ii) Make 10- to 50-μL aliquots in 0.5-mL microcentrifuge tubes and store at -20°C. Each culture will require 5 μL of the stock per medium change.

9.2.10. Chondrocyte Differentiation Medium (CDM)

High-glucose DMEM containing:

(i) Dexamethasone _____ 1×10^{-7} M (*see* Section 9.2.6)
(ii) Ascorbate-2-phosphate _____ 50 μg/mL
(iii) Proline _____ 40 μg/mL
(iv) Pyruvate _____ 100 μg/mL
(v) ITS Plus premix _____ 50 mg/mL
(vi) Filter sterilize.

9.3. ISOLATION OF HUMAN BONE MARROW FOR MSC EXPANSION

Iliac crest bone marrow aspirates are generally preferred for the isolation and expansion of hMSCs, although MSC-like cells have been recovered from trabecular bone [Sakaguchi et al., 2004], synovium [Sakaguchi et al., 2005], adipose tissue [Zuk et al., 2002], and even exfoliated teeth [Miura et al., 2003; *see also* Chapter 8]. A 2-mL bone marrow aspirate is adequate for the production of enough MSCs for most applications. Aspiration of bone marrow should be carried out by appropriately trained personnel with approved clinical protocols that are beyond the scope of this chapter. The bone marrow can be stored in heparinized blood drawing tubes charged with 3 mL of α-minimal essential medium (α-MEM) and stored on ice for up to 8 h before processing. Longer incubations at 4°C decrease the initial rate of propagation of MSCs and are therefore discouraged. Preparation of rat bone marrow and isolation of the MSCs are described in Section 9.7.

9.4. PROCESSING OF HUMAN BONE MARROW FOR MSC CULTURE

Human bone marrow is initially processed by enriching for the nucleated component of the bone marrow that contains the hematopoietic and mesenchymal stem cells by Ficoll-mediated discontinuous density gradient centrifugation as described in Protocol 9.1. The bone marrow is then cultured on 15-cm tissue culture plates, or after recovery on tissue culture plates, the cells can be expanded in Nunc Cell Factories® with frequent washes and medium changes. The nonadherent hematopoietic component of the culture is gradually washed away over a few days, resulting in an exclusively adherent MSC culture.

All work with human material should be carried out in a Class II microbiological safety cabinet.

Protocol 9.1. Discontinuous Density Gradient Centrifugation of Human Bone Marrow for MSC Production

Reagents and Materials

Sterile

- ❑ Complete culture medium (CCM; *see* Section 9.2.1)
- ❑ Phosphate- buffered saline without calcium or magnesium (PBSA)
- ❑ Hanks' balanced salt solution without calcium or magnesium (HBSS)
- ❑ Ficoll-Paque
- ❑ Polypropylene centrifuge tubes, 15 mL and 50 mL
- ❑ Plastic tissue culture Petri dishes, 15-cm diameter
- ❑ Plastic micropipettor tips for dispensing 10 μL

Nonsterile

- ❑ Trypan Blue solution in 0.85% saline
- ❑ Microcentrifuge

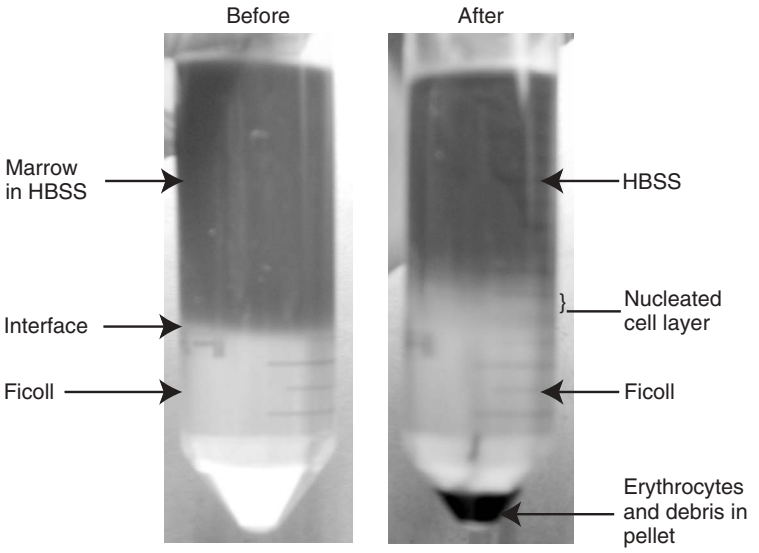

FIGURE 9.1. Processing of rodent and human bone marrow. Photographs before and after centrifugation of the discontinuous Ficoll gradient used to separate the mononuclear fraction of human bone marrow. The mononuclear layer is recovered from the white band at the interface between the two layers. (*See also* Plate 13.)

- ❑ Refrigerated bench top centrifuge with swinging bucket rotor
- ❑ Water bath set to 37°C
- ❑ Improved Neubauer hemocytometer
- ❑ Micropipettor, Eppendorf P20 or equivalent

Procedure

(a) Uncap the drawing tube of bone marrow and transfer to one 50-mL centrifuge tube. Make the volume up to 25 mL with room temperature HBSS.

(b) To another 50-mL centrifuge tube, add 20 mL of Ficoll-Paque and gently overlay the 25-mL cell solution on to the Ficoll. The interface between the HBSS and the Ficoll should not be disrupted.

(c) Centrifuge at 1800 *g* for 30 min at room temperature with the brake off.

(d) After centrifugation, collect the white cell layer at the interface of the Ficoll and HBSS (Fig. 9.1; Plate 13) and transfer to a fresh 50-mL centrifuge tube.

(e) Make the volume of the interface cell suspension up to at least 3 volumes with HBSS and centrifuge at 1000 *g* for 10 min at room temperature with the brake on. Repeat wash.

(f) Suspend the cell pellet in 30 mL of CCM prewarmed to 37°C.

(g) Add 10 μL of the cell suspension to 10 μL of Trypan Blue and assess the viability with a hemocytometer; viability should be above 80%.

(h) Transfer the 30-mL cell suspension to a 15-cm-diameter tissue culture Petri dish and culture in a humidified incubator under 5% CO_2 for at least 15 h.

(i) Remove the Petri dish from the incubator and remove the medium.

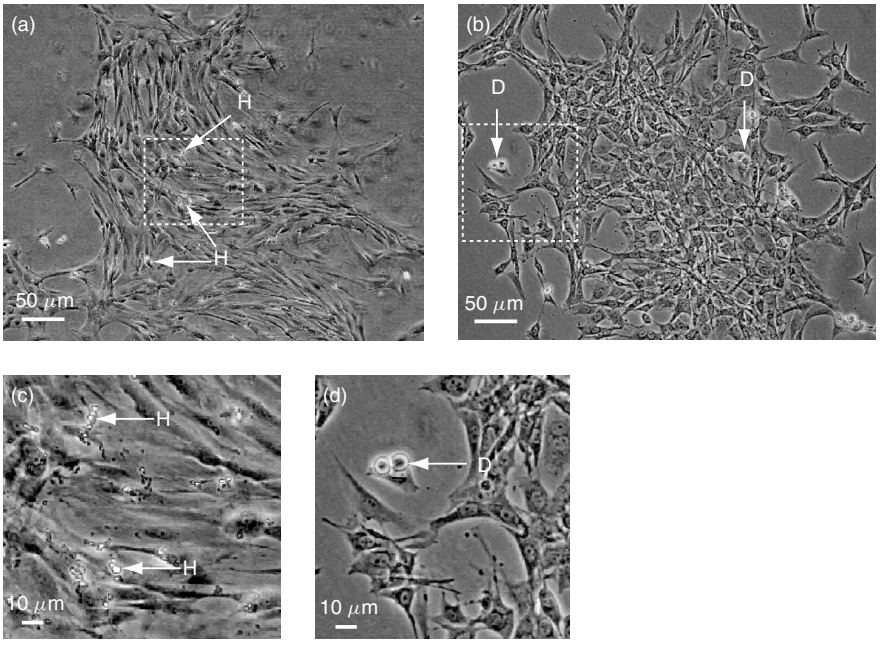

FIGURE 9.2. Colonies of MSCs established from plating of bone marrow. The micrographs in panels (**a**) and (**b**) depict colonies formed before plating of whole bone marrow mononuclear cells. Both colonies still harbor small, round cells of presumptive hematopoietic origin (arrowed H). Dividing MSCs that often appear as doublets are easily mistaken for contaminating hematopoietic cells (arrowed D), but the dividing MSCs are typically larger than hematopoietic cells. Panels (**c**) and (**d**) are enlargements of the dotted squares in (**a**) and (**b**), respectively. After washing of the monolayer, medium changes, and passaging, the contaminating cells will disappear, resulting in fibroblast-like, adherent MSCs with numerous dividing MSCs.

(j) Add and remove 20 mL of prewarmed PBSA to wash the monolayer.
(k) Repeat the wash procedure 3 times.
(l) Replace with 30 mL of fresh prewarmed CCM.
(m) Repeat this wash and medium replenishment every second day for 6 days.
(n) After 6 days, examine the monolayers with an inverted microscope. Adherent, fibroblast-like colonies of MSCs should be clearly visible in the Petri dish (Fig. 9.2, a and b). In some cases, there may be signs of hematopoietic contamination, but these cells will be depleted on passaging the cells. When the culture is 50–60% confluent, proceed to Section 9.5.

9.5. EXPANSION AND CRYOPRESERVATION OF MSC CULTURES

9.5.1. Expansion of MSCs from Human Bone Marrow

Although MSC preparations vary based on donor and species, under the correct culture conditions it is feasible to expand MSCs roughly 500-fold over about a 3-week period [Sekiya et al., 2002b; Gregory et al., 2005a,b]. This is achieved by a combination of

low-density plating and passaging. For small-to medium-scale experiments, the MSCs are best cultured in 15-cm diameter Nunclon or Corning plates with a growth area of between 140 and 150 cm^2. The final cell yield is dependent on the number of plates, but typically 5×10^5 cells per plate can be produced with this method of expansion. For large-scale production, Nunclon Cell Factories® with a total growth area of 6320 cm^2 are usually employed, allowing the rapid production of over 5×10^7 cells in about 10 days. Because Cell Factories can be used as a completely sealed unit, they are often the unit of choice for the expansion of cells for clinical use. This section focuses on the use of standard Petri dishes for the expansion of MSCs since this approach is best suited to general research-based applications, but the protocol is the same, in principle, for investigators choosing to expand their MSCs in Cell Factories.

The recovery of MSCs from different species and strains of rodents can vary, but reproducible results can be derived from Lewis rats [Javazon et al., 2001]. This chapter focuses on the isolation and expansion of rat cells only because of their relative ease of culture and similarity to hMSCs. The culture of murine MSCs is complex, and a standard protocol for their production has not been agreed upon by investigators in the field. The expansion of murine MSC-like cells, however, can be achieved by numerous protocols that differ extensively from each other and from the standard method used to propagate rat and human MSCs [Phinney et al., 1999; Jiang et al., 2002; Baddoo et al., 2003; Peister et al., 2004].

Protocol 9.2. Subculture of Mesenchymal Stem Cells (MSCs)

Reagents and Materials

Sterile

- ❑ Complete culture medium (CCM; *see* Section 9.2.1)
- ❑ PBSA
- ❑ Trypsin/EDTA (*see* Section 9.2.2)
- ❑ Polypropylene centrifuge tubes, 15 mL and 50 mL
- ❑ Plastic tissue culture Petri dishes, 15-cm diameter
- ❑ Plastic pipette tips to dispense 10–1000 μL

Nonsterile

- ❑ Trypan Blue solution in 0.85% saline
- ❑ Improved Neubauer hemocytometer
- ❑ Pipettors, Eppendorf P10, P100, and P1000 or equivalent

Procedure

(a) Inspect the MSC cultures generated by Protocol 9.1. If the cultures consist of small, adherent, spindle-shaped fibroblastoid cells that are approximately 60% confluent (see Fig. 9.3d), proceed. If the monolayer is sparse (see Fig. 9.3c), continue to wash and replenish medium as described in Protocol 9.1.

(b) Trypsinize the monolayer as follows:

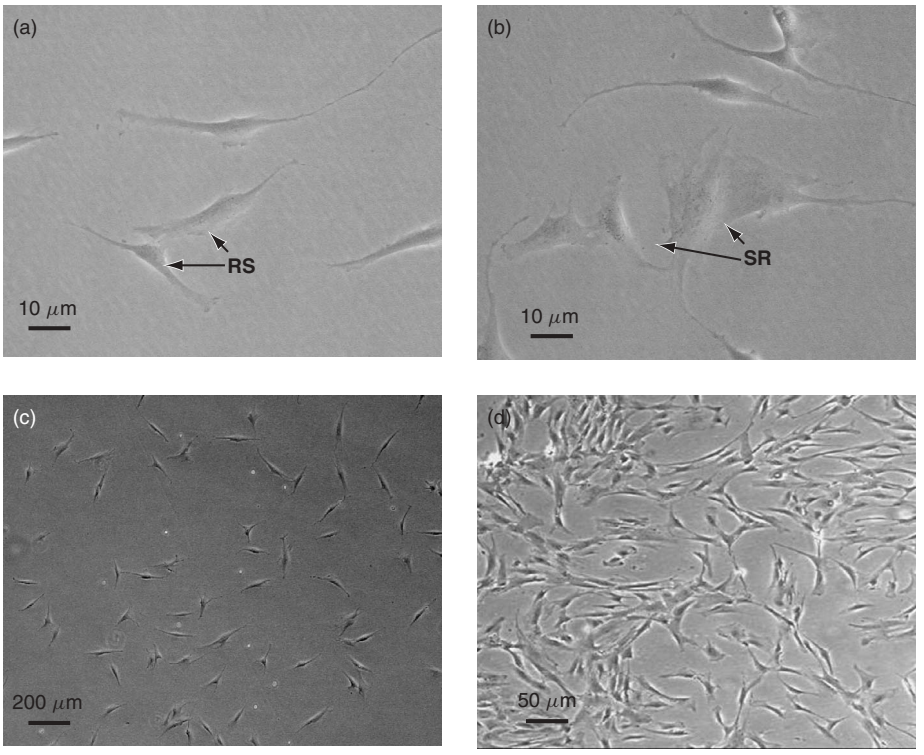

FIGURE 9.3. Morphology and optimal passaging density of MSCs. (**a**) RS-type MSCs with spindle-shaped morphology. (**b**) SR shaped MSCs that are larger, with more of a rhomboidal morphology. (**c**) An early-passage culture of MSCs with a prevalence of RS-type cells. (**d**) A monolayer at the appropriate density for passaging.

(i) Wash the monolayer with 20 mL of prewarmed PBSA and add 5 mL of trypsin/EDTA.

(ii) Place the plate at 37°C for 2 min, and then inspect the monolayer at 10× magnification. The adherent cells should be in the process of detaching from the plastic substratum.

(iii) Replace plate at 37°C for 2 min, then inspect again. Repeat inspection until 90% of the MSCs have detached from the plastic.

(iv) Add 5 mL of CCM, transfer the 10-mL suspension to a 15-mL conical tube, and centrifuge for 10 min at 500 *g*.

(v) After the centrifugation, remove the supernate from the cell pellet and resuspend the pellet in 1–2 mL of warm PBSA per tube. If necessary, combine multiple resuspended pellets for a single cell count.

(c) Add 10 μL of the cell solution to 10 μL of Trypan Blue and count with a hemocytometer. An adequate concentration for the cell suspension should be between 2 and 5×10^5 cells per mL, with a viability >80%. This trypsinized primary culture suspension, prepared for subculture, should also be used as starting material for colony-forming unit (CFU) assays (Protocol 9.5), cryopreservation (Protocol 9.3), or differentiation assays (Protocols 9.6, 9.7, and 9.8).

(d) The cells should then be plated at an initial density of 50–100 viable cells/cm^2 and maintained at low density to maintain the rapidly self-renewing, multipotential phenotype. Suspend the MSCs at a concentration of 7×10^3 (for a final density of 50 cells/cm^2) to 1×10^4 (for a final density of 100 cells/cm^2) cells/mL in prewarmed CCM.

(e) Prepare the appropriate number of plates by adding 25 mL of prewarmed CCM to each of the 15-cm plates.

(f) Seed the plates by adding 1 mL of the suspension prepared in Steps (b) and (c). Slide the plates from side to side (do not swirl) to distribute the cells evenly. Replace the plates in the incubator. These are designated *passage 1* cells.

(g) After 2–3 days of culture, inspect the plates and make an assessment of morphology. The MSCs should adopt a small, spindle-shaped morphology with frequent refractile doublets (for an example, *see* Fig. 9.2b). This is the sign of a healthy culture of MSCs (*see also* Section 9.8).

(h) Aspirate the medium from the plates, wash the MSCs with 20 mL of prewarmed PBSA, and replace with 25 mL of fresh prewarmed CCM.

(i) The number of subsequent expansion plates per passage is limited only by the number of cells available to seed the plates. The volumes quoted in the protocol above are suitable for a single 15-cm plate of MSCs. This can be expanded proportionally to accommodate multiple plates where necessary.

9.5.2. Cryopreservation and Recovery of MSCs

Another characteristic of MSCs is that they can be preserved in liquid nitrogen and easily recovered for further expansion. For cryopreservation, the cells are suspended in the presence of freezing medium that contains dimethyl sulfoxide (DMSO) and a high concentration of FBS. For successful freezing, the suspensions are gradually cooled to -80°C and then rapidly transferred to a liquid nitrogen freezer, where they are stored at approximately -150°C for periods up to at least 3 years.

Protocol 9.3. Cryopreservation of Mesenchymal Stem Cells (MSCs)

Reagents and Materials

Sterile

- ❑ α-MEM (*see* Section 9.2.1)
- ❑ FBS *(see* Section 9.2.1)
- ❑ Tissue culture-grade dimethyl sulfoxide (DMSO)
- ❑ Freezing medium (from above constituents): α-MEM with 30% FBS and 5% DMSO. Filter sterilize (for additional security and to clarify the medium, which can become cloudy with the high serum concentration combined with DMSO).
- ❑ Polypropylene centrifuge tubes, 15 mL and 50 mL
- ❑ PBSA
- ❑ Vacuum filter units with 0.22-μm^2 filtration membrane, 50 mL

- ❑ Cryovials, 2 mL
- ❑ Micropipettor tips for Eppendorf P10, P100, P1000, or equivalent

Nonsterile

- ❑ Isopropyl alcohol 99.5%
- ❑ Water bath or equivalent set to 37°C
- ❑ Micropipettors, Eppendorf P10, P100, P1000, or equivalent
- ❑ Nalgene Cryo−1°C freezing container
- ❑ Thermo ULT low-temperature freezer or equivalent set to −80°C
- ❑ Liquid nitrogen freezer filled with medical-grade liquid nitrogen

Procedure

(a) Centrifuge the remaining MSC suspension recovered by procedure described in Protocol 9.2, Steps (b) and (c), at 500 *g* for 10 min.

(b) Prelabel cryovials with the source of cells, date, and passage number.

(c) Resuspend the pellet at a concentration of 1×10^6 cells/mL in prewarmed freezing medium and aliquot 1 mL into the appropriate number of cryovials. Do not allow the MSCs to stand at room temperature for more than 20 min in the presence of DMSO.

(d) Fill the Cryo−1°C freezing container to the mark with isopropyl alcohol and place the tubes in the tube holders of the container.

(e) Place the container at −80°C for at least 15 h.

(f) Transfer the tubes to liquid nitrogen, where they can be stored for at least 3 years.

Protocol 9.4. Recovery of Cryopreserved Mesenchymal Stem Cells (MSCs)

Reagents and Materials

Sterile

- ❑ CCM: (*see* Section 9.2.1)
- ❑ Plastic tissue culture Petri dishes, 15 cm
- ❑ PBSA
- ❑ Pipettor tips for P10, P100, and P1000

Nonsterile

- ❑ Fisher Scientific Isotemp water bath or equivalent set to 37°C
- ❑ Micropipettors, Eppendorf P10, P100, and P1000, or equivalent

Procedure

(a) Prepare two 15-cm Petri dishes containing 25 mL of prewarmed CCM.

(b) Retrieve the required cryotube from the liquid nitrogen stock. Place the tube in the 37°C water bath to thaw. Take care at this point and wear a face shield since the thawing tube is prone to shatter if compromised during storage.

(c) When the cell suspension has thawed, transfer 450 μL to each of the plates and incubate for at least 18 h at 37°C under 5% CO_2.
(d) The following day, wash the cells with 30 mL of prewarmed PBSA, then add 30 mL of prewarmed CCM.
(e) Proceed to expand as described in Protocol 9.2.

9.6. CHARACTERIZATION

Since cultures of MSCs possess a degree of heterogeneity, they are not easily defined by their repertoire of cell surface antigens. Because of this limitation, cultures of MSCs are best appraised by their performance in clonogenic assays and trilineage differentiation into osteoblasts, adipocytes, and chondrocytes. However, cell surface phenotypic analysis is useful to measure the degree of hematopoietic contamination.

9.6.1. Flow Cytometric Analysis of MSCs

Although MSCs are not easily defined on the basis of their cell surface antigens, flow cytometry can be useful for MSC characterization in some cases. For instance, simple assays of forward and side scatter can provide information on the proportion of smaller, rapidly self-renewing (RS)-type cells to slowly replicating (SR) cells in a population of MSCs (*see* Section 9.8.1). RS cells typically exhibit a far lower forward and side scatter profile compared with the larger, and more complex, SR cells. See, for example, [Sekiya et al., 2002b] and [Smith et al., 2004], where detailed protocols for this procedure are provided.

The main utility of flow cytometry in MSC characterization is for confirmation that the cultures are not contaminated by cells of hematopoietic origin. In accordance with the International Society for Cell Therapy, human MSC cultures should be negative for the pan-leukocyte marker CD45 (Beckman Coulter, clone IgG1 J.33), the endothelial and hematopoietic marker CD34 (Beckman Coulter, clone IgG1 581), the monocyte/macrophage markers CD14 (Beckman Coulter, clone IgG2a RMO52) and CD11b (Beckman Coulter, clone IgG1 Bear1), and the B cell markers CD79a (clone IgG1 HM47, requires fixation and permeabilization) and CD19 (clone IgG1 J4.119). The MSCs should not express HLA class II molecules (BD Biosciences, clone IgG2a TÜ36) unless stimulated to do so by exposure to interferon-γ. In contrast, MSCs are known to express CD105 (also known as BD Biosciences clone IgG1 AD2) and CD90 (also known as Thy1, Beckman Coulter clone IgG1 Thy1/310).

9.6.2. Clonogenic Assays of MSCs

One of the most important assets of MSCs is their ability to self-renew in culture. Although the proliferative capacity of MSCs can be evaluated by a number of means, including labeled nucleotide incorporation, hemocytometer counts, and flow cytometry, the most widely accepted method is an appraisal of CFU potential [Digirolamo et al., 1999]. In the CFU assay, the culture of MSCs is recovered by trypsinization and the cells are counted by hemocytometer. One hundred cells are then plated into a 15-cm tissue culture dish containing CCM and allowed to adhere and proliferate under normal

conditions of expansion. After 3 weeks, the CFU cultures are washed, fixed, and stained with Crystal Violet. The colonies are then counted and the plating efficiency determined. Although there are more complex variations on the CFU assay utilizing single-cell plating in a 96-well format [Smith et al., 2004], the standard CFU assay remains a consistently reliable measure of replication potential for MSC cultures.

Protocol 9.5. Colony-Forming Unit Assay of Self-Renewal of MSCs

Reagents and Materials

Sterile

- ❑ CCM (*see* Section 9.2.1)
- ❑ PBSA
- ❑ Trypan Blue solution in 0.85% saline
- ❑ Trypsin/EDTA (*see* Section 9.2.2)
- ❑ Polypropylene centrifuge tubes, 15 mL or 50 mL
- ❑ Plastic tissue culture Petri dishes, 15 cm
- ❑ Pipettor tips

Nonsterile

- ❑ Crystal Violet solution (*see* Section 9.2.3)
- ❑ Distilled water
- ❑ Improved Neubauer hemocytometer
- ❑ Pipettors, P10, P100, and P1000

Procedure

(a) Recover the MSCs and passage as directed by Protocol 9.2. Re-count the remaining cells in the suspension to ensure accuracy.

(b) Prepare a suspension of MSCs in CCM at a concentration of 1000 cells/mL.

(c) Prepare three 15-cm Petri dishes per measurement containing 25 mL of fresh, prewarmed CCM.

(d) Transfer 100 μL of the suspension (100 cells) to each of the three plates, rock the plate to disperse the cells, and incubate for 3 weeks at 37°C under 5% CO_2.

(e) After 3 weeks, aspirate the medium from the CFU cultures and wash the dishes 3 times with PBSA.

(f) Add 10 mL of Crystal Violet solution to each plate and incubate for 5–10 min at room temperature.

(g) Aspirate the stain, then wash with excess distilled water until the background is clear.

(h) Count the number of colonies for each dish, derive the mean, and calculate the plating efficiency or ''CFU potential'' (% CFU formed relative to inoculum). A good culture of MSCs typically has a CFU potential of over 40%.

9.6.3. Osteogenic Differentiation of MSCs

MSCs are defined by their potential to differentiate into mineralizing osteoblast-like cells in vitro. This is achieved by incubation of a confluent monolayer of MSCs in the presence of osteogenic medium for 10–21 days, after which time the mineralized monolayer can be evaluated by Alizarin Red S (ARS) staining, which permits visualization of calcified matrix (Fig. 9.4a; Plate 10A). For semiquantitative assays, the ARS can be extracted from

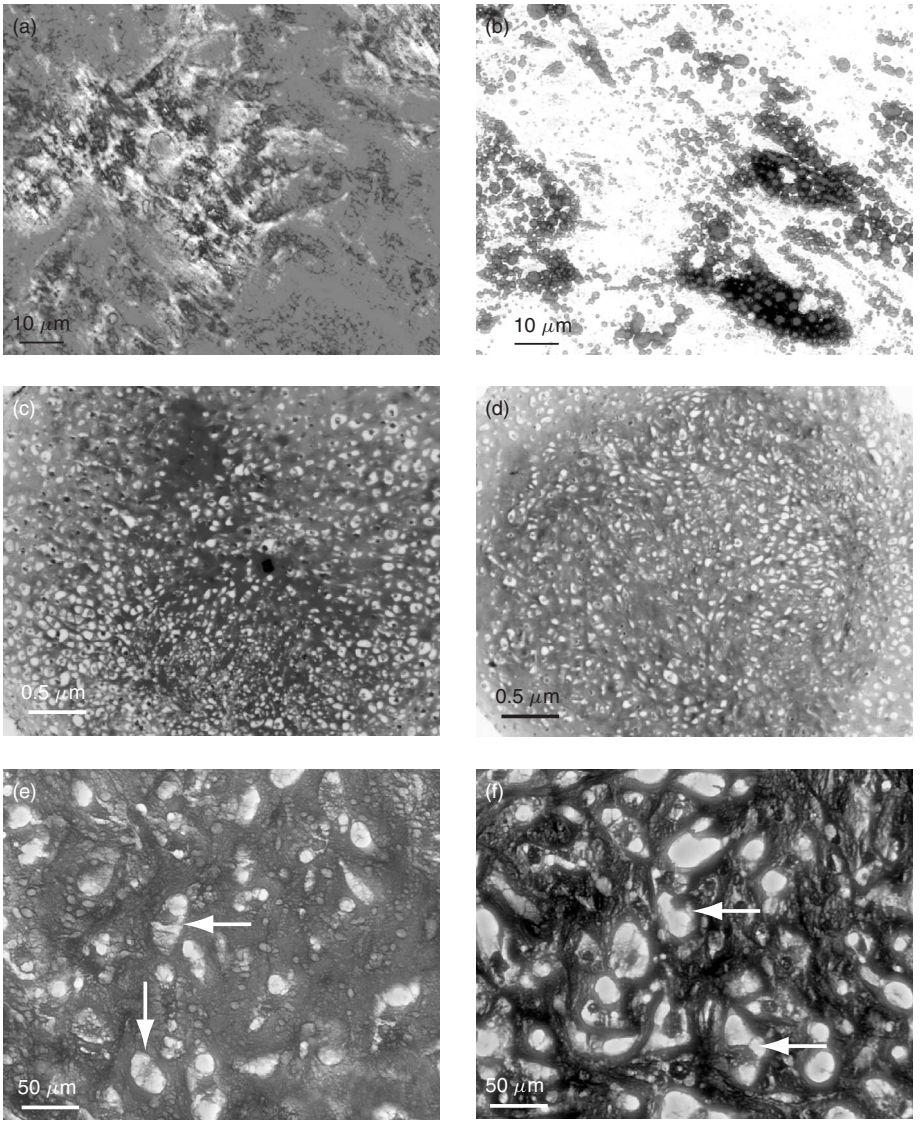

FIGURE 9.4. Results of differentiation assays. (**a**) Alizarin Red S-stained monolayer of osteogenic MSCs. (**b**) Oil Red O-stained monolayer of adipogenic MSCs. (**c–f**) Stained 10-μm sections of chondrogenic pellets: (**c, e**) Toluidine blue, (**d, f**) Safranin O. High-power images (**e, f**) show the morphology of the cartilage and the lacunae (arrowed) populated by chondrocytes. (*See also* Plate 10.)

the monolayer and measured spectrophotometrically [Gregory et al., 2004]. Other methods for the assessment of osteogenesis such as alkaline phosphatase measurements [Gunn et al., 2005] and direct calcium measurement [Gregory et al., 2004] are also described in the literature.

Protocol 9.6. Differentiation of MSCs into Mineralizing Osteoblasts

Reagents and Materials

Sterile

- ❑ CCM (*see* Section 9.2.1)
- ❑ BDM (*see* Section 9.2.4)
- ❑ PBSA
- ❑ Trypsin/EDTA (*see* Section 9.2.2)
- ❑ Polypropylene centrifuge tubes, 15 mL and 50 mL
- ❑ Tissue culture plates, 6 well, with 9.6-cm^2 wells

Nonsterile

- ❑ ARS (*see* Section 9.2.5)
- ❑ Buffered formalin, 10%
- ❑ Improved Neubauer hemocytometer

Procedure

(a) Recover MSCs from the monolayer as described in Protocol 9.2.

(b) Add 2 mL of CCM (for human and rat) containing 1×10^4 cells to each of the 6 wells of a 6-well plate. The final density will be approximately 1×10^3 cells/cm^2.

(c) Label the upper 3 wells ''osteogenic'' and the lower 3 wells ''negative''.

(d) Incubate at 37°C under 5% CO_2 with changes of medium every 2 days until the cells reach 70–80% confluence.

(e) On reaching the desired level of confluence, aspirate the complete medium from the wells and add 2 mL of BDM to the upper wells of the 6-well plate (labeled ''osteogenic'') and 2 mL of CCM to the lower wells (labeled ''negative'').

(f) Incubate at 37°C under 5% CO_2 with changes of medium every 2 days. For ARS assays, stain the cells at day 21:

  (i) Take the differentiated MSCs from the incubator and wash the monolayers twice with 2 mL per well of PBSA.

  (ii) Add 2 mL of formalin to each well and fix the monolayers at room temperature for 10 min.

  (iii) Aspirate the formalin and wash twice with 2 mL of PBSA per well, then once with 2 mL of distilled water per well.

  (iv) Add 2 mL of ARS solution and incubate at room temperature for 30 min.

  (v) Wash the wells with excess distilled water until the background staining on the ''negative'' wells is maximally cleared.

(g) Assess degree of intense red staining of the monolayers labeled ''osteogenic'' by microscopy. A satisfactory level of osteogenic differentiation results in over 50% of the surface area of the monolayer stained with ARS. At this point, the monolayers can be stored at 4°C for up to 7 days in water. Alternatively, the ARS on the stained monolayers can be extracted and measured by a previously described protocol [Gregory et al., 2004].

9.6.4. Adipogenic Differentiation of MSCs

Under the appropriate conditions, MSCs can be induced to form adipocytes that harbor fat droplets. The method for achieving adipogenic differentiation is similar to that used for osteogenesis, but the media composition is different. After 14–21 days of exposure to adipogenic medium fat droplets become faintly visible with phase-contrast microscopy, but on staining with the lipophilic dye Oil Red O (ORO) the droplets are clearly distinguishable from the surrounding cell bodies. As with ARS, the ORO dye can be extracted for semiquantitative assays and measured spectrophotometrically [Gregory et al., 2005c] (Fig. 9.4b; Plate 10B).

Protocol 9.7. Differentiation of MSCs into Adipocytes

Reagents and Materials

Sterile

- ❑ CCM (*see* Section 9.2.1)
- ❑ FDM (*see* Section 9.2.6)
- ❑ PBSA
- ❑ Polypropylene centrifuge tubes, 15 mL and 50 mL
- ❑ Tissue culture plates, 6 well with 9.6-cm^2 wells
- ❑ Trypsin/EDTA (*see* Section 9.2.2)

Nonsterile

- ❑ Trypan Blue solution in 0.85% saline
- ❑ Neutral buffered formalin
- ❑ ORO working solution (*see* Section 9.2.7)
- ❑ Improved Neubauer hemocytometer

Procedure

(a) Recover MSCs from the monolayer as described in Protocol 9.2.

(b) Add 2 mL of CCM containing 1×10^5 cells to each well of a 6-well plate. The final density will be approximately 1×10^3 cells/cm^2.

(c) Label the upper 3 wells ''adipogenic'' and the lower 3 wells ''negative''.

(d) Incubate at 37°C under 5% CO_2 with changes of medium every 2 days until the cells reach 70–80% confluence.

(e) On reaching the desired level of confluence, aspirate the complete medium from the wells and add 2 mL of FDM to the upper wells of the 6-well plate (labeled ''osteogenic'') and 2 mL of CCM to the lower wells (labeled ''negative'').

(f) Incubate at 37°C under 5% CO_2 with changes of medium every 2 days. For assays of ORO staining, stain the cells at day 21 as follows:

  (i) Take the differentiated MSCs from the incubator and wash the monolayers twice with 2 mL per well of PBSA.

  (ii) Add 2 mL of formalin to each well and fix the monolayers at room temperature for 10 min.

  (iii) Aspirate the formalin and wash twice with 2 mL of PBSA per well, then once with 2 mL of distilled water per well.

  (iv) Add 2 mL of ORO working solution and incubate at room temperature for 30 min.

  (v) Wash the wells with excess PBSA until the background staining on the ''negative'' wells is maximally cleared.

(g) Assess degree of red staining of the droplets labeled ''adipogenic'' by microscopy. A satisfactory level of adipogenic differentiation results in over 30% of the surface area of the monolayer stained with ORO. At this point, the monolayers can be stored at 4°C for up to 7 days in PBSA. Alternatively, the ORO on the stained monolayers can be extracted and measured by a previously described protocol [Gregory et al., 2005c]. An example is presented in Fig. 9.4b and Plate 10B.

9.6.5. Chondrogenic Differentiation of MSCs

(After Johnstone et al. [1998] and Sekiya et al. [2002a, 2005])

MSCs will form a cartilaginous pellet when exposed to chondrogenic medium in a simple micro-mass culture system. In this assay, 2×10^5 MSCs are suspended in 500 μL of defined chondrogenic medium and centrifuged into a pellet. After 2–3 days in the tissue culture incubator, the flattened pellet forms a roughly spherical construct. After 21 days, on histological inspection, the pellet consists of hyaline-like cartilage containing type II collagen and an abundance of sulfated proteoglycans. The MSCs are present in distinct lacunae. The chondrogenic medium can be supplemented with either bone morphogenic protein 2 or 6, for satisfactory chondrogenesis. Although some investigators report that BMP2 is more efficient than BMP6 in inducing chondrogenesis [Sekiya et al., 2005], the MSCs probably exhibit a preferential response to either BMP in a donor-dependent manner. With newly prepared MSC stocks, it is advisable to carry out chondrogenic trials with both BMP2 and BMP6.

Protocol 9.8. Differentiation of MSCs into Chondrocyte Constructs

Reagents and Materials

Sterile

- ❑ CCM (*see* Section 9.2.1)
- ❑ Stock solutions (100×) of BMP2 and -6 (*see* Section 9.2.8: Each culture will require 5 μL of the stock per medium change.

- ❑ Stock solution (100×) of TGF-β3 (*see* Section 9.2.9): Each culture will require 5 μL of the stock per medium change.
- ❑ CDM (*see* Section 9.2.10)
- ❑ Polypropylene centrifuge tubes, 15 mL and 50 mL
- ❑ PBSA
- ❑ Trypan Blue solution in 0.85% saline
- ❑ Trypsin/EDTA (*see* Section 9.2.2)

Nonsterile

- ❑ Toluidine Blue borate and Safranin O stains
- ❑ Improved Neubauer hemocytometer

Procedure

(a) Recover MSCs from the monolayer as described in Protocol 9.2. Suspend cells at a concentration of 4×10^5 cells/mL in prewarmed CDM and aliquot 500 μL of the suspension into the appropriate number of 15-mL polypropylene tubes.

(b) Add 5 μL of the BMP stock and 5 μL of the TGF-β3 stock to each of the tubes and swirl gently.

(c) Pellet the cells by centrifugation at 500 *g* for 10 min and incubate at 37°C under 5% CO_2 for 2–4 days, after which time the flattened pellet should become cohesive and adopt a roughly spherical shape.

(d) At this point, gently aspirate the medium from the pellet with a micropipettor and replace with CDM supplemented with BMP and TGF-β3. Replace the cultures in the incubator.

(e) Change the medium every 2–3 days for 21 days.

(f) After 21 days, wash the pellet in PBSA and fix with 10% buffered formalin.

(g) Embed the fixed pellets in paraffin, make 10-μm sections, and stain with Toluidine Blue borate and Safranin O according to standard histological procedures. The cartilage-bound lacunae should be clearly visible with both histological stains. Sulfated proteoglycans will appear purple when stained with Toluidine Blue borate and pinkish red with Safranin O. Examples are presented in Fig. 9.4, c–f and Plate 10, C–F.

9.7. ADDITIONAL PROTOCOLS FOR RECOVERY AND EXPANSION OF RAT MSCs

9.7.1. Recovery of Rat Bone Marrow

Rat MSCs are cultured in essentially the same manner as human MSCs with just a few exceptions. The main distinction between the protocols for expansion of human and rat MSCs is that rat MSCs are best passaged at a monolayer density of about 40–50% confluence whereas human cells can be passaged later at about 50–60% confluence. This is because the proliferation potential and multipotency of rat MSCs deteriorate significantly after excessive cell–cell contact in culture. For the production of rat MSCs,

the bone marrow is recovered from the long bones of freshly euthanized animals. The marrow can be extracted from the bone by flushing with growth medium.

Protocol 9.9. Recovery of Rat Bone Marrow for MSC Production

Reagents and Materials

Sterile

- ❑ CCM (*see* Section 9.2.1) supplemented with 0.2 μg/mL amphotericin B (fungizone)
- ❑ Gauze
- ❑ PBSA with 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.2 μg/mL amphotericin B
- ❑ Hanks' balanced salt solution without magnesium or calcium (HBSS)
- ❑ Plastic tissue culture Petri dishes, 15-cm diameter
- ❑ Surgical scalpels fitted with #10 and #15 blades
- ❑ Surgical scissors with 1- to 2-inch blades
- ❑ Forceps
- ❑ Hemostat
- ❑ Rongeur bone cutters with 15-cm blades
- ❑ Syringes with Luer lock, 20 mL
- ❑ Hypodermic needles, 23 gauge and 18 gauge
- ❑ Microcentrifuge tubes, 1.5 mL and 0.5 mL
- ❑ Polypropylene centrifuge tubes, 15 mL and 50 mL

Nonsterile

- ❑ Adult Lewis rats
- ❑ CO_2 rodent euthanasia chamber
- ❑ Eppendorf 5417C microcentrifuge or equivalent

Procedure

(a) Euthanize animal with CO_2 asphyxiation; do not use drugs.

(b) Swab the hindlimbs with ethanol and dissect out the hindlimbs without breaking the femurs. Detach the femurs from the rest of the limb.

(c) Cut away muscle and tendons from the femurs. Use sterile gauze to wipe remaining tissue from the bones.

(d) Swab the cleaned bones with ethanol and place them in a plastic 50-mL tube.

(e) Cover bones with ice cold CCM containing antimycotic until ready for marrow extraction. The time taken from dissection to bone marrow extraction should be kept to a minimum.

(f) Transfer tubes containing femurs to the microbiological safety cabinet and place bones in 15-cm Petri dish.

(g) Extract the bone marrow as follows:
  (i) Cut the top and bottom of the bones with the bone cutters, exposing the marrow inside.
  (ii) Flush each femur with 5 mL of CCM containing antimycotic, using a 10-mL syringe fitted with a 23 G needle into a 50-mL centrifuge tube.
  (iii) Recover the flushed CCM and pass through the bone three times before transferring to a 15-mL centrifuge tube.
  (iv) Centrifuge at 1000 *g* and resuspend the pelleted bone marrow in 1 mL of CCM containing antimycotic.
  (v) Transfer the 1 mL of suspended bone marrow into 4 mL of fresh PBSA containing antibiotics and antimycotic in a 15-mL centrifuge tube. The 5-mL cell suspensions from each femur can be combined into the same 15-mL tube.

(h) Centrifuge the bone marrow at 1000 *g* for 10 min at 10°C and wash the marrow three times in 10 mL of PBSA containing antibiotics and antimycotic.

(i) Finally, resuspend the bone marrow in 5 mL of CCM and place on ice until culture according to Protocol 9.10.

9.7.2. Culture of Rat Bone Marrow

Rodent bone marrow is usually directly suspended in medium for MSC recovery without any additional purification. Rat bone marrow can be cultured in the same medium used for human cells but initially containing antimycotic for the first few days of culture (*see* Protocol 9.10).

Protocol 9.10. Primary Culture of Rat Bone Marrow for MSC Production

Reagents and Materials

Sterile

- ❑ CCM (*see* Section 9.2.1) supplemented with 0.2 μg/mL amphotericin B
- ❑ Polypropylene centrifuge tubes, 15 mL or 50 mL
- ❑ PBSA
- ❑ Plastic tissue culture Petri dishes, 15 cm

Nonsterile

- ❑ Trypan Blue solution in 0.85% saline
- ❑ Eppendorf 5417C micro centrifuge or equivalent
- ❑ Improved Neubauer hemocytometer

Procedure

(a) Remove 10 μL of the resuspended marrow from one femur and mix with 10 μL of Trypan Blue (*see* Protocol 9.9). Assess viability and ensure that it is above 80%.

(b) Pellet the bone marrow by centrifugation at 1000 *g* for 10 min.
(c) Suspend the bone marrow in 25 mL of prewarmed CCM with antimycotic (antimycotic for rat marrow but not for human).
(d) Transfer the 25-mL cell suspension to a 15-cm-diameter tissue culture plate and incubate at 37°C under 5% CO_2 for at least 15 h.
(e) Take the plates from the incubator and remove the medium.
(f) Wash the monolayer with 20 mL of prewarmed PBSA. Repeat the wash 3 times, then replace the final wash with 30 mL of fresh prewarmed CCM with antimycotic (antimycotic for rats only).
(g) Repeat Step (f) every second day for 6–14 days as necessary.
(h) Every 3 days, examine the monolayers with an inverted microscope. Adherent, fibroblast-like colonies of MSCs should be clearly visible on the plate. In some cases, there may be signs of hematopoietic contamination, but these cells will be depleted on passaging of the cells (Fig. 9.2). When the culture is 40–50% confluent (*see* Fig. 9.3d), proceed to Section 9.5. These MSCs are designated *passage 0*.

9.8. TECHNICAL NOTES ON MSC EXPANSION

9.8.1. A Note on MSC Morphology

MSCs are extremely responsive to their environment and can rapidly deteriorate in culture if treated improperly. Also, in rare cases, MSCs deteriorate spontaneously in a donor-dependent manner. While in culture, the most convenient means of assessing the quality of a culture of MSCs is by a simple morphological inspection. Cells in a rapidly dividing, healthy culture of MSCs adopt a small, spindle-shaped morphology with frequent refractile doublets of newly dividing cells (Fig. 9.2b). These cells are referred to as RS or "rapidly self-renewing cells" (Fig. 9.3a) and are usually highly clonogenic with far better potential for differentiation into osteoblasts, adipose, and cartilage [Sekiya et al., 2002b] (*see also* Section 9.6.1). As cultures of MSCs deteriorate, through mistreatment, extensive passage, or high-density plating, or because of donor-dependent reasons, the cells adopt what is regarded an SR or "slowly replicating" morphology (Fig. 9.3b). These MSCs are generally larger and rhomboidal in shape and are accompanied by very few, if any, refractile doublets of newly replicated cells. These cells are frequently poorly clonogenic and fare badly in differentiation assays [Sekiya et al., 2002b].

9.8.2. A Note on MSC Culture Density

The MSCs should be cultured at low density to preserve the multipotential phenotype. Confluent cultures of MSCs frequently become SR cells and thus lose their clonogenicity and differentiation potential [Sekiya et al., 2002b; Gregory et al., 2005a,b]. Sustained confluence can actually induce spontaneous and irreversible differentiation into mineralizing cells in the absence of inductive media [Gregory et al., 2004, 2005b]. Human MSCs should be passaged at 50–60% confluence at a density of approximately 8×10^3 cells/cm^2. An example is presented in Fig. 9.3d. Rat MSCs grow faster and should be passaged earlier at less than 50% confluence.

9.9. SUMMARY AND CONCLUDING REMARKS

This chapter provides a collection of basic protocols and guidelines for the successful preparation, expansion, and in vitro characterization of MSCs from bone marrow. The MSCs generated by these protocols are inherently heterogeneous, with a proportion of smaller, spindle-like RS cells and a proportion of the larger, more slowly proliferating SR cells. As the MSCs are passaged in culture, the proportion of RS cells is reduced and the proliferative and multipotency of the culture decreases. The protocols described in this chapter are designed to preserve the proliferative potential and the multipotentiality of the MSCs for as long as possible through low-density culture and specifically selected tissue culture substrates. Although the SR cells do not perform well in differentiation assays; they may secrete essential growth factors for the sustenance of the culture. Indeed, the SR cells seem to be the most efficient in expressing proteins such as Wnts that are mitogenic for a number of tissues including hematopoietic stem cells [Austin et al., 1997; Gregory et al., 2003]. Therefore, it is likely that a successful culture of MSCs is comprised of both RS and SR cells, with the greatest majority of MSCs falling in the RS category.

9.10. SOURCES OF MATERIALS

Item	Supplier
Antibiotic solution 100× containing 10,000 units/mL penicillin G sodium, 10,000 μg/mL streptomycin sulfate in 0.85% saline	Invitrogen
Ascorbate-2-phosphate	Sigma
Automated pipette filler, Hoodmate 300	Drummond
B cell markers CD79a, clone IgG1 HM47 and CD19, clone IgG1 J4.119	Beckman Coulter
Bench top centrifuge	Eppendorf
Bone morphogenic proteins (BMP)2 and -6	R & D Systems
CD105 (also known as SH2 or endoglin), clone IgG3 IG2	Beckman Coulter
CD11b, clone IgG1 Bear1	Beckman Coulter
CD14, clone IgG2a RMO52	Beckman Coulter
CD19, clone IgG1 J4.119	Beckman Coulter
CD34 , endothelial and hematopoietic marker, clone IgG1 581	Beckman Coulter
CD45, pan-leukocyte marker, clone IgG1 J.33	Beckman Coulter
CD73 (also known as SH3 or -4), clone IgG1 AD2	Becton Dickinson
CD90 (also known as Thy1), clone IgG1 Thy1/310	Beckman Coulter
Cell Factory	Nunc
CO_2 incubator, water jacketed	Forma Scientific
Cryo−1°C freezing containers, 5100-0001	Nalgene
Dewar flask, 2000	Cryosystem
Dexamethasone	Sigma
Fetal bovine serum (FBS) hybridoma qualified and not heat inactivated	Atlanta Biologicals or Hyclone

Item	Supplier
Ficoll-Paque	Amersham Pharmacia Biotech
Filter units with 0.22-μm^2 filtration membrane	Millipore
HLA class II molecules, clone IgG2a TÜ36	BD Biosciences
Insulin-transferrin-selenium premix plus (ITS+)	BD Biosciences
Inverted microscope, Eclipse TS100	Nikon
Liquid nitrogen freezer	Cryosystem
Microbiological safety cabinet class IIA/B3	Forma Scientific
Microcentrifuge, 5417C	Eppendorf
Micropipettors	Eppendorf
Monocyte/macrophage markers CD14, clone IgG2a RMO52 and CD11b, clone IgG1 Bear1	Beckman Coulter
Multiwell plates	Corning
Petri dishes	Corning or Nunc
Pipettors	Eppendorf
Proline	Sigma
Refrigerated bench top centrifuge, 5810R	Eppendorf
Six-well plates	Corning
Sodium pyruvate	Sigma
Sterilizing filters	Millipore
Tissue culture plates, 6 well, with 9.6-cm^2 wells	Corning
Transforming growth factor-β3 (TGF-β3)	R & D Systems
Trypsin/EDTA	Invitrogen
Vacuum filter units with 0.22-μm^2 filtration membrane	Millipore
Water bath, Isotemp	Fisher Scientific

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CHAPTER 10

ISOLATION, CHARACTERIZATION, AND CULTURE OF SOFT CONNECTIVE TISSUE STEM/PROGENITOR CELLS

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Culture of Human Stem Cells, Edited by R.I. Freshney, G.N. Stacey and J.M. Auerbach

10.1. BACKGROUND

Our laboratory's main interests lie in the development of the early skeleton and, more recently, in developing joints and articular cartilage. We strive to apply knowledge that we gain from studying mechanisms of cartilage development to repair situations. Cartilage has a very poor repair potential due, in part, to its avascular nature and lack of innervation. Hence, when cartilage is injured and the trauma does not compromise the underlying subchondral bone, a normal inflammatory wound response does not ensue because of the lack of bleeding and the absence of infiltration by inflammatory elements. If, however, the subchondral bone is compromised, then there is bleeding through into the defect and a clot is formed, which eventually leads to the population of the defect by marrow stromal elements that give rise to a fibrocartilaginous repair tissue (*see* Chapter 9 for details on bone marrow stromal stem cells). In addition, the lack of innervation also affects the tissue since, if the damage is only to the cartilage, the patient may be unaware and may exacerbate existing damage through continued use in heavy load-bearing activities or perhaps in contact sports, thus leading to further and progressive tissue damage [*see* Redman et al., 2005 for review].

Currently, there are a few biological treatments of defects in articular cartilage. One treatment is microfracture, where small holes are drilled through the subchondral plate into the underlying marrow cavity [Beiser and Kanat, 1990]. This procedure allows bleeding into the defect to occur and facilitates both blood-borne and marrow mesenchymal stem cells (MSCs) to enter and contribute to a fibrocartilaginous repair tissue. Periosteal transplantation has also been used clinically with some encouraging results [Alfredson and Lorentzon, 1999]. Mosaicplasty involves taking osteochondral plugs from the peripheral regions of the joint and transplanting them into regions where the cartilage has been damaged or eroded [Hangody et al., 2004]. Finally, there is autologous chondrocyte implantation (ACI), where again, in cases of damage in load-bearing areas, cartilage is harvested from the joint periphery and the chondrocytes released and expanded in monolayer culture. The cells are then implanted back into the defect and held in place by a periosteal or collagenous flap [Brittberg, 1999]. However, because the cells lose both their phenotype and their ability to reexpress it in permissive environments as a function of the number of cell divisions they progress through (usually about 7 population doublings), the size of the defect that can be treated is limited. Thus a technique that can provide a much larger cell source would be of considerable benefit.

To this end, we were the first to isolate and characterize a progenitor cell population from immature bovine articular cartilage [Dowthwaite et al., 2004] and to demonstrate that it could be expanded up to 50 population doublings and still maintain a chondrogenic potential through maintenance of expression of Sox-9, one of the major transcription factors regulating chondrogenesis [Lefebvre et al., 2001]. We further demonstrated that these cells showed phenotypic plasticity within the connective tissue lineage by injecting them

into developing chick limbs before the overt differentiation of the endogenous tissues and analyzing them 7 days postimplantation, when the host tissue had differentiated. We found that the articular cartilage progenitor cells had engrafted into all the connective tissue types of the limb. Furthermore, we tested for functional engraftment by using a collagen type I antibody that was specific for bovine collagen but did not recognize chick collagen. We found that under fluorescence microscopy parallel arrays of collagen fibers within tendons and subperiosteal bone fluoresced brightly, indicating functional engraftment of these cells.

We then asked the question of whether a progenitor cell population resided in human cartilage and, if so, at what ages. Surprisingly, based on the criteria that are described below, we have been able to identify cells with progenitor-like properties from aged (>70 year old) articular cartilage and from cell clusters (chondrones) in osteoarthritic cartilage in addition to those expected from fetal cartilage.

Interestingly, in terms of potential clinical use in articular repair procedures, there has been an intensive quest for alternative tissue sources (due possibly to the only recent discovery of progenitors from the tissue itself) of cells with chondrogenic potential from a variety of other connective tissues. These have included periosteum [O'Driscoll and Fitzsimmons, 2001], perichondrium [Arai et al., 2002], bone marrow stromal cells [Vaananen, 2005], adipose tissue [Guilak et al., 2004], and dermal stem cells [Sorrell and Caplan, 2004], to name but a few, and they have been compared in an animal defect model [Hui et al., 2005]. More recently, umbilical cord blood has also been demonstrated to contain cohorts of cells with chondrogenic potential [Moise, 2005] (*see* Chapter 7). In terms of cartilage, it may be the case that virtually any stem cell isolated from connective tissues can be induced to become chondrogenic but the strength and stability of the phenotype vary. Even progenitor cells from articular cartilage vary in their chondrogenic capacity. Therefore, it appears that chondrocytes generated from stem/progenitor cells either from the same source or differing sources are not equivalent.

The techniques outlined in this chapter are all based on differential adhesion to fibronectin. It is common that many stem/progenitor cells have high affinity for fibronectin, and our initial isolation of these cells in articular cartilage was partly based on the specific expression of the extra domain A (EDA) splice variant of fibronectin in the surface of cartilage [Dowthwaite et al., 2004]. Admittedly, to date, we do not know what the relevance of this particular splice variant is to the progenitor cells. Nonetheless, since we are interested in the clinical application of these cells, it is a useful method of isolation for the following reasons. If we were to isolate cells based on cell surface-expressed molecules, we would require a fluorescence-activated cell sorter (FACS). However, none of these machines (to date) has been accredited for clinical use, and it seems unlikely that this will be the case for some time to come. While the use of purified ligands offers a better prospect, quality control still remains a problem. However, potentially, by employing recombinant molecules, such as those containing RGD sequences (the arginine-glycine-aspartic acid motif that acts as ligand for the integrin receptors), this offers the availability of highly purified ligands that can be used in the clinical application of these cells. Together with this aspect, other current efforts are being directed toward finding predictor markers of chondrogenic potential of individual clonal cell lines that are isolated from cartilage and other connective tissues types.

10.2. PREPARATION OF MEDIA AND REAGENTS

10.2.1. For Isolation of Chondroprogenitor Cells from Human Articular Cartilage

10.2.1.1. Digestion Media.

(i) Pronase digestion medium
DMEM/Ham's F-12 (1:1) with:

FBS	5%
Ascorbic acid	50 μg/mL
Glucose	1 mg/mL
Gentamicin (50 mg/mL)	0.2% (100 μg/mL final)
Pronase	70 U/mL
HEPES	10 mM

(ii) Collagenase digestion medium
DMEM/Ham's F-12 (1:1) supplemented as follows:

FBS	5%
Ascorbic acid	50 μg/mL
Glucose	1 mg/mL
Gentamicin (50 mg/mL)	0.2% (100 μg/mL final)
Collagenase, type I	300 U/mL
HEPES	10 mM

10.2.1.2. Differential Adhesion Assay Medium.

DMEM/Ham's F-12 (1:1) supplemented as follows:

Ascorbic acid	50 μg/mL
Glucose	1 mg/mL
Gentamicin (50 mg/mL)	0.2% (100 μg/mL final)

10.2.1.3. Growth Medium.

DMEM/Ham's F-12 (1:1) with

FBS	10%
Ascorbic acid	50 μg/mL
Glucose	1 mg/mL
Gentamicin (50 mg/mL)	0.2% (100 μg/mL final)

10.2.1.4. Differentiation Medium.

DMEM/Ham's F-12 (1:1) with

FBS	2%
HEPES	10 mM
ITS (insulin, transferrin, selenium; *see* Section 10.6)	1% (v/v)
TGF-β1	1 ng/mL

10.2.2. For Isolation of Chondroprogenitor Cells from Human Synovium

10.2.2.1. High-Collagenase Digestion Medium.

DMEM supplemented with:

FBS	5%
Collagenase	1500 U/mL
HEPES	10 mM
Antibiotic/antimycotic (10,000 U/mL penicillin, 10 mg/mL streptomycin, 25 µg/mL amphotericin B)	1% (v/v; 100 U/mL, 100 µg/mL, and 0.25 µg/mL, respectively, final concentrations)
Gentamicin (50 mg/mL)	0.2% (v/v; 100 µg/mL final)

10.2.2.2. Expansion Medium.

DMEM supplemented with:

FBS	20%
bFGF	5 ng/mL
TGF-β1	2.5 ng/mL
Gentamicin, 50 mg/mL	0.2% (100 µg/mL final)

10.2.2.3. Differentiation Medium.

DMEM supplemented with:

ITS (insulin, transferrin, selenium; *see* Section 10.6)	1% (v/v)
TGF-β1	15 ng/mL
HEPES, 1 M	1% (10 mM final)
Gentamicin, 50 mg/mL	0.2% (100 µg/mL final)

10.2.2.4. Phosphate-Buffered Saline.

(i) PBSC: Dulbecco's phosphate-buffered saline complete with 1 mM $CaCl_2$, 1 mM $MgCl_2$

(ii) PBSA: Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+}

10.3. ISOLATION OF CHONDROPROGENITOR CELLS FROM NORMAL AND OSTEOARTHRITIC HUMAN ARTICULAR CARTILAGE

This isolation procedure involves what is termed a differential adhesion assay (Fig. 10.1). The assay is based on the cells' differing affinities for a particular extracellular ligand that is coated onto the Petri-dish. For articular cartilage, the ligand used is fibronectin. Several plates are coated with the ligand while control plates are coated with a nonadhesive ligand such as bovine serum albumin (BSA). The cells are plated at low density onto the ligands for a specified short period of time. Those cells with high affinity for the ligand will adhere first, whereupon the nonadherent cells are aspirated into a second dish for a similar period of time. Again, nonadherent cells are aspirated and placed into a third dish, where they remain (Fig. 10.2). The cultures are then maintained until such a time that colonies can be distinguished and isolated. If known to have arisen from one cell, these can be confirmed as clones (Fig. 10.3).

Protocol 10.1. Isolation of Chondroprogenitor Cells from Human Articular Cartilage

Reagents and Materials

Sterile or aseptically prepared

- ❑ Articular cartilage from any synovial joint excluding the temporomandibular and clavicular joints as they are fibrocartilaginous. The age range is largely immaterial; we have obtained clonable populations from cartilage of an 83-year-old patient. At present, normal cartilage is difficult to obtain so we often use cartilage from

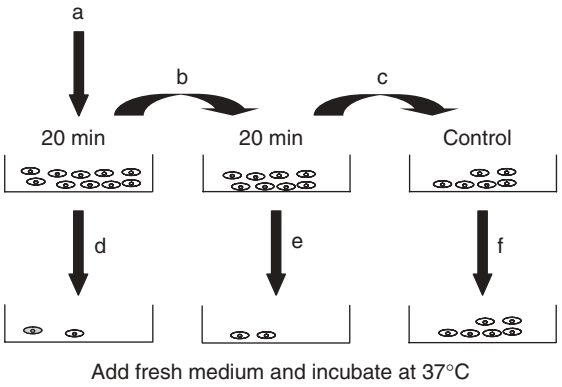

FIGURE 10.1. Differential adhesion to fibronectin. Isolated cells (typically 4000 cells per 3.5-cm dish) are plated onto fibronectin-coated dishes (10 μg/mL) for 20 min (**a**), nonadherent cells are removed to a second fibronectin-coated dish (**b**) and incubated for a further 20 min, and nonadherent cells removed to another fibronectin-coated dish (**c**). Remaining cells are covered with culture medium and incubated at 37°C for initial adhesion and colony-forming counts to be performed. Note that with chondrocytes, colonies (gray in **d**) will only form in cells adhered to fibronectin for the first 20 min.

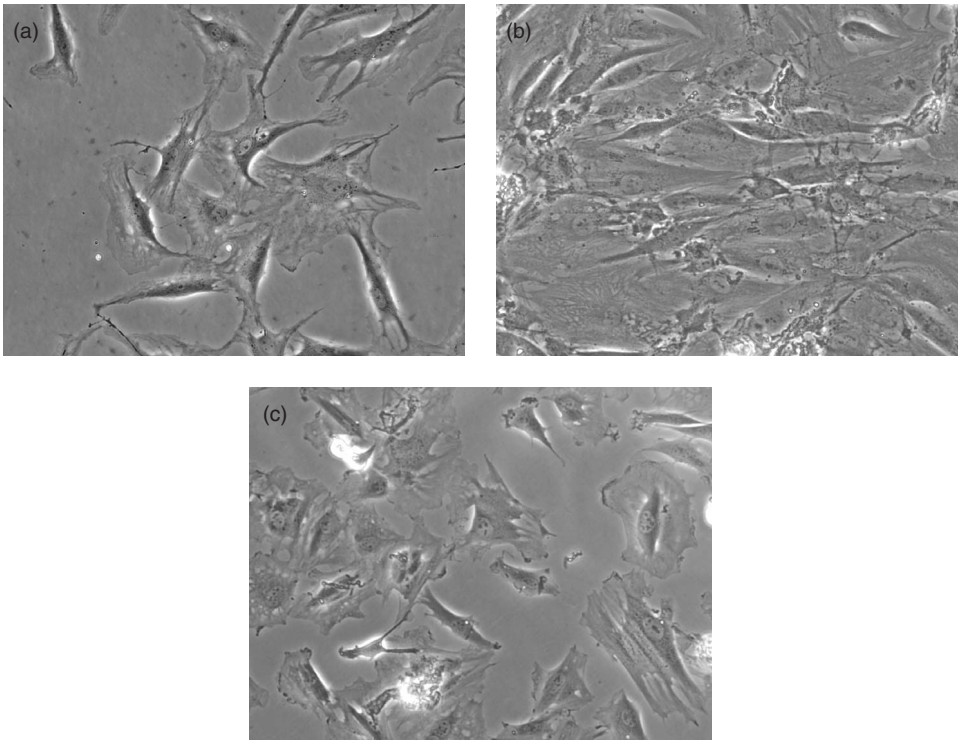

FIGURE 10.2. Normal human chondrocytes were isolated by sequential pronase and collagenase digestion and subjected to differential adhesion to fibronectin. Colonies consisting of more than 32 cells (**a**) were selected and extensively subcultured. Early-passage (**b**, 12 population doublings) and late-passage (**c**, >30 population doublings) cells are illustrated.

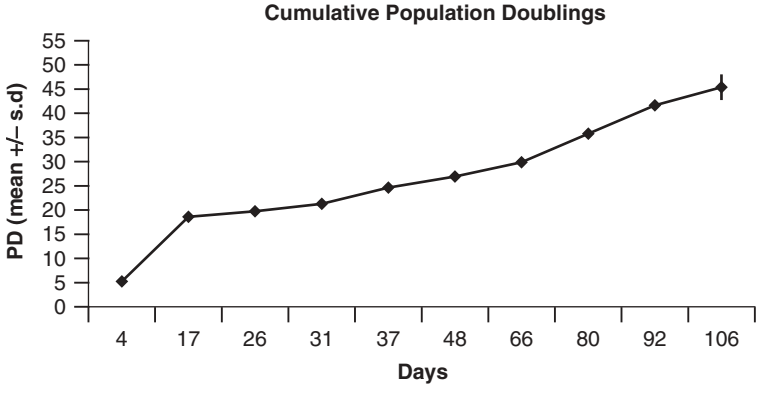

FIGURE 10.3. Graph representing cumulative population doublings of a single clonal cell line isolated from a patient 67 years of age.

the noninvolved compartment of patients undergoing a hemiarthrotomy of the knee for osteoarthritis. Although the cartilage is derived from a diseased joint, frequently the noninvolved condyle is macroscopically and histologically normal in appearance.

- ❑ Pronase digestion medium (*see* Section 10.2.1.1)
- ❑ Collagenase digestion medium (*see* Section 10.2.1.1)
- ❑ Trypsin/EDTA: trypsin (0.05%) with EDTA (0.53 mM) in PBSA
- ❑ PBSC (*see* Section 10.2.2.4)
- ❑ PBSA (*see* Section 10.2.2.4)
- ❑ Fetal bovine serum
- ❑ Bovine serum albumin (BSA), 10 μg/mL and 1 mg/mL in PBSC
- ❑ Serum fibronectin, 10 μg/mL in PBSC
- ❑ Nylon filter, 40-μm mesh (''Cell Strainer'')
- ❑ Universal containers, 30 mL, or centrifuge tubes, 50 mL, with conical bases for centrifuging cells
- ❑ Scalpel blades, no. 10
- ❑ Scalpel handle, no. 3
- ❑ Forceps, 2 pairs (1 large, 1 small)
- ❑ Petroleum jelly (Vaseline)
- ❑ Cloning rings, 6 mm

Nonsterile

- ❑ Standard bench-top centrifuge
- ❑ Microcentrifuge
- ❑ Chain mail glove for dissection of cartilage
- ❑ Felt pens

Procedure

A. Tissue collection and cell isolation

(a) Under aseptic conditions, carefully dissect the cartilage from the underlying subchondral bone with a no. 10 scalpel blade (see Note 1 below this protocol). Place cartilage slices in 9-cm Petri dish containing PBSA.

(b) Dice cartilage finely with forceps and scalpel (blade size of choice) into 1-mm^3 pieces.

(c) Transfer cartilage pieces to a Universal container or centrifuge tube in 18 ml of pronase digestion medium.

(d) Place on roller-mixer at 37°C for 1 h.

(e) Remove digestion mixture and discard.

(f) Add 18 ml of collagenase digestion medium and replace on roller-mixer at 37°C for 3 h.

(g) Filter through 40-μm cell filter.

(h) Count cells on a hemocytometer.

(i) Wash in adhesion assay medium by centrifugation for 10 min at 620 *g* and resuspend in adhesion medium at 2000 cells/mL (4000 cells).

B. Differential adhesion assay (see Fig. 10.1)

(a) Coat 3.5-cm Petri dishes with 10 μg/mL fibronectin overnight at 4°C. As negative controls, use 10 μg/mL BSA.
(b) Aspirate liquid and block dishes with PBSC containing 1 mg/mL BSA for 30 min.
(c) Plate 2 mL of medium containing cells for 20 min.
(d) Remove medium and nonadherent cells and place in second dish for 20 min.
(e) Put 2 mL of growth medium in first dish and place in incubator.
(f) Remove medium and nonadherent cells from second dish and transfer to third dish.
(g) Put 2 mL of growth medium in second dish and place in incubator.
(h) Add 200 μL of FBS into last dish and place in incubator.
(i) After 3 h, count initial adhesion of cells with phase-contrast microscope.
(j) Culture cells until colonies of over 32 cells (*see* Note 2) are apparent, which usually occurs by 10 days.
(k) A colony-forming efficiency (CFE) can be calculated by the formula:

$$\text{CFE} = \frac{\text{colonies at } X \text{ days}}{\text{Initial number of adherent cells at day 0}}$$

Clonal expansion

(a) Identify cell colonies of over 32 cells with phase microscopy and, if possible, circle them with a felt marker under the Petri dish (*see* Note 3).
(b) Count and record the number of cells for each colony (*see* Note 4).
(c) Aspirate medium from the Petri dish and wash in PBSA.
(d) Smear petroleum jelly under the cloning ring aseptically with a scalpel or dip the ring into the jelly directly with sterile forceps.
(e) Place the cloning ring over the colony (*see* Note 5), add the trypsin/EDTA solution into the ring and leave for 5–10 min — check under phase-contrast microscope that the cells have detached.
(f) Resuspend the cells, place in an Eppendorf tube, and centrifuge at 300 *g* for 5 min (*see* Note 6).
(g) Wash in growth medium and centrifuge again as above.
(h) Dilute in growth medium and plate out in 12-well plates at 1 colony per well.
(i) When confluent, transfer to 3.5-cm Petri dishes (or 6-well plates) and from there to larger culture flasks such as 25 cm^2, 75 cm^2, and finally, 175 cm^2.

Notes

(1) If you are dissecting a small piece of cartilage or a single condyle, grip it with large forceps to hold it steady during dissection. If, however, you are dissecting a whole epiphysis, use the chain mail glove (washed in ethanol) to hold the joint. Cartilage can be very slippery, and since condyles are curved it is very easy to

cut yourself! Use sterile PBSA to keep the cartilage moist by pipetting over the surface.

(2) By choosing colonies of greater than 32 cells, the intention is to avoid selecting cells that form the transit amplifying cohort of cells (the immediate progeny of the progenitor cells) that are normally capable of undergoing 5 population doublings (PDs).

(3) There are a variety of ways to mark colonies that are both operator dependent and microscope dependent, for example, objective magnification. Some people place the cloning ring directly on the colony, using a dissecting microscope within the laminar flow hood. A ring marker, which fits on the objective nosepiece of an inverted microscope, is available from Nikon.

(4) Counting the cells in each colony is important when you require accurate assessments of the number of population doublings the cells have progressed through.

(5) Make sure there is enough space between colonies for the ring so as to avoid contamination; otherwise, use smaller rings if available.

(6) If using cells from small colonies (35–50 cells), the Eppendorf washing stage can be omitted and the cells with the trypsin/EDTA transferred directly into 12-well plates containing a minimum of 2 mL of growth medium, which is enough to neutralize the trypsin.

(7) As with any extracellular matrix, that in cartilage becomes increasingly cross-linked with age. Consequently, the digestion time will vary with age of the donor, and tissue from younger donors will require less time for full digestion. It should be remembered that the minimum digestion time for cell release should be striven for in order to maximize cell viability. Dissolution of the tissue can also be expedited by sharp agitation at hourly intervals when in collagenase. As a guide, use a minimum of 7 mL of digestion medium per gram of cartilage weight, although excess volume also works but is wasteful of reagents. In the case of very young donors, refer to the fetal protocol that follows.

(8) When the clonal cell lines have been transferred to 6-well plates or 3.5-cm Petri dishes, and if the culture is growing well, that is, is confluent within a few days, then further passaging can involve splitting at a 1:2 dilution rate. Greater dilutions (1:4 or 1:8) can be made with more mature cultures.

(9) It is important when using collagenases that you work in units of activity since there can be variations of some threefold between batches. Batch testing is also advised.

10.4. ISOLATION OF CHONDROPROGENITOR CELLS FROM FETAL HUMAN CARTILAGE

This technique is a variation of the one above but takes due cognizance of the immature status of the tissue, which will be largely resorbed during development, is only lightly load-bearing, and has a poorly cross-linked matrix.

Tissue source: We usually use cartilage from the developing appendicular skeleton of fetuses between 9 and 12 weeks of gestation. The soft tissues should be dissected away with sharp sterile forceps under a dissecting microscope and the cartilaginous anlagen

placed in a Petri dish with PBSA before the top half of the epiphyses are removed to a fresh Petri dish and finely diced with a scalpel.

The protocol is essentially the same as for adult tissue, but the tissue is sometimes fully digested after only an hour in collagenase. Alternatively, the pronase and collagenase concentrations can be reduced to a third (i.e., 23 U/mL pronase and 100 U/mL collagenase) and then normal digestion times followed as for adult cartilage.

10.4.1. Progenitor Markers

Like many other tissues, as yet there is no one single marker for the progenitor cells found in articular cartilage. Instead, we are reliant on a combination of markers that are membrane bound. These include CD105 (endoglin), CD157 (BST1), CD166 (ALCAM), Stro-1, and Notch-1.

10.4.2. Differentiation

For differentiation of these progenitor cells into chondrocytes, they must be moved to culture conditions that allow the cells to assume a rounded configuration, as this will promote chondrogenesis. As mentioned above, there is an integral relationship between cell shape and chondrogenesis that involves the status of the actin cytoskeleton [Benya et al., 1988].

Protocol 10.2. Differentiation of Human Chondroprogenitor Cells

Reagents and Materials

Sterile

- ❑ Differentiation medium (*see* Section 10.2.1.4)
- ❑ PBSA (*see* Section 10.2.2.4)
- ❑ Trypsin/EDTA: trypsin (0.05%) with EDTA (0.53 mM) in PBSA
- ❑ Centrifuge tubes, 15 mL
- ❑ Universal containers (30 mL) or centrifuge tubes (50 mL) with conical base

Nonsterile

- ❑ Hemocytometer
- ❑ Microfuge

Procedure

(a) Aspirate growth medium from flasks and discard.
(b) Wash in PBSA and discard wash.
(c) Add enough trypsin/EDTA to cover the base of the flask.
(d) Incubate for 5–10 min at 37°C (check under microscope to see if cells are detached).
(e) Resuspend cells, place in a 15-mL centrifuge tube, and centrifuge for 5 min at 300 *g*.

(f) Wash in 1 mL of growth medium and transfer to a Universal container or centrifuge tube.
(g) Count cells on a hemocytometer.
(h) Centrifuge again (620 *g* for 10 min) and discard the supernate.
(i) Dilute in differentiation medium to 1×10^6 cells/mL.
(j) Aliquot 1 mL into new Eppendorf tubes.
(k) Centrifuge the tubes at 300*g* for 5 min and leave the supernatant medium in place.
(l) Place in 37°C incubator
(m) Change medium every 2–3 days (*see* Note 1 below).
(n) Culture for between 2 and 3 weeks to obtain chondrogenesis (*see* Note 2).

Notes

(1) Great care should be taken when changing the medium for the first couple of changes, as it is very easy to disperse the pellets at these stages when there is little matrix elaborated to hold the pellets together.
(2) Although we have concentrated here on chondrogenesis, appropriate media can be used to induce other phenotypes. With bovine cartilage progenitor cells, we have been successful in obtaining osteogenic, adipogenic, and neurogenic phenotypes either as pellet cultures or as monolayers.

10.5. ISOLATION OF PROGENITOR CELLS FROM HUMAN SYNOVIUM

The synovium surrounds the joint cavity, which contains synovial fluid. One of the functions of the synovium is to contain and manufacture synovial fluid components such as hyaluronan and lubricin. The synovial membrane has two layers. The outer subintima can be of almost any type: fibrous, fatty, or loosely "areolar." The intimal cells are of two types, fibroblasts and macrophages, both of which are different in certain respects from equivalent cells in other tissues.

This procedure uses an abbreviated form of differential adhesion assay to select the stem/progenitor cell population.

Protocol 10.3. Isolation, Culture, and Differentiation of Chondroprogenitor Cells from Human Synovium

Sterile or aseptically prepared

- ❑ Tissue source: Tibial synovium from patients undergoing total joint replacement for osteoarthritis or from fresh cadaveric material if normal tissue is required
- ❑ DMEM (containing glucose at 4.5 g/L and L-glutamine, 4 mM)
- ❑ High-collagenase digestion medium (*see* Section 10.2.2.1)
- ❑ Adhesion medium: DMEM with gentamicin, 100 μg/mL

- ❑ Colony growth medium: DMEM, 10% FBS and gentamicin, 100 μg/mL
- ❑ Expansion medium (*see* Section 10.2.2.2)
- ❑ Differentiation medium (*see* Section 10.2.2.3)
- ❑ PBSC (*see* Section 10.2.2.4)
- ❑ Fetal bovine serum
- ❑ Bovine serum albumin (BSA), 10 μg/mL in PBSC
- ❑ Serum fibronectin, 1 mg/mL
- ❑ Nitex cell filter, 40-μm mesh
- ❑ Scalpel blades, no. 10 and 23
- ❑ Scalpel handles, no. 3 and 4
- ❑ Forceps, 2 pairs
- ❑ Universal containers or centrifuge tubes, 50 mL
- ❑ Petroleum jelly (Vaseline)
- ❑ Cloning rings, 6 mm

Nonsterile

- ❑ Chain mail glove for protection when removing synovium from periphery of joint

Procedure

A. Tissue collection and cell isolation

(a) Under aseptic conditions, carefully trim the synovium from the periphery of the joint, holding the plateau with a chain mail gloved hand.

(b) Transfer synovium to a Petri dish containing PBSC. Remove fatty tissue with the aid of forceps under a dissecting microscope *(see* Note 1 below).

(c) Transfer cleaned synovium to a fresh Petri dish containing PBSC *(see* Note 2).

(d) Dice tissue with scalpel and forceps and transfer to the Universal with 10 mL of digestion medium *(see* Note 3). Leave overnight on a roller-mixer at 37°C.

(e) Wash twice in adhesion medium by centrifugation for 10 min at 300*g*. Resuspend in adhesion medium at 4000 cells in 2 mL.

(f) Filter through 40-μm Nitex cell filter.

B. Differential adhesion assay (see Fig. 10.1)

(a) Coat 3.5-cm Petri dishes (or a 12-well plate) *(see* Note 4) with fibronectin overnight at 4°C. As negative controls, use 10 μg/mL BSA.

(b) Pipette 2 mL of cell suspension onto the coated dishes and leave for 20 min in a 5% CO_2 incubator.

(c) Aspirate nonadherent cells and discard.

(d) Add 2 mL of growth medium and culture for 10 days; feed at day 5.

C. Cloning and cell expansion (see Note 5)

(a) Colonies (>32 cells) are cloned as described for cartilage progenitor cells (*see* Protocol 10.1, C).

(b) Transfer cells to 12-well plates at 1 colony/well in expansion medium containing bFGF and TGF-β1.

(c) Expansion is then continued from a 12-well plate to a 6-well plate through 25-cm^2 and up to 75-cm^2 flasks as in Protocol 10.1 Procedure C.

D. Differentiation

The procedure for inducing differentiation is essentially the same as for chondroprogenitor cells described above (*see* Protocol 10.2). However, note that the medium is different (*see* Section 10.2.2.3). In our hands, by 14 days the immature isoform of collagen type II (IIa) is present in the matrix. Longer culture periods are required for the mature (type IIb) collagen to be expressed.

Notes

(1) If there is a lot of adherent fat on the tissue, it may be easier to use a no. 23 blade and a no. 3 handle.

(2) Hanks' balanced salt solution (HBSS) can be substituted for PBSC.

(3) Larger tissue quantities can be accommodated in a 50-mL tube with 30 mL of digestion medium.

(4) If using plates for the differential adhesion assay, colonies can be located by placing a dot with a felt tip pen on the plate cover centered within the light beam when viewing the colony. This can then be aligned with a second dot placed on the base. Alternatively, a Nikon ring marker could be used.

(5) If the clonal cell lines are expanding slowly, it may be useful to add 5 ng/mL PDGF to the medium.

10.6. SOURCES OF MATERIALS

Item	Catalog No.	Supplier
Ascorbic acid	A4034	Sigma Aldrich
Antibiotic/antimycotic	15240-062	Invitrogen
bFGF	F0291	Sigma Aldrich
BSA	A3059	Sigma Aldrich
Cell strainer, 40 μm	352340	BD Biosciences
Centrifuge tubes, 50 mL	430828	Corning
Chain mail gloves		BRB Industrial Services
Cloning rings	C7983	Sigma Aldrich
Collagenase, type I	C0130	Sigma Aldrich
DMEM	41965-062	Invitrogen
DMEM/Ham's F-12	21331-046	Invitrogen
Dishes, 3.5 cm	430165	Corning
Fetal bovine serum (FBS)	10106-169	Invitrogen
Fibronectin	F1141	Sigma Aldrich

Item	Catalog No.	Supplier
Filter, 40 μm (*see* Cell strainer)		
Flasks, 25 cm^2	3056	Corning
Flasks, 75 cm^2	3376	Corning
Flasks, 175 cm^2	431079	Corning
Gentamicin	15750-045	Invitrogen
Glucose	G6152	Sigma Aldrich
HEPES	15630-056	Invitrogen
ITS	41400-045	Invitrogen
Phosphate-buffered saline, without Ca^{2+} and Mg^{2+} tablets (PBSA)	P4417	Sigma Aldrich
Plates, 6 well	3516	Corning
Plates, 12 well	3513	Corning
Pronase	1459643	Roche
TGF-β1	100B	R&D systems
Trypsin/EDTA	25300-062	Invitrogen
Universal containers, 30 mL	128B	Sterilin

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CHAPTER 11

CULTURE OF HUMAN CORNEAL STEM CELLS

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Culture of Human Stem Cells, Edited by R.I. Freshney, G.N. Stacey and J.M. Auerbach

11.1. GENERAL INTRODUCTION

11.1.1. Aim of the Chapter

The cornea is a relatively simple organ, composed of three distinct tissue layers. One of these tissues is a self-renewing epithelium long believed to harbor a resident stem cell population. The other two corneal tissues are largely quiescent after infancy, and until recently they were not considered to undergo self-renewal or maintain resident stem cells. Over the last decade these views have changed. The location and characteristics of the corneal epithelial stem cells have now been described by a number of research groups, and populations of these cells have been expanded and used therapeutically. In addition, cell populations with characteristics of adult stem cells have been isolated and characterized from the stoma and endothelium of corneas. This chapter describes the methods used in identification, isolation, and culture of these three populations of cells. We also review data that speak to the stem cell character of these cells and their potential for use in therapeutic and tissue engineering applications.

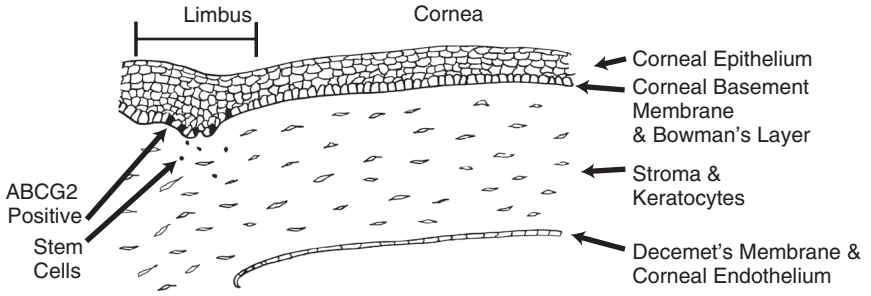

FIGURE 11.1. Diagrammatic section through cornea.

11.1.2. Structure and Cells of the Cornea

The cornea is the window of the visual system. As the outermost layer of the eye, the cornea both serves as a barrier and provides the essential optical function of transmitting light to the retina. In addition to the transmission of light, the cornea provides 70–75% of the refractive power required to focus the light into an image [Zieske, 2004]. The cornea is made up of three unique differentiated cell types separated by basal laminae (Fig. 11.1). The outermost layer consists of a stratified squamous, nonkeratinized epithelium. This tissue is supported by a basement membrane overlying an acellular zone of connective tissue known as the Bowman layer. The stroma, a collagenous connective tissue, makes up 90% of the cornea. It is populated with keratocytes, neural crest-derived mesenchymal cells that secrete the unique transparent tissue of the corneal stroma. The most posterior boundary of the cornea is the endothelium, comprised of a single layer of flattened cuboidal cells that maintain corneal transparency by regulating corneal hydration. Separating the corneal endothelium and the stroma is a basal lamina known as the Descemet membrane.

Formation of the human cornea begins at approximately 5 to 6 weeks of gestation [Zinn and Mockel-Pohl, 1975]. After the lens vesicle separates from the surface ectoderm, the

latter forms a layer of cuboidal epithelial cells, which develop into the corneal epithelium. Neural crest cells migrate between this epithelium and the lens, forming the corneal endothelium. A second wave of migration of cells from neural crest subsequently forms the stroma [Johnston et al., 1979; Wulle, 1972].

The three cellular layers of the cornea differ markedly in mitotic and self-renewal abilities. In the corneal epithelium, mitotically active basal cells continuously renew the nonmitotic population of suprabasal cells, which subsequently flatten as they migrate to the surface, where they are lost by desquamation. The stromal keratocytes, on the other hand, show little cell division in the normal adult. They undergo rapid cell division after localization in the cornea in late embryogenesis, but after birth the keratocyte cell number stabilizes and little or no mitosis can be detected throughout the lifetime. In the case of inflammation or wounding, however, the stromal keratocytes become activated and mitotic. The phenotype of the activated keratocytes changes to resemble that of fibroblasts and myofibroblasts, and connective tissue matrix secreted by these cells during wound-healing becomes opaque scars. After healing the cells become quiescent, but human corneal scars are very slow to resolve and it is not clear whether the resident cells return to a fully keratocytic phenotype. These properties suggest only a limited means of tissue renewal in the corneal stroma.

Renewal of corneal endothelial cells is even more limited than that of the keratocytes. After childhood, human corneal endothelial cells do not divide. Compensation for endothelial damage is accomplished by flattening of the remaining cells to cover the posterior surface of the cornea. In vitro, human corneal endothelial cells show only limited ability to divide after infancy [Engelmann et al., 1988, 2004; Ide et al., 2006; Joyce, 2003; Joyce and Zhu, 2004; Konomi et al., 2005; Sumide et al., 2006; Wilson et al., 1995; Yokoo et al., 2005; Zhu and Joyce, 2004].

These characteristics have led to the conventional view that the corneal epithelium is maintained by a stem cell population, but that the stroma and the endothelium, with limited ability for self renewal, are not products of tissue-resident stem cells.

11.1.3. Evidence for Stem Cells in the Cornea

Stem cells, by definition, undergo asymmetric cell division, that is, they undergo self-renewal while giving rise to differentiated daughter cells. Embryonic stem cells derived from the inner cell mass of the blastocyst are pluripotent, giving rise to most cells of the body. In culture, embryonic stem cells can be propagated indefinitely in an undifferentiated state. Historically, self-renewing adult tissues such as dermis have been thought to be generated by tissue-resident stem cells capable of generating only one type of cell, making them unipotent. The corneal epithelium is such a tissue, a rapidly regenerating stratified squamous layer covering the external surface of the cornea. The epithelium contains 5 layers of cells in the center and 10–11 layers in the transition zone between cornea and conjunctiva known as the limbus (Fig. 11.1). Although the anatomic structure termed the limbal palisades of Vogt is implicated as the site responsible for corneal epithelial self-renewal [Davanger and Evensen, 1971; Goldberg and Bron, 1982], the exact location of the self-renewing progenitor cells remained obscure until Schermer and co-workers [Schermer et al., 1986] demonstrated that corneal epithelial stem cells reside in the limbal basal epithelium. This location was determined by analysis of the expression pattern of a major corneal epithelial keratin, K3, which is absent in a small population of these basal limbal epithelial cells. Injury or disease destroying the basal

limbal cells causes a loss of corneal epithelium and its replacement with conjunctival epithelium, resulting in loss of corneal transparency. The best current marker for stem cells is a long-term retention of DNA-labeling by [^{3}H]thymidine or bromodeoxyuridine. This property is indicative of a slow-cycling population of cells and distinguishes the stem cells from the more rapidly cycling basal "transient amplifying" daughter cells, which are found centrally as well as in the limbus. Despite the strong evidence for the existence of limbal stem cells, methods have not been yet developed for isolation and culture of pure populations of these cells. Explants of limbal tissue containing presumptive stem cells can be expanded in culture and have been used in experimental animal models of limbal stem cell deficiency and clinically in patients with limbal stem cell deficiency to restore healthy corneal epithelial function. Such stem cell grafts are most successful with autologous tissue obtained from the contralateral eye of the same patient. Allografts usually fail without continuous antirejection therapy [Espana et al., 2004].

Over the past 10 years it has become apparent that stem cells in adult tissues are not restricted to self-renewing epithelia or hematopoietic tissues. Cells with properties of stem cells, including multipotential differentiation capability and extended life span, have been isolated from a number of adult mesenchymal tissues [Verfaillie, 2002]. The corneal stroma is a mesenchymal connective tissue making up 90% of the corneal thickness, with physical properties that provide the cornea its essential character. During development the stroma is produced by mesenchymal neural crest cells as they differentiate into keratocytes and begin to synthesize and secrete an extracellular matrix composed of collagens I, V, and VI and proteoglycans [Funderburgh, 2000; Funderburgh et al., 1986; Hart, 1976; Hay et al., 1979; Linsenmayer et al., 1986, 1998]. As maturation proceeds, the stroma dehydrates, becomes thin and transparent, and contains flattened and interconnected keratocytes [Jester et al., 1994]. In late embryogenesis, chicken keratocytes appear to retain neural crest progenitor properties after transplantation into a new environment along cranial neural crest migratory passageways [Lwigale et al., 2005]. In adult mammals, however, numerous in vitro experiments show that keratocytes rapidly lose their characteristic phenotype after several population doublings. Such a loss of phenotype occurs in healing wounds in vivo as well. Recently, the authors found that the stroma of bovine and human corneas contain small populations of cells exhibiting self-renewal ability for an extended number of population doublings in culture [Du et al., 2005; Funderburgh et al., 2005]. These stromal progenitor cells demonstrated potential for differentiation into several noncorneal cell types [Du et al., 2005], a characteristic similar to that of adult stem cells from other mesenchymal tissues. These corneal stromal stem cells can be cloned and proliferate in vitro for more than 100 doublings. Currently the function of the stromal stem cells in vivo, especially during wound healing, is unclear. Some researchers have suggested that in corneal stroma there are stem cells derived from bone marrow [Sosnova et al., 2005] that are CD34 positive. Nakamura et al. [2005] found that lethally irradiated mice, rescued by tail vein injection of bone marrow cells from green fluorescent protein (GFP)-expressing mice, exhibited a resident population of green cells in the cornea. The function of bone marrow-derived stem cells in cornea and the relationship between bone marrow-derived cells and keratocytes remain unknown.

The corneal endothelium is a single layer of flat hexagonal cells forming a boundary between the corneal stroma and anterior chamber and functioning as a pump to regulate stromal hydration. The endothelium permits the passage of nutrients from the aqueous humor into the avascular cornea through a leaky barrier formed by focal tight junctions. The endothelium simultaneously removes water and CO_2 from the stroma via the activity

of Na^+/K^+-ATPase and bicarbonate-dependent ionic pump. The pump protein is located mainly on the lateral plasma membranes [Joyce, 2003]. Although, like the keratocytes, the corneal endothelium is derived from neural crest, the endothelial cell characteristics are different from those of keratocytes. The human corneal endothelium comprises a monolayer of polygonal cells, which are arrested in G_1 phase in vivo and do not normally replicate to replace dead or injured cells [Joyce et al., 1996a,b]. This relative lack of cell division results in a physiological reduction of cell density of about 0.3–0.5% per year [Bourne and Kaufman, 1976; Hollingsworth et al., 2001]. Scattered evidence, however, suggests the potential for some mitotic events in human corneal endothelium. Mitotic figures were observed in vivo by specular microscopy after rejection reaction of a corneal graft [Laing et al., 1984]. Clusters of cells smaller than surrounding cells suggested that at least under some circumstances mitosis occurs in the endothelium of the adult human cornea. Recently Yokoo et al. [2005] identified cells in the human corneal endothelium that can form cell spheres in attachment-independent culture. Cells in these spheres, which form under conditions similar to those used for isolation of neural stem cells, can be expanded and can generate daughter cells expressing neuronal and mesenchymal molecular markers. These properties suggest a stem cell origin for the cells forming the spheres. The sphere-forming cells also adapted the polygonal morphology characteristic of endothelial cells, suggesting that these cells are endothelial progenitors. Mimura et al. [2005b,c] showed that these precursors were effective in vivo in restoring endothelial function in an animal model of corneal endothelial deficiency. The same group [Mimura et al., 2005a] observed peripheral and central rabbit corneal epithelia to contain a significant number of precursors, but the peripheral endothelium contains more precursors and has a stronger self-renewal capacity than the central region by sphere-forming assay. Because the long-term culture of human endothelial cells has not been carried out and because of the lack of both stem cell and endothelial markers, positive identification of the proposed endothelial stem cells in situ has yet to be carried out.

11.1.4. Identification of Corneal Stem Cells

Although the concept that corneal epithelial stem cells reside in the limbus is widely accepted, identification of these cells has been difficult because of the lack of unique molecular markers. Label retention has been the most robust means of stem cell identification in experimental animals, but it has not been feasible for human epithelial stem cells. One candidate marker, p63 protein, a transcription factor homolog of the tumor suppressor p53, is highly expressed in the basal or progenitor layers of many epithelial tissues and is essential for regenerative proliferation [Yang et al., 1999]. Whether p63 is a marker of limbal stem cells, however, is still controversial. Pellegrini et al., [1999, 2001] investigated p63 as a specific marker of keratinocyte stem cells based on its localization to a subset of basal limbal cells and clonal analysis showing preferential p63 expression in colonies with the greatest clonogenic potential. Wang et al. [2003] suggested that p63 is not a stem cell-specific marker, based on its coexpression with Ki-67 (a proliferating cell marker) in suprabasal nuclei of rabbit corneal limbal sections and limbal explants on amniotic membrane. Moore et al. [2002] and Joseph et al. [2004] described the p63 expression pattern of mouse and rat keratoepithelial grafts and human limbal explants, respectively, both noting that p63 is not exclusive to the stem cells, based on the continued p63 expression of cells migrating out from cultured explants and its expression in sections from the central cornea. Based on the coexpression of connexin-43 (a negative marker for

limbal stem cells) and p63 in a monolayer of cultured human corneal limbal cells formed after 2–3 weeks of culture, Du et al. [2003] concluded that p63 is a marker of stem cells as well as transient amplifying (TA) cells in limbal cultures. Chen et al. [2004] identified p63 as one of a few markers that could define a putative stem cell phenotype based on its differential expression in the basal cells of human limbal sections, bringing the controversy of the utility of p63 as a corneal epithelial stem cell marker to the foreground. More recently, Salehi-Had et al. [2005] suggested that p63 expression in culture cannot be used as a marker for stem cells, based on the observation that the majority of corneal limbal epithelial cells express p63 in colonies derived from single cells and in subconfluent cultures regardless of time in culture or continuance of cell division. Keratins 3 and 12 (K3 and K12) are widely used to identify differentiated corneal epithelial cells, and connexin-43 is expressed in basal epithelial (TA) cells but not basal limbal (stem) cells. Thus these antigens currently serve as negative markers to identify the differentiated epithelial cells [Grueterich et al., 2002; Schermer et al., 1986; Tseng, 1989].

The most recent and currently best candidate for a marker of corneal epithelial stem cells is a drug-resistance transporter protein known as ABCG2. Adult stem cells have the ability to efflux fluorescent dye Hoechst 33342, leading to reduced red and blue fluorescence in fluorescence-activated cell sorting (FACS) [Goodell et al., 1996]. These cells are referred to as "side population" (SP) cells because in the two-dimensional display of red and blue fluorescence, cells having reduced Hoechst dye appear as a small tail to the left side of the mass representing live somatic cells. SP cells from a number of adult tissues have been shown to exhibit many characteristics of stem cells. The SP cells are lost after treatment with verapamil, a drug that blocks action of the ATP-binding cassette transporter G family member ABCG2. This transporter protein has been identified as the Hoechst efflux pump [Kim et al., 2002; Scharenberg et al., 2002; Zhou et al., 2001] and as a specific marker for many kinds of stem cells such as hematopoietic, mesenchymal, muscle [Zhou et al., 2001], neural [Cai et al., 2004; Jang et al., 2004], cardiac [Martin et al., 2004], islet [Lechner et al., 2002], and keratinocyte [Terunuma et al., 2003; Triel et al., 2004] stem cells. Recent studies have shown the presence of side population cells present in corneal epithelial and stromal tissue. In these cells, ABCG2 protein and mRNA expression has been found to be correlated with the SP phenotype and with stem cell characteristics [Budak et al., 2005; de Paiva et al., 2005; Schlotzer-Schrehardt and Kruse, 2005; Watanabe et al., 2004]. The ABCG2-positive cells of cornea are located in the limbus (Fig. 11.1). In the epithelium these cells are localized in unique crypts associated with the palisades of Vogt [Dua et al., 2005]. In the stroma ABCG2-staining cells are seen just posterior to the limbal basement membrane [Du et al., 2005].

11.2. PREPARATION OF MEDIA AND REAGENTS

11.2.1. PBSA/GASP

PBSA containing antibiotics: gentamicin, 50 μg/mL; amphotericin B, 1.25 μg/mL; streptomycin, 100 μg/mL; penicillin, 100 U/mL (GASP)

11.2.2. CMF-Saline G

(i) NaCl _____ 8 g/L

(ii) KCl _____ 0.4 g/L

(iii) $Na_2HPO_4 \cdot 7H_2O$ _____ 0.29 g/L

(iv) KH_2PO_4 — 0.15 g/L
(v) Glucose — 1.1 g/L
(vi) pH — 7.2

11.2.3. CMF/GASP

CMF-Saline G with GASP antibiotics (*see* Section 11.2.1)

11.2.4. Trypsin/EDTA

Trypsin 0.25%, Na_2EDTA 0.5 mM, in CMF-Saline G

11.2.5. DMEM/F-12/GASP

DMEM/F-12 with GASP antibiotics (*see* Section 11.2.1)

11.2.6. LSC Culture Medium

DMEM/F-12 containing:

(i) FBS — 5%
(ii) Human epidermal growth factor (EGF) — 20 ng/mL
(iii) Glutamine — 4 mM
(iv) Triiodothyronine — 2 nM
(v) ITS
  Insulin — 5 μg/mL
  Transferrin — 5 μg/mL
  Selenous acid — 5 ng/mL
(vi) Hydrocortisone — 0.5 μg/mL
(vii) Cholera toxin — 30 ng/mL
(viii) Adenine — 0.18 mM
(ix) GASP antibiotics (*see* Section 11.2.1)

11.2.7. DMEM/10FB/GASP

DMEM containing 10% FBS and GASP antibiotics (*see* Section 11.2.1)

11.2.8. Modified Jiang Medium (MJM) for Culture of Human Stromal Stem Cells

DMEM/MCDB-201, 60:40, with:

(i) Fetal bovine serum (FBS) — 2%
(ii) Epidermal growth factor (EGF) — 10 ng/mL
(iii) Platelet-derived growth factor (PDGF-BB) — 10 ng/mL
(iv) ITS (*see* Section 11.2.5)
(v) Leukemia inhibitory factor (LIF) — 200 U/mL
(vi) Linoleic acid–bovine serum albumin (LA-BSA), 1 mg/mL

(vii) Ascorbic acid-2-phosphate — 0.1 mM
(viii) Dexamethasone — 1×10^{-8} M
(ix) GASP antibiotics (*see* Section 11.2.1)

11.2.9. HBSS/2FB

Hanks' BSS with 2% FBS

11.2.10. DMEM/2FB

DMEM with 2% FBS

11.2.11. Keratocyte Differentiation Medium

Advanced D-MEM (Invitrogen) with:

(i) Basic fibroblast growth factor (FGF-2) — 10 ng/mL
(ii) Ascorbic acid-2-phosphate — 0.1 mM

11.2.12. Chondrocyte Differentiation Medium (CDM)

DMEM/MCDB-201, 60:40 with:

(i) FBS — 2%
(ii) Ascorbic acid-2-phosphate — 0.1 mM
(iii) Dexamethasone — 1×10^{-7} M
(iv) TGF-β — 10 ng/mL
(v) Sodium pyruvate — 100 μg/mL

11.2.13. Neural Differentiation Medium (NDM)

Advanced D-MEM with:

(i) Epidermal growth factor (EGF) — 10 ng/mL
(ii) FGF-2 — 10 ng/mL
(iii) All-*trans* retinoic acid — 1 μM

11.2.14. Corneal Endothelial Cell Medium (CECM)

DMEM/F-12, 1:1, with:

(i) FBS — 2%
(ii) FGF-2 — 10 ng/mL
(iii) EGF — 10 ng/mL
(iv) PDGF-BB — 10 ng/mL
(v) ITS, 1× (*see* Section 11.2.6)
(vi) Ascorbic acid-2-phosphate — 0.1 mM
(vii) GASP antibiotics (*see* Section 11.2.1)

11.2.15. Endothelial Spheres Medium (ESM)

DMEM/F-12, 1:1, with:

(i) Methylcellulose 1.5%
(ii) B27 supplement, 1×
(iii) FGF-2 10 ng/mL
(iv) EGF 20 ng/mL
(v) GASP antibiotics (*see* Section 11.2.1)

11.2.16. SDS Sample Buffer (6×)

(i) Tris·HCl, 0.5 M, pH 6.8 7 mL
(ii) Glycerol 3 mL
(iii) SDS 1 g
(iv) Bromophenol blue 1.2 mg

Concentrations in 6× stock: 0.35 M Tris, 30% glycerol (v/v), 10% (w/v) SDS, 0.012% (w/v) Bromophenol Blue.

Use at final concentrations: 0.058 M Tris, 5% glycerol (v/v), 1.67% (w/v) SDS, 0.002% (w/v) Bromophenol Blue.

11.2.17. Blocking Buffer

PBSA + 0.5% BSA + 2% normal goat serum

11.3. CULTURE OF HUMAN CORNEAL LIMBAL STEM CELLS (LSC)

Although side population cells can be identified and isolated from the limbal epithelium by FACS, this purification method has not been adopted widely as a means for isolation of pure populations of viable LSC for culture. Typically, unfractionated populations of human corneal limbal cells are cultured and passaged in vitro under conditions favoring stem cell expansion. These cultures have been transplanted in vivo for treatment of limbal stem cell deficiency. For in vitro culture of LSC, there are three methods: (1) culture directly on plastic, (2) culture on amniotic membrane, and (3) culture with a feeder layer. The authors compared culture procedures on plastic and amniotic membrane and concluded that culture on human amniotic membrane suppresses differentiation of limbal progenitor cells and promotes their proliferation [Du et al., 2003]. Tseng and co-workers concluded that the coculture system with a feeder layer of mitomycin C-treated mouse 3T3 cells promoted more clonal growth of limbal progenitor cells [Tseng et al., 1996]. We introduce these different culture systems below.

Safety Note: Human tissues should be handled in Biosafety Level 2 laboratories, using a laminar flow hood (*see* Chapter 1) and protective personal apparel as approved by the institutional review board as appropriate for this purpose. Serology should be obtained from each donor to exclude possibility of contamination with HIV and hepatitis.

11.3.1. Preparation of Substrata

Protocol 11.1. Preparation of Human Amniotic Membrane (HAM)

Reagents and Materials

Sterile or aseptically prepared

- ❑ Human placenta. ***Note:*** Human tissue for research must be obtained with informed consent of the donor, using a protocol approved by the institutional review board. Preservation of the anonymity of donors, procedures in accordance with the Declaration of Helsinki, and biosafety concerns will need to be addressed.
- ❑ PBSA/GASP (*see* Section 11.2.1)
- ❑ Glycerol
- ❑ DMEM containing 50% glycerol
- ❑ Trypsin/EDTA (see Section 11.2.4)
- ❑ Millicell microporous membrane tissue culture insert
- ❑ Plastic spatula and sterile cotton swab

Procedure

(a) Wash the tissue with PBSA/GASP.

(b) Separate the HAM, a thin sheet consisting of the epithelium, basement membrane, and some underlying compact stroma, manually, using a gloved hand to slide under the HAM and separate it from the stromal tissue of the placenta.

(c) Use the sterile cotton swabs to remove the underlying compact stroma from the basement membrane to keep the amniotic membrane as thin as possible.

(d) The crude HAM can be stored in DMEM containing 50% glycerol at -70°C, pending testing of donor sera for disease.

(e) Immediately before use, thaw the HAM, wash it with PBSA, and cut it into pieces approximately 2 cm in diameter.

(f) Digest the pieces with Trypsin/EDTA at 37°C for 30 min.

(g) Scrub the digested HAM gently with a plastic spatula to remove the epithelium without breaking the basement membrane.

(h) Wash the denuded membrane with PBSA and allow it to adhere onto a Millicell microporous membrane tissue culture insert with the basement membrane side (from which epithelial cells have been removed) facing up.

Protocol 11.2. Preparation of Mouse NIH 3T3 Fibroblast Feeder Layer

Reagents and Materials

Sterile or aseptically prepared

- ❑ Mouse NIH 3T3 fibroblasts

- ❑ DMEM/10FB/GASP (*see* Section 11.2.7)
- ❑ Trypsin/EDTA (*see* Section 11.2.4)
- ❑ Mitomycin C (MMC), 500 μg/mL in water
- ❑ Tissue culture-treated plastic flasks, 25 or 75 cm^2
- ❑ Tissue culture plates, 6-well

Nonsterile

- ❑ Optional (alternative to mitomycin C treatment): gamma or X-ray irradiator
- ❑ Low-speed refrigerated centrifuge

Procedure

(a) Seed NIH 3T3 fibroblasts in 25-cm^2 or 75-cm^2 tissue culture flasks in DMEM10FB/GASP).

(b) When the cells just reach confluence, add MMC (5 μg/mL) for 2 h at 37°C.

(c) Trypsinize and plate the cells at a density of 2×10^4 cells/cm^2 in 3.5-cm dishes or six-well plates.

(d) As an alternative to MMC, confluent cultures of 3T3 cells may be irradiated with 60 Gy, using ^{60}Co or an X-ray source. The cells are amitotic but still living and can be used as a feeder layer.

Protocol 11.3. Harvesting Corneal Epithelium and Stem Cell Isolation

Reagents and Materials

Sterile or aseptically prepared

- ❑ Whole human cornea (*see* safety and regulatory note in Section 11.3)
- ❑ CMF-Saline G (*see* Section 11.2.2)
- ❑ Dispase II, 1.2 U/mL (2.4 U Dispase II diluted in DMEM/F-12/GASP)
- ❑ Trypsin/EDTA (*see* Section 11.2.4)
- ❑ DMEM/F-12/GASP (*see* Section 11.2.5)
- ❑ DMEM/F-12/2FB/GASP: DMEM/F-12/GASP with 2% FBS
- ❑ CMF/GASP (*see* Section 11.2.3)
- ❑ LSC culture medium (*see* Section 11.2.6)
- ❑ Curved iris scissors, 11 cm (4-3/8 in.)
- ❑ Jeweler's forceps, 10 cm (4 in.)
- ❑ Corneal scissors, 19-mm blades, sharp tip
- ❑ Colibri suturing forceps, 0.1 mm

Nonsterile

- ❑ Variable-speed rocking mixer
- ❑ Dissecting microscope
- ❑ Low-speed refrigerated centrifuge
- ❑ Hemocytometer

Procedure

(a) Wash the cornea 3 × 5 min in CMF-Saline G
(b) Trim off the residual sclera, conjunctiva, and iris.
(c) Add 2 ml of Dispase II and leave at 4°C, overnight, with gentle agitation.
(d) Rock the cornea in Dispase II at 4°C for 30 min in a rocking mixer.
(e) Wash the cornea once in DMEM/F-12/GASP.
(f) Under a dissecting microscope, carefully remove the central corneal epithelium (at this point, most of the central epithelium is already exfoliated) and peel off the limbal pigmented epithelium, which contains the palisades of Vogt.
(g) Digest the limbal epithelial sheets in Trypsin/EDTA for 10 min at 37°C.
(h) Add one volume of DMEM/F-12/2FB/GASP.
(i) Centrifuge at 400 *g* for 10 min and discard the supernate.
(j) Wash once with DMEM/F-12/2FB/GASP, centrifuge, and discard the supernate.
(k) Add 1 mL of LSC culture medium, disperse the cell pellet, and determine the cell number with a hemocytometer.
(l) Seed the cells at 1×10^4/ cm^2 into a tissue culture plate with a 3T3 feeder layer or on prepared HAM.
(m) Change the medium every 3 days.
(n) When the cells are 90% confluent, passage by trypsinization:
    (i) Remove the medium.
    (ii) Wash 1× with CMF-Saline G.
    (iii) Add trypsin/EDTA for 10 min at 37°C.
    (iv) When the cells are released, add an equal volume of DMEM/F-12/2FB/GASP.
    (v) Count the cells.
    (vi) Centrifuge 400 *g* for 10 min.
    (vii) Discard the supernate and add sufficient new medium to seed at a density of 1×10^4 cells/cm^2.
(o) Alternatively, primary cells may be frozen in cryopreservation medium (*see* Section 11.4.4).

11.3.2. LSC Characterization and Differentiation

As mentioned above, LSC express ABCG2 and p63 but do not express connexin-43 and keratins 3 and 12. Thus these cells can be identified by immunostaining, RT-PCR, and immunoblotting.

11.3.2.1. LSC Differentiation. LSC can differentiate into stratified epithelial layers after culture at an air-liquid interface [Espana et al., 2003; Wolosin et al., 2000]. Passage the cells on a Millipore Millicell culture insert, submerged in LSC medium. When confluent, reduce the volume of culture medium in the well so that the layer of cells is bathed from the bottom by medium but the top is covered only by a thin film of medium. The level of medium must be checked daily. After 10–14 days, the cells will form stratified epithelial layers. These can be identified by standard histological staining and by immunostaining for connexin-43 and keratin 3.

Protocol 11.4. Immunostaining Limbal Stem Cell Cultures

Reagents and Materials

Nonsterile

- ❑ Primary antibodies:
  - ❑ ABCG2 _____ mouse monoclonal BXP-21 ____ 1:100
  - ❑ Connexin-43 _____ rabbit polyclonal Cx43 _____ 1:100
  - ❑ Cytokeratin 3 (K3) _____ mouse monoclonal AE5 _____ 1:500
  - ❑ Cytokeratin 12 (K12) _____ rabbit polyclonal J7 _____ 1:200
- ❑ Secondary antibodies:
- ❑ Anti-mouse IgG _____ Alexa-488 _____ 1:2500
- ❑ Anti-rabbit IgG _____ Alexa-546 _____ 1:1500
- ❑ Blocking antibody: 10% heat-inactivated goat serum in PBSA
- ❑ Triton X-100
- ❑ PBSA
- ❑ Paraformaldehyde (PFA), freshly made from 16% stock, 4% in PBSA.
- ❑ Immu-Mount antifade mounting solution
- ❑ Slides and #1 coverslips
- ❑ Confocal microscope

Procedure

(a) Culture cells either on amniotic membrane or on plastic.
(b) Wash with CMF-Saline G once.
(c) Fix in freshly made 4% PFA in PBSA for 15 min at room temperature.
(d) Wash once in PBSA.
(e) Permeabilize with 0.1% Triton X-100 in PBSA for 10 min.
(f) Block nonspecific binding with 10% heat-inactivated goat serum in PBSA.
(g) Incubate samples with primary antibodies for 1 h at room temperature.
(h) Wash twice with PBSA.
(i) Add secondary antibodies and incubate for 1 h at room temperature.
(j) Wash the samples twice in PBSA.
(k) Mount in a minimal volume of antifade mounting medium under a #1 coverslip.
(l) Photograph the samples with an epifluorescence or confocal microscope using a 40× oil objective.

Protocol 11.5. Characterization of Limbal Stem Cells with Reverse Transcription-PCR

Reagents and Materials

- ❑ Primers:

ΔN p63	Forward: CAGACTCAATTTAGTGAG
	Reverse: AGCTCATGGTTGGGGCAC
K12	Forward: CTA CCT GGA TAA GGT GCG AGC T
	Reverse: TCT CGC ATT GTC AAT CTG CA
β-Actin	Forward: GAG GCG TAC AGG GAT AGC AC
	Reverse: GTG GGC ATG GGT CAG AAG

- ❑ RNA extraction kit, e.g., RNeasy (Qiagen)
- ❑ Superscript II (Invitrogen)
- ❑ DNase I (Ambion)
- ❑ HotStarTaq (Qiagen)
- ❑ Materials for 6% acrylamide gel electrophoresis
- ❑ SYBR Gold (Invitrogen)

Procedure

(a) Extract RNA from stem cells from one 3.5-cm dish by a standardized procedure, such as RNeasy.

(b) Transcribe cDNA from 400 ng of total RNA, using random hexamers and Superscript II followed by DNase I treatment.

(c) Carry out PCR on cDNA from 20 ng of RNA, using HotStarTaq.
Cycling conditions: 94°C for 2 min, 94°C for 30 s, 55°C for 45 s, 72°C for 45 s, for 35 cycles, 72°C for 10 min

(d) Separate PCR products on 6% acrylamide gel and detect by SYBR Gold.

11.3.2.2. Immunoblotting. Connexin-43 and keratins 3 and 12 are detected by immunoblotting of proteins from cell lysates with procedures described in Section 11.4. ABCG2 can be immune precipitated from cell lysates after cell surface biotinylation as described below. Antibodies used are the same as previously described.

11.3.3. Cryopreservation

Briefly, for LSC cells, trypsinize, count, pellet by centrifugation, and resuspend the cells in freezing medium at $2–5 \times 10^6$ cells/mL with 1 mL per vial. Chill at a controlled rate of 1°C/h, using a commercial freezing box filled with isopropanol in a −80°C freezer overnight. On the next day, transfer the vials to liquid nitrogen. Freezing medium: 70% culture medium (DMEM/F-12), 20% FBS, 10% DMSO. Make fresh each time before freezing cells.

11.3.4. Variations and Applications of the Method

LSC can be cultured as limbal explants or purified by FACS for side population cells.

11.3.4.1. Explant Culture. After Dispase II digestion of human cornea (*see* Protocol 11.3), isolate the limbal epithelium and cut it into pieces about 1 mm in diameter. Seed these pieces on 3T3 feeder layers (*see* Protocol 11.2) or on amniotic membrane (*see* Protocol 11.1) with the epithelial side up. The cells will migrate out from the tissue over a period of 2–4 weeks.

11.3.4.2. FACS Isolation of Limbal SP Cells. This procedure is identical to that used for isolation of stroma side population cells by cell sorting, described in Section 11.4 below. For epithelial cells, both primary cells and passaged cells can be sorted if enough cells can be obtained after tryptic digestion.

11.4. CULTURE OF HUMAN CORNEAL STROMAL STEM CELLS

Corneal stromal cells, known as keratocytes, have a quiescent phenotype characterized by a unique dendritic morphology and a very low to nonexistent rate of proliferation in vivo [Jester et al., 1994; Zieske, 2001]. In response to acute injury, keratocytes become mitotic, adopt a fibroblastic phenotype, and move to the injured area [Carlson et al., 2003; Jester et al., 1999]. In vitro, primary keratocytes can be maintained in serum-free or low mitogen serum-containing culture medium in a quiescent state, exhibiting cellular morphology and matrix secretion similar to those of keratocytes in vivo [Beales et al., 1999; Jester et al., 1996]. Fetal bovine serum can induce keratocytes to proliferate but also causes keratocytes to become fibroblasts and myofibroblasts [Masur et al., 1996].

11.4.1. Isolation of Stromal Cells

The isolating and culturing of stromal stem cells provides an important source of keratocytes for in vitro and in vivo research. We have demonstrated a method for obtaining human corneal stromal stem cells using FACS to isolate the SP cell population from primary stromal cell cultures. The isolated stem cells express ABCG2 and grow in vitro for more than 100 population doublings without loss of the ability to differentiate into keratocytes [Du et al., 2005]. Like quiescent primary keratocytes, the cells secrete the keratan sulfate proteoglycans lumican, keratocan, and mimecan, often identified as molecular markers for keratocytes [Funderburgh et al., 2001, 2003].

Protocol 11.6. Isolation of Primary Human Corneal Stromal Cells

Reagents and Materials

Sterile or aseptically prepared

- ❑ Whole human cornea (*see* Section 11.3 and Chapter 1)
- ❑ CMF-Saline G (*see* Section 11.2.2)
- ❑ Dispase II
- ❑ Collagenase type L, 1 mg/mL in DMEM/F-12/GASP
- ❑ TrypLE Express or 0.25% trypsin in CMF-Saline G
- ❑ DMEM//F-12/GASP (*see* Section 11.2.5)
- ❑ DMEM/F-12/2FB/GASP: DMEM/F-12/GASP with 2% FBS
- ❑ CMF/GASP (*see* Section 11.2.3)
- ❑ Tissue culture-grade plastic dishes, 3.5 cm, or 6-well plates
- ❑ Scalpel or single-edge razor blades

- ❑ Cell strainer, 70-μm nylon mesh
- ❑ Plastic spatula, ''Cell Lifter'' or ''Cell Scraper''
- ❑ Curved iris scissors, 11 cm (4-3/8 in.)
- ❑ Jeweler's forceps, 10 cm (4 in.)
- ❑ Corneal scissors, 19-mm blades, sharp tip
- ❑ Colibri suturing forceps, 0.1 mm

Nonsterile

- ❑ SDS sample buffer (*see* Section 11.2.16). Use at final concentrations: 0.058 M Tris, 5% glycerol, 1.67% SDS, 0.002% bromophenol blue
- ❑ Variable-speed rocking mixer
- ❑ Tube roller apparatus
- ❑ Dissection microscope
- ❑ Centrifuge, low speed, refrigerated

Procedure

(a) Wash the cornea 3 × 5 min in CMF-Saline G.
(b) Trim off the residual sclera, conjunctiva, and iris.
(c) Add 2 mL of 1.2 U Dispase II (2.4 U Dispase II diluted in DMEM/F-12/GASP) 4°C, overnight on a rocking mixer.
(d) Rotate the cornea in Dispase II for 30 min at 4°C.
(e) Wash the cornea once in DMEM/F-12/GASP.
(f) Under the dissection microscope, carefully peel off the epithelium and endothelium.
(g) Use a plastic spatula to scrape both epithelial side and endothelial side of the stroma. Observe through microscope to make sure all of these cellular layers are removed.
(h) Wash the corneal stroma in new medium once.
(i) Mince the stroma into 2-mm cubes, using scalpel, razor blades, or fine scissors.
(j) Digest up to 3 h at 37°C in collagenase in DMEM/F-12/GASP until most of the tissues disperse.
(k) Centrifuge at 400*g* for 10 min and discard the supernate.
(l) Resuspend the cells in fresh DMEM/F-12/GASP, filter the digest through a 70-μm Cell Strainer, and repeat the centrifugation.
(m) Repeat this wash a second time. Count the cell number after each spin.
(n) Resuspend the primary stromal cells in MJM (*see* Section 11.2.8) and seed into tissue culture coated plastic dishes at a density of 1×10^4 cells/cm^2.
(o) Change the medium every 3 days.
(p) When the cells are 90% confluent, passage by trypsinization.
    (i) Aspirate the medium.
    (ii) Wash in CMF-Saline G.
    (iii) Add trypsin or TrypLE to barely cover cells for 10 min at 37°C.
    (iv) Add DMEM/F-12/2FB to terminate the digestion.

(v) Count the cell number.
(vi) Centrifuge resuspended cells and discard the supernate.
(vii) Resuspend in sufficient fresh MJM to seed cultures at a density of 1×10^4 cells/cm^2.

11.4.2. Isolation of Stromal SP Cells with FACS

Protocol 11.7. Selection of Corneal Stromal Stem Cells by Hoechst 33342 Efflux

Reagents and Materials

Sterile or aseptically prepared

- ❑ Stromal cells at passages 2–4
- ❑ HBSS/2FB (*see* Section 11.2.9)
- ❑ Hoechst 33342 dye, 1 mg/mL in water
- ❑ Propidium iodide, 2 mg/mL in water
- ❑ Verapamil, 500 μg/mL in water
- ❑ DMEM/2FB (*see* Section 11.2.10)
- ❑ Trypsin/TrypLE: TrypLE Express or 0.25% trypsin in CMF-Saline G
- ❑ MoFlo (or similar) high-speed cell sorter, with 350-nm excitation

Procedure

(a) Trypsinize passage 2–4 stromal cells.
(b) Count the cell number and dilute to 1.0×10^6 cells/mL in DMEM/2FB.
(c) Add 5 μg/mL Hoechst 33342 dye for 90 min at 37°C; agitate every 20 min.
(d) As a control, some cells are preincubated for 20 min with 50 μg/mL verapamil before Hoechst 33342 incubation.
(e) After staining, wash the cells twice by centrifugation in HBSS/2FB at 4°C and then resuspend and store them in cold HBSS/2FB on ice.
(f) Immediately before sorting, add 2 μg/mL propidium iodide to identify nonviable cells.
(g) Sort cells on a sterile, high-speed cell sorter, using 350-nm excitation. Collect the cells showing reduced fluorescence of both blue (670 nm) and red (450 nm). This ''side population'' is collected separately from dead cells and from fully labeled cells. As a control, confirm that the side population is eliminated by verapamil preincubation.

Alternatively, cells can be sorted according to expression of ABCG2 protein, although this procedure may not yield a population with the same level of "stemness" as side population cells.

Protocol 11.8. Immunoselection of Corneal Stromal Stem Cells by ABCG2 Expression

Reagents and materials

Sterile

- ❑ Stromal cells at passages 2–4
- ❑ PBSA/BSA: PBSA + 0.5% BSA
- ❑ PBSA/2FB: PBSA with 2% FBS
- ❑ Blocking buffer (*see* Section 11.2.17)
- ❑ Antibody: MAB 4155F, Clone 5D3 anti-ABCG2-FITC, or Isotype-FITC

Nonsterile

- ❑ High-speed cell sorter with a 488-nm argon laser and a band-pass filter of 525/20

Procedure

(a) Trypsinize passage 2–4 cells and count the cell number.
(b) Spin down at 400 $\times$ *g*, 10 min.
(c) Wash once with PBSA/BSA.
(d) Block in 50 μL of blocking buffer for 10 min on ice.
(e) Add 10 μL of antibody (MAB 4155F, Clone 5D3 anti-ABCG2-FITC, or Isotype-FITC), 30 min, on ice.
(f) Wash once by centrifugation in PBSA/BSA.
(g) Gently resuspend the cells in 1 mL of PBSA/2FB. Keep the cells on ice until flow cytometry is performed.
(h) Perform fluorescence-activated cell sorting (FACS), using a high-pressure, high-speed cell sorter. A 488-nm argon laser is used to excite the fluorescein isothiocyanate, and a band-pass filter of 525/20 is used to measure emitted light. Gates in the right angle scatter versus forward scatter diagrams are used to exclude debris. Collect at least 100,000 events for analysis.
(i) The sorted ABCG2-positive and -negative cell populations can be used to evaluate their colony-forming efficiency and gene expression of stem cell markers and to passage for further investigation and in vivo transplantation.

11.4.3. Characterization and Differentiation

11.4.3.1. Stem Cell Characterization. Stromal stem cells in MJM will exhibit expression of PAX6 and ABCG2, genes not expressed by differentiated keratocytes.

PAX6: Stem cells in sparse conditions in MJM will express high levels of nuclear PAX6. Cells seeded at 1×10^4/ cm^2 in MJM are fixed in 4% PFA in PBSA for 10 min, permeabilized in 0.1% Triton X-100 for 10 min, and blocked in 10% goat serum as described above (*see* Protocol 11.4). The cells are stained with anti-PAX6, diluted 1:100, and counterstained with cytoplasmic myosin (CMII25) followed by secondary antibodies

of Alexa-546 anti-rabbit and Alexa-488 anti-mouse for 1 h. The cells are photographed by epifluorescence microscopy as described above.

ABCG2: ABCG2 can be detected by immune precipitation after cell surface biotinylation. Cell layers are rinsed in PBSA and incubated with Sulfo-NHS-LC-Biotin at 1 mg/mL in PBSA for 15 min on ice. Cell layers are washed again in PBSA, and the cells are scraped with a cell lifter and pelleted. Cells are lysed in 0.5 mL of M-PER and cleared with 10 μL of protein G magnetic beads. ABCG2 antibody (clone BXP-21) is preincubated with protein G beads, and then the loaded beads are incubated with samples overnight. The beads are collected and rinsed in PBSA, and the bound protein is eluted by heating in SDS sample buffer. Proteins are separated on 4%–20% SDS-PAGE gel and transferred to polyvinylidene difluoride (PVDF) membranes, and biotinylated protein is detected with streptavidin-horseradish peroxidase, using a luminescent substrate.

11.4.3.2. Keratocyte Differentiation. As the stem cells differentiate to keratocytes they will lose expression of PAX6 and ABCG2 and express molecular markers unique to keratocytes. The most reliable of these markers are the proteoglycan keratocan and the glycosaminoglycan keratan sulfate. These both can be detected by immunostaining and Western blot. Keratocan mRNA can be quantified as well.

To induce differentiation, passage the stem cells at 1×10^4 cells/cm^2 in keratocyte differentiation medium (*see* Section 11.2.11). Change the medium every 2–3 days. After 1–2 weeks, the cells will be induced into keratocytes.

Immunodetection of Proteoglycans. Proteoglycans are recovered from culture media by passage over ion exchange columns (SPEC-NH2 microcolumns). These are rinsed in 0.2 M NaCl, 6 M urea, 0.02 M Tris, pH 7.4, and eluted in 0.5 mL of 4 M guanidine-HCl, 0.02 M Tris, pH 7.4. The samples are dialyzed against water and lyophilized. Samples are resuspended in 100 μL of 0.1 M ammonium acetate, pH 6.5, and divided into half. One half is digested overnight at 37°C with 2 mU/mL Keratanase II and 2 mU/mL endo-β-galactosidase. Digested and undigested samples, normalized for cell number, are run on a 4%–20% SDS-PAGE gradient gel, transferred to PVDF membrane, and subjected to immunoblotting with Kera-C polyclonal antibody against keratocan and monoclonal antibody J36 against keratan sulfate. The digested samples will not react with J36 but will show a sharp band of 55 kDa for the keratocan.

Quantitative Reverse Transcription–Polymerase Chain Reaction (RT-PCR). Quantitative RT-PCR is performed with SYBR Green RT-PCR Reagents according to the manufacturer's instructions. The reaction is carried out for 40 cycles of 15 seconds at 95°C and 1 minute at 60°C after initial incubation at 95°C for 10 minutes. Reaction volume is 50 μL, containing 1× SYBR Green PCR buffer, 3 mM Mg^{2+}, 200 μM dATP, dCTP, and dGTP and 400 μM dUTP, 0.025 units/mL AmpliTaq Gold polymerase, 5 μL cDNA, and forward and reverse primers (*see* Table 11.1) at optimized concentrations. A dissociation curve for each reaction is generated on the Gene-Amp ABI Prism 7700 Sequence Detection System to confirm the absence of nonspecific amplification. Amplification of 18S rRNA is performed for each cDNA (in triplicate) for normalization of RNA content. Threshold cycle number (C_t) of amplification in each sample is determined by ABI Prism Sequence Detection System software. Relative mRNA abundance is calculated as the C_t for amplification of a gene-specific cDNA minus the average C_t for 18S expressed as a power of 2, that is, $2^{-\Delta Ct}$. Three individual gene-specific values thus calculated are averaged to obtain means ± SD.

TABLE 11.1 Human Primers for Detection of Expression of Keratocyte Differentiation Genes

Keratocan	Forward: ATCTGCAGCACCTTCACCTT
	Reverse: CATTGGAATTGGTGGTTTGA
ABCG2	Forward: TGCAACATGTACTGGCGAAGA
	Reverse: TCTTCCACAAGCCCCAGG
Pax6	Forward: CAATCAAAACGTGTCCAACG
	Reverse: TAGCCAGGTTGCGAAGAACT
18S	Forward: CAATCAAAACGTGTCCAACG
	Reverse: TAGCCAGGTTGCGAAGAACT

Immunostaining. As described in Section 11.4.3.2, the cells will have lost ABCG2 and PAX6 staining but will become positive for keratocan when stained with Kera C antibody.

11.4.4. Cryopreservation

This procedure is identical as that for limbal stem cells.

11.4.5. Variations and Applications of the Method

The corneal stromal stem cells have clonogenic and multipotent differentiation potential. These properties can be used to confirm the stem cell character of isolated cells.

11.4.5.1. Clonal Growth. Stromal stem cells grow clonally in MJM. Trypsinized cells are counted and diluted in MJM to a concentration of 3 cells/mL. Plate 0.1 mL per well in half-area (A/2) 96-well microtitration plates. The ratio of 0.3 cells/well provides a very low chance that any well will have two cells. After 2 weeks, wells with colonies are marked and medium is changed. When confluent, the cells are trypsinized and expanded at 10^4 cells/cm^2. Cloning is recommended before differentiation.

11.4.5.2. Chondrogenic Potential. Chondrocytes are never observed in mammalian eyes. The ability of cells to express cartilage-specific genes and gene products is therefore a strong marker for their multidifferentiation potential (and hence multipotent stem cell character). Chondrocyte differentiation medium (CDM) contains DMEM/MCDB-201, 2% FBS, 0.1 mM ascorbic acid-2-phosphate, 10^{-7} M dexamethasone, 10 ng/mL TGF-β 1, and 100 μg/mL sodium pyruvate. Cells (2×10^5) are resuspended in CDM and are pelleted in a 15-mL conical centrifuge tube. The medium is changed every 3 days. Pellets are cultured for 2 to 3 weeks. Messenger RNA for collagen II, aggrecan, and cartilage oligomatrix protein (COMP) can be detected by RT-PCR (for primers see Table 11.2) in stem cells cultured under the chondrogenic conditions but not in similar cultures of fibroblasts or keratocytes. Collagen II and COMP protein expression can also be detected with immunoblotting of pellet extracts.

11.4.5.3. Neural Differentiation. Stem cells are incubated under conditions that induce neural differentiation in Advanced D-MEM with 10 ng/mL epithelial growth factor (EGF), 10 ng/mL FGF-2 and 1 μM all-*trans* retinoic acid. Retinoic acid is added every 3 days, and the cells are kept 2 to 3 weeks to induce neurogenesis. RT-PCR from

TABLE 11.2 Human Primers for Detection of Cartilage-Specific Gene Expression by RT-PCR

Collagen II	Forward: CCGGGCAGAGGGCAATAGCAGGTT
	Reverse: CAATGATGGGGAGGCGTGAG
COMP	Forward: ACAATGACGGAGTCCCTGAC
	Reverse: AAGCTGGAGCTGTCCTGGTA
Aggrecan	Forward: TGAGGAGGGCTGGAACAAGTACC
	Reverse: GGAGGTGGTAATTGCAGGGAACA

TABLE 11.3 Human Primers for Detection of Neural-Specific Gene Expression by RT-PCR

Neurofilament protein	Forward: GAGGAACACCAAGTGGGAGA
	Reverse: CTCCTCCTCTTTGGCCTCTT
GFAP	Forward: ACTACATCGCCCTCCACATC
	Reverse: CAAAGGCACAGTTCCCAGAT

these cells will detect mRNA upregulation of both glial fibrillary acidic protein (GFAP) and neurofilament protein. Increases in GFAP and neurofilament proteins can also be detected by Western blotting. Immunofluorescent staining shows cells positive for neurofilament, GFAP, and β-tubulin III. Procedures are similar to those discussed above using the primers in Table 11.3.

11.5. CULTURE OF HUMAN CORNEAL ENDOTHELIAL STEM CELLS

Scientists first attempted to isolate and culture human corneal endothelial cells (HCEC) about 40 years ago. At that time these cells were found to exhibit a very limited ability to divide in vitro. Contamination with stromal keratocytes caused technical problems with these studies. More recently, Joyce and colleagues cultured HCEC by stripping the Descemet membrane with HCEC intact to avoid contamination by stromal keratocytes [Chen et al., 2001; Joyce and Zhu, 2004; Zhu and Joyce, 2004]. The presence of stem cells in human corneal endothelium remains controversial. Whikehart and co-workers [2005] suggested that endothelial stem cells may reside at the junctional region where the corneal endothelium meets the trabecular meshwork. Telomerase activity and labeling by bromodeoxyuridine (BrdU) were detected in cells of this region after wounding [Whikehart et al., 2005]. Yokoo et al. demonstrated that cells from human endothelium form spheres under conditions in which neural stem cells are known to form spheres, known as neurospheres [Yokoo et al., 2005]. Mimura et al. [2005b,c] showed that presumptive stem cells isolated under these neurosphere-inducing conditions were effective for treatment of bullous keratopathy and corneal endothelium deficiency in rabbit models. These authors found that peripheral and central rabbit corneal endothelium contains a significant number of presumptive stem cells but the peripheral endothelium contains more and has a stronger self-renewal capacity than the central region as determined by sphere-forming assay [Mimura et al., 2005a]. The search for precursors/stem cells that can differentiate into corneal endothelium is of high clinical relevance. HCEC show little

or no growth in vivo, and failure of endothelial function is a common cause of corneal opacity, thus generating the need for many corneal grafts.

An important tool in determining the ability of presumptive stem cells to differentiate into corneal endothelium is the presence of easily identifiable molecular markers of the HCEC cells. Foets et al. [1992a, b] showed that human corneal endothelial cells express neural cell adhesion molecules (N-CAM), intercellular adhesion molecule-1 (ICAM-1), and the transferrin receptor CD71 detected by monoclonal antibody OKT9. Cell border-associated zonula occludens-1 (ZO-1), a tight junction protein, has been extensively used to identify corneal endothelial morphology and integrity [Chen et al., 2001, 2005; Ide et al., 2006; Joyce, 2003; Petroll et al., 1997, 2001; Senoo and Joyce, 2000; Zhu and Joyce, 2004]. Antibodies against some of these proteins, particularly N-CAM, anti-transferrin receptor monoclonal antibody OKT9, and tight junction protein ZO-1, are commercially available and thus provide the requisite tools to identify human corneal endothelial cells.

The most important identification for differentiated corneal endothelial cells is function. The endothelium has a specific barrier and pump function for maintaining cornea transparency. The pump function of corneas as reconstructed in vitro with transplanted cells or endothelial sheet can be measured with an Ussing chamber [Amano, 2003; Mimura et al., 2004, 2005c; Wigham et al., 2000]. The most effective assay for pump function is the observation of corneal transparency in vivo in animals with cultured cells transplanted into the anterior chamber and allowed to settle on the Descemet membrane [Mimura et al., 2005b,c].

11.5.1. Isolation and Culture of Human Corneal Endothelial Cells

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Protocol 11.9. Isolation of Human Corneal Endothelial Cells

Reagents and Materials

Sterile or aseptically prepared

- ❑ Whole human cornea (see Section 11.3)
- ❑ CMF-Saline G (*see* Section 11.2.2)
- ❑ Dispase II, 2.4 U/mL: dilute to 1.2 U/mL in DMEM/F-12/GASP for use
- ❑ Trypsin/EDTA (*see* Section 11.2.4)
- ❑ DMEM/F-12/GASP (*see* Section 11.2.5)
- ❑ DMEM/F-12/2FB: DMEM:Ham's F-12, 1:1, with 2% FBS
- ❑ CMF/GASP (*see* Section 11.2.3)
- ❑ Scalpel or single-edged razor blades
- ❑ Curved iris scissors, 11 cm (4-3/8 in.)
- ❑ Jeweler's forceps, 10 cm (4 in.)
- ❑ Corneal scissors, 19-mm blades, sharp tip
- ❑ Colibri suturing forceps, 0.1 mm

Nonsterile

- ❑ Variable-speed rocking mixer

- ❑ Centrifuge, low speed, refrigerated
- ❑ Tube roller apparatus

Procedure

(a) Wash the cornea 3 times in PBSA, 5 min each time.
(b) Trim off the residual sclera, conjunctiva, and iris.
(c) Add 2 ml of 1.2 U/mL Dispase II, shaking gently at 4°C overnight.
(d) Rotate the cornea in Dispase II at 4°C for 30 min.
(e) Wash the cornea once in DMEM/F-12/GASP.
(f) Under a dissection microscope, carefully remove trabecular meshwork, peel off the endothelium, and digest it in trypsin/EDTA at 37°C, 30 min.
(g) Add the same volume of DMEM/F-12/2FB and disperse the cells.
(h) Centrifuge at 400*g* for 10 min and discard the supernate.
(i) Add 1 ml culture medium, gently resuspend the cells, and count in a hemocytometer.

Protocol 11.10. Conventional Culture of Human Corneal Endothelial Cells

Reagents and Materials

- ❑ Culture Medium: CECM (*see* Section 11.2.14)
- ❑ Trypsin/EDTA (*see* Section 11.2.4)
- ❑ Multiwell plate, 6-well

Procedure

- ❑ Seed the cells into 1 well of 6-well plate precoated with FNC.
- ❑ Change the medium every 3 days.
- ❑ When the cells are 90% confluent, passage by trypsinization.

Protocol 11.11. Culture of Endothelial Spheres

Reagents and Materials

- ❑ Culture medium: ESM (*see* Section 11.2.15)
- ❑ Trypsin/EDTA (*see* Section 11.2.4)
- ❑ Non-tissue culture-treated 24-well plate

Procedure

(a) Trypsinize cells from Protocol 11.10.
(b) Resuspend dissociated cells in culture medium at 10 cells/μL and plate on non-tissue culture coated 24-well plate.
(c) After 7 days, cells are collected by centrifugation, dissociated by trypsin, and replated.

11.5.2. Characterization and Differentiation

The endothelial cells have very special morphology, forming a flat hexagonal cell sheet. H&E staining defines the cell shape. For ZO-1, N-CAM, OKT9 immunostaining, wash the dishes with PBSA once, fix with 100% acetone at -20°C for 10 min, wash with PBSA, then stain or store in 50% glycerol-PBSA at 4°C until staining.

11.5.3. Variations and Applications of the Method

Endothelial cells can also be cultured as explants. After peeling off the endothelium, cut it into small pieces of about 1-mm diameter and put the pieces into culture dishes with the endothelium side down. The cells will migrate out of the tissue. After confluence, passage the cells as described above.

11.6. CONCLUSIONS

Keratoplasty is currently the only effective method providing recovery of vision after corneal blindness. Although donated corneal tissue currently meets the needs of most recipients in the U.S., worldwide, 8 to 10 million individuals suffer from corneal blindness without access to therapy. Additionally, numerous individuals reject allogeneic corneal tissue, and the supply of donated corneas may soon be reduced by the increasing number of refractive surgeries, which render the corneas unsuitable for transplantation. Hence, there is significant interest in development of artificial and bioengineered corneas. Griffith et al. [1999] demonstrated that corneal equivalents generated from the three corneal cell layers mimic human corneas in key physical and physiological functions. These studies used immortalized cell lines transformed with retrovirus, making the engineered tissue unsuitable for transplantation. Focus has therefore turned to stem cells as a source of tissues for use in cell-based therapy and corneal tissue engineering. If we can use the stem cells from corneal epithelium, stroma, and endothelium to make artificial corneas suitable for clinical use, millions of patients suffering from corneal blindness will benefit.

11.7. LIST OF MATERIALS AND SUPPLIERS

Product	Catalog Number	Supplier
A/2 half area microtitration plates	3696	Corning
ABI Prism Sequence Detection System software		Applied Biosystems
Acrylamide/Bis 30% solution (37.5:1)	161-0158	Bio-Rad Laboratories
Advanced D-MEM	12491-015	Invitrogen
Amphotericin B	A2411	Sigma-Aldrich
Antibody to ABCG2, FITC-conjugated, clone 5D3	MAB4155F	Chemicon
Antibody FITC-conjugated mouse IgG2b isotype control	CBL 602F	Chemicon
Antibody to ABCG2, clone BXP-21	MAB4146	Chemicon

(*continued*)

Product	Catalog Number	Supplier
Antibody to connexin 43 (Cx43)	71-007	Invitrogen
Antibody to cytoplasmic myosin	CMII 25	Developmental Studies Hybridoma Bank
Antibody to GFAP	MAB360	Chemicon
Antibody to K3	5551-3059	Biogenesis
Antibody to mouse IgG Alexa-488	A11001	Invitrogen
Antibody to p63	ab3239	Abcam
Antibody to PAX6	PRB-278P	Covance
Antibody to rabbit IgG Alexa-546	A11034	Invitrogen
Antibody to β-tubulin	MAB1637	Chemicon
Antibody to transferrin receptor, OKT9/CD-71	14-0719	eBioscience
Antibody to ZO-1	40-220	Invitrogen
Antibody to N-CAM	C9672	Sigma-Aldrich
Antibody, anti-human nuclear	MAB1287	Chemicon
Ascorbic acid-2-phosphate	A8960	Sigma-Aldrich
B27 growth supplement	17504-044	Invitrogen
Cell Strainer, 70-µm nylon mesh	352350	BD Falcon
Cholera toxin	227037	Calbiochem
Colibri suturing forceps, 0.1 mm	555063 FT	World Precision Instruments
Collagenase type L	C8176	Sigma-Aldrich
Corneal scissors, Wescott, 19 mm blades, sharp tip	55541S	World Precision Instruments
Dexamethasone	D1756	Sigma-Aldrich
Dimethyl sulfoxide		Sigma-Aldrich
Dispase II, 100 mL, 2.4 U/mL	10295825001	Roche Applied Science
DNase I, RNase free	222	Ambion
Dulbecco's modified Eagle's medium (high glucose)	11995065	Invitrogen
Dulbecco's modified Eagle's medium (low glucose)	D5521-1	Sigma-Aldrich
Dulbecco's modified Eagle's medium/Ham's Nutrient Mixture F-12 (DMEM/F-12)	D8900	Sigma-Aldrich
Dynabeads Protein G	100-03D	Invitrogen
EGF, human recombinant	E9644	Sigma-Aldrich
Endo-β-galactosidase	100812-1	Seikagaku
EZ-Link Sulfo-NHS-LC-Biotin	21225	Pierce Biotechnology Inc
Fetal bovine serum	SV30014	HyClone
FGF-2	F0291	Sigma-Aldrich
FNC Coating Mix	0407	Athena Environmental Sciences Inc
GeneAmp RNA-PCR kit	N8080143	Applied Biosystems Inc.

Product	Catalog Number	Supplier
Gentamicin	G1379	Sigma-Aldrich
Hanks' balanced salt solution 10×	H4641	Sigma-Aldrich
Hoechst 33342	B2261	Sigma-Aldrich
HotStar *Taq* polymerase	203203	Qiagen
Hydrocortisone	H0396	Sigma-Aldrich
Immu-Mount	9990402	ThermoShandon
Iris Scissors, curved, 4-3/8 in.	#54-6503	Codman
ITS (insulin, transferrin, and selenium)	354350	BD Biosciences
Keratanase II	100455-1	Seikagaku
Leukemia inhibitory factor	ESG1106	Chemicon
Linoleic acid-bovine serum albumin	L8384	Sigma-Aldrich
MCDB-201	M6770-1L	Sigma-Aldrich
Methylcellulose	HSC001	R&D Systems
Millicell microporous membrane tissue culture insert	PixO01250	Millipore Corp.
Mitomycin C	M4287	Sigma-Aldrich
Mixer, variable-speed tilting	M48725	Barnstead/Thermolyne
M-PER lysing agent		Pierce
NIH/3T3 fibroblasts	CRL-2752	ATCC
Paraformaldehyde, 16% solution	M48725	Electron Microscopy Sciences (EMS)
Penicillin-streptomycin solution	P0781	Sigma-Aldrich
Phosphate-buffered saline (10×)	70011-044	Invitrogen
Plastic spatula, "Cell Lifter"	08-773-1	Fisher Scientific
Platelet-derived growth factor	P4056	Sigma-Aldrich
Propidium iodide	P4170	Sigma-Aldrich
Protein G magnetic beads		Invitrogen (Dynal)
all-*trans* retinoic acid	R2625	Sigma-Aldrich
RNeasy Mini kit	74104	Qiagen
sodium pyruvate	S8636	Sigma-Aldrich
SPEC, 3-m NH2 columns	531-07-20	Ansys Diagnostics
Streptavidin-horseradish peroxidase		BD Biosciences (Pharmingen)
Sulfo-NHS-LC-Biotin		Pierce
Superscript II reverse transcriptase	18064-022	Invitrogen
SYBR Gold nucleic acid gel stain (10,000×)	S11494	Invitrogen
SYBR Green RT-PCR reagents	4304886	Applied Biosystems Inc.
TBE (10×)	161-0741	Bio-Rad Laboratories
TE (100×)	BP1338-1	Fisher Scientific
TEMED	17919	Pierce Biotechnology Inc
TGF-β1	T1654	Sigma-Aldrich

(*continued*)

Product	Catalog Number	Supplier
Tissue culture dishes and flasks	various	BD Biosciences (Falcon)
Triiodothyronine	T6397	Sigma-Aldrich
Triton X-100	BP151-500	Fisher Scientific,
TrypLE Express	12604-013	Invitrogen
Verapamil	V4629	Sigma-Aldrich

ACKNOWLEDGMENTS

The authors wish to acknowledge Martha L. Funderburgh for creating the artwork in Fig. 11.1 and for help in proofreading the manuscript. The work was supported by National Institutes of Health Grants EY09368 and EY016415. JLF is a Jules and Doris Stein Research to Prevent Blindness Professor.

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CHAPTER 12

CULTURING MAMMARY STEM CELLS

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Culture of Human Stem Cells, Edited by R.I. Freshney, G.N. Stacey and J.M. Auerbach

12.1. THE NECESSITY OF CULTURING STEM CELLS

Adult stem cells, by the traditional definition, are tissue-specific; they stay dormant until there is a need for repair, expansion, or regeneration and thus, in normal circumstances they are the major guardians of organ homeostasis. To be classified as stem cells, they must meet the basic requirements of self-renewal and differentiation into at least one cell type [Till et al., 1964], giving rise to cells that function appropriately in their tissue of residence. By and large, the ultimate proof that a cell is a stem cell requires the demonstration that a single cell, by clonal expansion, can give rise to a functional tissue in the physiological context of an animal, most often achieved through transplantation experiments in vivo. Studying the biology of adult stem cells in mammals, particularly in humans, is notoriously difficult. These cells are rare constituents within any tissue, difficult to isolate, and in many cases intractable to expansion in culture. Rodents and other animal models, in addition to providing a copious source of genetically homogeneous stem cells, also provide excellent physiological microenvironments to test for their function. In contrast, access to live tissue specimens from humans can be difficult and laborious to obtain, and typically the genetic heritage and history of the specimen is unknown. Perhaps most importantly, short of clinical trials, it is exceedingly difficult to test the function of a putatively isolated stem cell from a human in its native environment, and using immunologically impaired or humanized rodents, while useful, does not always work or present an accurate picture of what happens in humans. Therefore, to facilitate the study of human stem cell biology, one can make the argument that physiologically functional culture models are essential.

Because traditional two-dimensional (2D) cell culture has many artifacts, and the conditions have been compared to a wound healing state [Bissell, 1981], its usefulness is often and understandably challenged by some stem cell biologists [Joseph and Morrison, 2005]. Indeed, culturing primary human cells is difficult, as they are prone to selection and senescence. Nearly four decades of novel work and methods adapted from other fields of study have addressed and solved many of these problems for the study of mammary gland stem cells. Using three-dimensional (3D) laminin-rich extracellular matrices (lrECM) and fibroblast feeder layers or other 3D assays, investigators have successfully recapitulated many key components of stem cell niches in culture models that are more physiologically relevant. In addition to some successful 2D culture approaches, methods for passaging primary mammary stem cells as mammospheres, which were modeled after methods for maintaining neural stem cells in culture, have been described [Dontu et al., 2003]. Methods for immortalizing different subsets of mammary epithelia that seem to allow cells to retain an acceptable normal phenotype, while still bypassing senescence in 2D culture, represent an invaluable advance in this field. Such techniques have fostered more detailed functional and morphological analysis in 3D cultures and in murine implants [Wazer et al., 1995; Gudjonsson et al., 2002b; Kuperwasser et al., 2004]. Together, this panoply of assays has allowed for a highly tractable experimental model system with which to unravel the mysteries of mammary stem cell biology. This

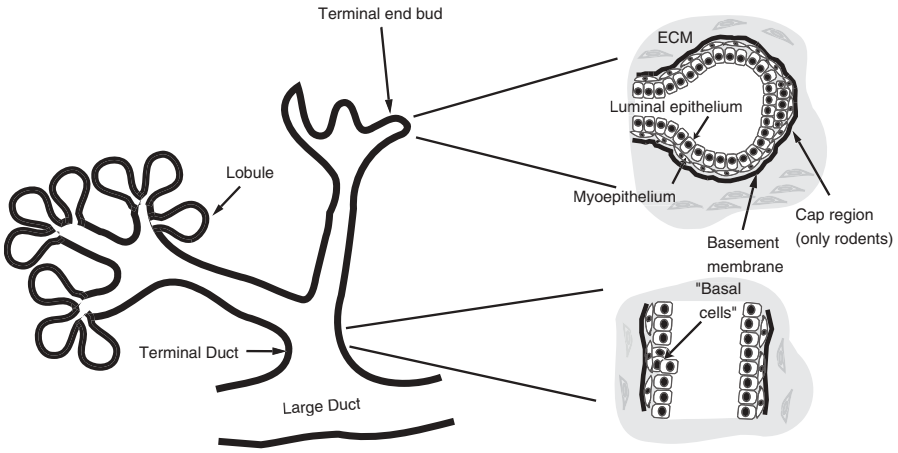
chapter primarily discusses the evidence that mammary stem cells can be cultured and describes the different culture assays that have been used to prove that it can be done.

12.2. EVIDENCE THAT MAMMARY STEM CELLS EXIST

The mammary gland is interesting from the stem cell biologist's point of view because it is an estrogen-dependent organ and the majority of its development occurs during adulthood. Therefore, one can recapitulate the gland's entire developmental history experimentally in adult animals. The mammary gland is a bilayered arbor structure that has at least two basic cell types, keratin $K8^+/18^+$, sialomucin $(Muc)^+$, and epithelial-specific antigen $(ESA)^+$ luminal epithelial cells and $K14^+/5^+$ and α-smooth muscle actin $(\alpha\text{-SMA})^+$ myoepithelial cells [Taylor-Papadimitriou and Lane, 1987; Taylor-Papadimitriou et al., 1989; Petersen and van Deurs, 1988] (Fig. 12.1). During pregnancy, the gland achieves its most functionally differentiated state when the luminal epithelial cells perform copious secretory functions to facilitate the production and delivery of milk. The outermost layer is made of myoepithelial cells, which provide a contractile apparatus that facilitates milk ejection and has been shown to play a crucial role in establishing tissue polarity and, by implication, to act as an architectural tumor suppressor [Gudjonsson et al., 2002a]. A third distinct cell type has also been described. Based on its surface marker profile (MUC^-/ESA^+), presumably it is located in between the luminal epithelial (MUC^+/ESA^+) and myoepithelial (MUC^-/ESA^-) layer [Stingl et al., 1998; Gudjonsson, et al., 2002b]. Evidence for such a location has been provided also from studies of the mouse mammary gland, where they are referred to as small light cells, are abluminal in location, and rest either directly on the basement membrane or on the luminally oriented surface of a myoepithelial cell [Smith and Chepko, 2001]. In the human breast, $K14^+/19^+$ and $\alpha\text{-SMA}^-$ multipotent cells were isolated and were shown to have a suprabasal location in situ [Pechoux et al., 1999]. These suprabasally located cells may be the only cell type that should be referred to as "basal" (much of the literature is confusing in this point, and many refer to myoepithelial cells also as basal) and are thought to represent mammary stem cells [Gudjonsson et al., 2002b].

That mammary stem cells were suspected to exist in the first place was due to observations of the gland's impressive cellular dynamics. In humans, mammary gland growth in adulthood commences at puberty, when the parenchymal cells branch from a few blunt ending primary and secondary ducts into an elaborate tree with multiple terminal ducts and lobules [Medina and Daniel, 1996; Russo and Russo, 2004]. Cell proliferation fluctuates with the menstrual cycle [Potten et al., 1988; Shetty et al., 2005], but during pregnancy there is a 10-fold increase in the number of alveoli as well as de novo formation of lobules by lateral budding from existing terminal ductules [Russo and Russo, 2004]. After pregnancy and following weaning, the gland undergoes a controlled but complete remodeling during involution, resulting in a return to a size close to the prepregnant state. This process can be repeated through multiple pregnancies. These cellular dynamics led [Taylor-Papadimitriou et al. 1983] to postulate that there exists a population of precursor cells in the adult human breast, which can give rise to new lobules.

The methods for prospective identification and isolation of stem cells were pioneered by those studying hematopoietic lineages [Till et al., 1964; Spangrude et al., 1988], and these techniques and concepts have established a paradigm for stem cell discovery and isolation in many other tissues. Studies performed in multiple species have observed



Common identifying markers of mammary epithelial cells

Luminal	"Basal"	Myoepithelial
Keratin 7	Keratin 5	Keratin 5
Keratin 8	Keratin 6a	Keratin 14
Keratin 18	Keratin 14	Vimentin
Keratin 19	Keratin 19	CD10
Muc1	Vimentin	αSMA
ESA	ESA	p63
Sca-1 (mouse)	CD10	
	Sca-1 (mouse)	

FIGURE 12.1. Mammary gland diagram. Both lobules and terminal end buds (not prominent in humans) are shown in the mammary gland diagram. Lobules are prominent during lactation (left branch), while terminal end-buds are more prominent during puberty (right branch). Enlargements of a terminal end-bud and terminal ducts show the putative regions that contain stem cells. Cap regions are primarily found in mice and are absent in humans. "Basal cells" are suprabasally located cells found throughout the gland and are thought to be stem or progenitor cells, but they are less well defined than luminal epithelial myoepithelial cells.

that adult stem cells in general are very focal in distribution and not necessarily colocalized with the bulk of transiently amplifying cells [for review *see* Fuchs et al., 2004]. The location and biochemical identity of mammary stem cells have been perplexing, in part because of the observation that any portion of the mammary gland regenerated an entire new gland when transplanted into a mammary fat pad divested of its epithelium [Deome et al., 1959]. Kordon and Smith [1998] used retroviral insertion mapping to suggest that murine mammary glands could be generated by one cell. Recently this was shown unequivocally in mice at single-cell resolution by two reports demonstrating that populations of $CD29^{+}/CD24^{+}$ [Shackleton et al., 2006] or $CD49f^{+}/CD24^{+}$ [Stingl et al., 2006] mammary epithelial cells, which are depleted for cells expressing blood lineage markers, are enriched for cells capable of clonal repopulation of a cleared fat pad with elaborate bilayered glands that produce milk. Importantly, the clonally derived glands can be serially transplanted into naive hosts, thus demonstrating the self-renewal capacity of the putative stem cell clones. Shackleton et al. [2006] also observed that whereas CD29 and CD24 immunofluorescence colocalized in the cap region of the terminal end-buds,

they were basolateral in the ducts, a finding consistent with previous studies suggesting that the cap region contains the majority of stem cells in mice [*see also* Kenney et al., 2001]. With transgenic mice that express green fluorescent protein (GFP) under the control of the Sca-1 (stem cell antigen-1) promoter, Sca-1^{hi} GFP^{+} mammary cells were enriched for gland-forming ability in cleared fat pads, were more numerous in the distal tips of the growing end-buds, and were observed in the luminal and basal (see first paragraph, this section) positions [Welm et al., 2002]. Using a label-retaining method, Kenney et al. observed putative stem cells in the ducts as well as the terminal end-buds of mice [Kenney et al., 2001]. In mice, therefore, one can conclude that mammary stem cells are $CD24^{+}$/$CD49f^{+}$/$CD29^{+}$/Sca-1^{+}/Lin^{-} cells that are most likely residents of the cap region of terminal end-buds, and potentially in the ducts as well. Once the mammary gland is fully developed around week 12—depending somewhat on the strain—end-buds disappear and little is known about the location of stem cells at this time. In humans, end-buds are transient and at no time are prominent structures [Howard and Gusterson, 2000]. In addition, there is no cap region, and Sca-1 has no reliable human homolog, necessitating the use of different techniques to identify the human mammary stem cell.

For nearly four decades, teams of investigators have been orbiting around the existence and identity of the human mammary stem cells, unable to draw strong conclusions because of the experimental constraints described above. Nevertheless, a number of investigators have isolated mammary epithelial cells, placed them into culture, and subsequently observed putative stemlike behavior. These reports individually were not conclusive, and they typically afforded very little information about the location of the stem cells in the gland or provided a biochemical means to isolate them. Because human stem cells cannot be transplanted into another human to confirm their gland-forming ability, much of our present knowledge about human mammary stem cells has been derived from several different cell culture models that together paint a comprehensive picture; these models are discussed below (*see* Section 12.4).

12.3. MAMMARY CELL CULTURE MEDIA COMPOSITION

12.3.1. CDM3 Basal Medium

From [Petersen et al., 1990]
Add to DMEM/F-12, 1:1:

(i) Sodium selenite — 2.6 ng/mL
(ii) Epidermal growth factor (EGF) — 100 ng/mL
(iii) Hydrocortisone — 0.5 μg/mL (1.38 μM)
(iv) Triiodothyronine — 10 nM
(v) Fibronectin — 100 ng/mL
(vi) Glutamine — 2 mM
(vii) Transferrin — 25 μg/mL
(viii) Dibutyryl cAMP — 10 nM
(ix) Phosphoethanolamine — 0.1 mM
(x) Fetuin — 20 μg/mL
(xi) Ascorbic acid — 0.06 mM (10 μg/mL)

(xii) Bovine serum albumin (fraction V)—— 0.01%
(xiii) HEPES buffer—— 10 mM
(xiv) GIBCO trace element mix—— 1:100
(xv) Insulin—— 3.0 μg/mL
(xvi) 17β-Estradiol—— 0.1 nM
(xvii) Ethanolamine—— 0.1 mM

12.3.2. CDM4

Modify CDM3 basal medium according to [Petersen and van Deurs, 1988] as follows:

(i) Reduce EGF concentration to 20 ng/mL.
(ii) Increase cAMP to 10 μM.
(iii) Add 10 ng/mL cholera toxin.

12.3.3. CDM6

Modify CDM3 basal medium according to [Pechoux et al., 1999] as follows:

(i) Add 10 ng/mL human recombinant hepatocyte growth factor.
(ii) Reduce EGF concentration to 20 ng/mL.

12.3.4. H14

Add to DMEM/F-12, 1:1:

(i) Insulin—— 250 ng/mL
(ii) Transferrin—— 20 μg/mL
(iii) Sodium selenite—— 2.6 ng/mL
(iv) Estradiol—— 0.1 nM
(v) Hydrocortisone—— 1.4 μM
(vi) Prolactin—— 5 μg/mL

12.3.5. Mammosphere Growth Medium

Modify mammary epithelial growth medium (Cambrex MEGM) according to [Dontu et al., 2003] as follows:

(i) B27 supplement—— 2%
(ii) EGF—— 20 ng/mL
(iii) bFGF—— 20 ng/mL
(iv) Heparin—— 4 μg/mL

12.3.6. Mammosphere Differentiation Medium

To Ham's F-12 add the following [Dontu et al., 2003]:

(i) FBS — 5%
(ii) Insulin — 5 μg/mL
(iii) Hydrocortisone — 1 μg/mL
(iv) Cholera toxin — 10 μg/mL
(v) EGF — 10 ng/mL

12.3.7. MCF10A Medium

To DMEM/F-12, 1:1 add:

(i) Horse serum — 5%
(ii) EGF — 20 ng/mL
(iii) Hydrocortisone — 0.5 μg/mL (1.4 μM)
(iv) Cholera toxin — 100 ng/mL
(v) Insulin — 10 μg/mL

12.4. CELL CULTURE MODELS FOR MAMMARY STEM CELLS

12.4.1. Mammary Stem Cells from Rodents

Culturing primary mammary stem cells has served as a gateway technique that subsequently enabled genetic modifications, subtype enrichments, and lineage tracing experiments in cells of the mammary gland. Studies of rodent-derived mammary epithelia, perhaps because of the relative ease of establishing them in culture, paved the way for experiments performed on human tissues. However, many conclusions drawn from the earliest attempts to study mammary stem cell biology in culture are difficult to translate to the situation in vivo because the judgment of multipotency was made based on morphological criteria alone.

Using such criteria, Rudland and colleagues may have isolated, cultured, and characterized one of the first stem cell lines, albeit from rat [Bennett et al., 1978]. Designated RAMA25, these cells were isolated from dimethylbenzanthracene-induced tumors and cultured in serum-containing media, were cuboidal in appearance, and were shown to be capable of giving rise to elongated and droplet-shaped cells (for example, *see* Fig. 12.2). Based on morphological comparison to the mammary gland cell types observed in vivo, the different cells in culture were thought to represent the luminal epithelial, myoepithelial, and secretory luminal cells, respectively. Subsequently, the RAMA25-derived elongated cells were clonally isolated and expanded, designated RAMA29, and shown to give rise only to more elongated cells. Confluent cultures of the RAMA25 cells in medium containing prolactogenic hormones would also yield dome-forming cells that were shown to express casein proteins with polyclonal antisera. When RAMA25 cells were reinjected into mice to form tumors, the authors observed that they gave rise to duct-containing tumors with sarcoma-like and adenocarcinoma-like regions. The tumor heterogeneity suggested a multipotent cellular phenotype, although lacking the specific markers available in

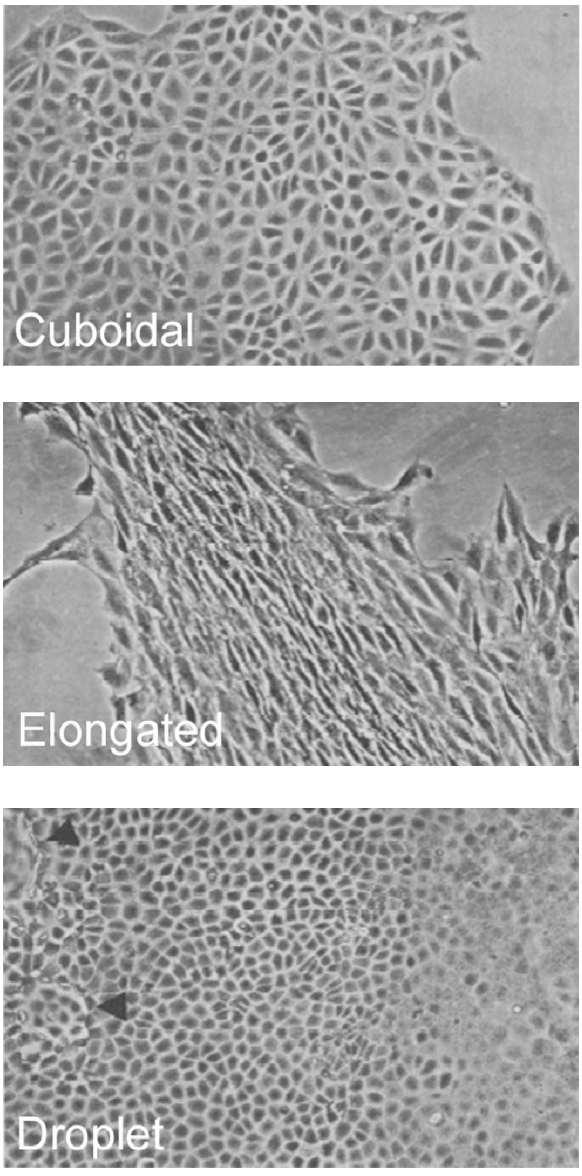


FIGURE 12.2. RAMA25-derived mammary cell types. Colonies of these three morphological phenotypes were described by Bennett et al. [1978]. Domes (arrows) were observed among colonies of droplet-shaped cells that expressed casein proteins (not shown).

more recent mammary stem cell transplant experiments. Interestingly, the RAMA29 cells did not form tumors in mice, which is consistent with the observation that myoepitheliomas in humans are exceedingly rare and differentiated myoepithelial cells generally do not passage well in culture [Petersen and van Deurs, 1988]. The logic represented in this early attempt to define mammary stem cells in culture has persisted and served as a model

for many subsequent studies. However, the techniques have evolved considerably and there has been a consistent movement away from using malignant or transformed cells.

One of the distinctive advantages of using rodent cells in culture experiments is that they can spontaneously immortalize and retain a relatively normal phenotype. The murine COMMA-D cell line was isolated from normal midpregnant mice and displayed the ability to form bilayered glandular outgrowths in cleared mammary fat pads [Medina et al., 1986]. A subline was cloned, designated COMMA-Dβ, that highly expresses Sca-1 and has basal characteristics, that is, K8/18 negative, K5 and K6 positive, and α-SMA negative [Deugnier et al., 2006]. These cells gave rise to luminal and myoepithelial cells in 2D culture, at higher efficiency than their parent line; they could repopulate cleared fat pads, and in 3D lrECM culture assays [Gudjonsson et al., 2002b], they clonally generated spheroids with a distinctive $K8^+$ interior and a $K5^+$ exterior. Whether or not these spheroids had basal polarity or contained lumina was not reported. The spheroids, once dissociated and replated in 3D lrECM, could generate new spheroids, and were thus able to self-renew. The latter point is critical in that there is an important difference between cells proliferating in culture and the concept of self-renewal of stem cells. While cells in culture do divide to make more of themselves, the special property of a stem cell is the ability to maintain itself (self-renewal) while simultaneously generating or regenerating the whole tissue (differentiation). The COMMA-Dβ cell line is a good example of a nearly normal, immortalized murine cell line that retains many characteristics expected of stem cells.

12.4.2. Mammary Stem Cells from Humans

The most common source of normal human mammary epithelia is tissue harvested from cosmetic reduction mammoplasty, which provides a comprehensive starting material consisting of every cellular constituent present in the mammary gland. An alternative cell source is breast milk, which is thought to contain exclusively differentiated cells of the luminal epithelial lineage. Cells from this source have a very limited life span in cell culture, typically undergoing only 1–5 doublings, although some success has been reported with SV40 immortalization [for reviews *see* Ratsch et al., 2001; Dimri et al., 2005]. Because of these limitations, cells from milk are not considered in this chapter, but it is important to know that the source exists. Similar to those described by Rudland and co-workers [Bennett et al., 1978], typically three morphologically distinct cell types will emerge from dissociated mammoplasty tissue in primary culture, but not every preparation will produce all three types every time [Dimri et al., 2005]. Initial successful attempts to culture primary human mammary epithelial cells from reduction mammoplasty tissue used serum-containing medium that was supplemented with hydrocortisone, epidermal growth factor (EGF), estrogen, and progesterone, but the cells were reported to undergo senescence after 1–4 passages [Stampfer et al., 1980]. Refinements in culture media enabled researchers to grow subpopulations of mammary epithelia selectively, either exclusively myoepithelial-like or luminal-like [Petersen and van Deurs, 1988; Taylor-Papadimitriou et al., 1989], and to move toward using serum-free, defined media. Two major advances were the use of cholera toxin as an additive, because of its ability to cause an increase in cAMP [Stampfer, 1982], and reduction of the concentration of calcium $\sim$17-fold in the medium, to $\sim$0.06 mM [Soule and McGrath, 1986]. With these refinements clones could grow as many as 50 generations [Soule and McGrath, 1986]. With

these conditions, the MCF10 cell line was derived from a benign fibrocystic breast disease lesion due to an apparently spontaneous immortalization [Soule et al., 1990; reviewed by Villadsen, 2005] (*see* Section 12.3.7 for medium formulation). Similar to the RAMA25 cell line, MCF10A cells that additionally expressed HA-*ras* exhibited tumor histology that included bilayered ducts, suggesting some stem cell characteristics [Dawson et al., 1996]. A relative newcomer that was derived by means similar to the MCF10 cell line is the MCF15 cell line, which also exhibits multilineage commitment in 2D cultures and in the tumors they form in nude mice [Shen et al., 2006]. However, because they are malignant, these cell lines represent more of a technological advance in culture techniques than a good example of human stem cell biology.

12.4.2.1. Disaggregation and Culture of Cells from Reduction Mammoplasty Tissue. Specimens of reduction mammoplasty tissue can be disaggregated in collagenase. The resulting crude digest can then be separated by centrifugation and sedimentation into three main fractions (Fig. 12.3; Plate 14), each of which can be seeded on Vitrogen-coated 25-cm^2 flasks.

Protocol 12.1. Preparation of Epithelial Cells from Reduction Mammoplasty Specimens

Reagents and Materials

Sterile or aseptically prepared

- ❑ DMEM/F-12, 50:50, with 1.2 mg/mL bicarbonate
- ❑ CMD3 medium (*see* Section 12.3.1)
- ❑ Collagenase, 900 IU/mL in DMEM/F-12
- ❑ Vitrogen
- ❑ Scalpels
- ❑ Culture flasks, 25 cm^2, Vitrogen-coated at 8 $\mu g/cm^2$

Nonsterile

- ❑ Rotary shaker
- ❑ Swinging bucket centrifuge

Procedure

(a) Transfer the tissue specimens to DMEM/F-12 (1:1) immediately after surgery.
(b) Using two scalpels (one in each hand), mince the tissue into ~2-mm cubes.
(c) Digest the minced fragments in collagenase for 24 to 48 h on a rotary shaker (60 rpm) at 37°C.
(d) Centrifuge the crude digest at 175 *g* for a few seconds:
    (i) Excess lipid and most cells with high lipid content float at the top of the supernate and should be discarded.
    (ii) The supernate is comprised of smaller organoids of vascular origin and of single cells.

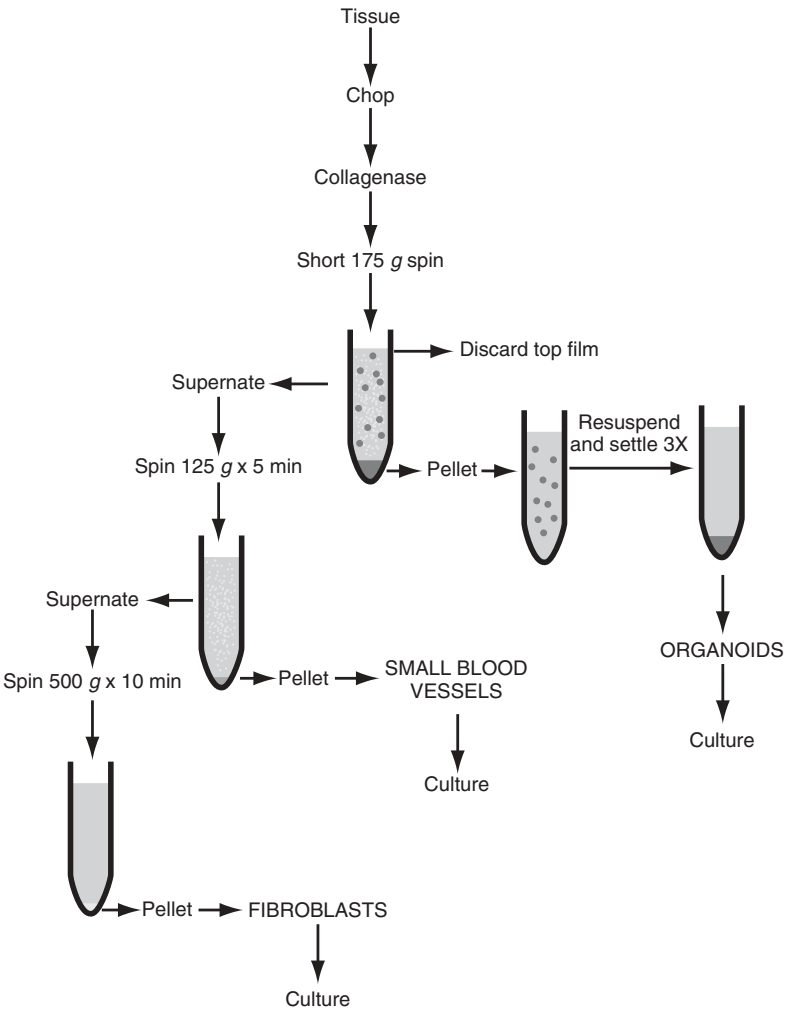


FIGURE 12.3. Selection of organoids. Flow chart of isolation of different fractions by selective sedimentation and centrifugation following collagenase digestion. (*See also* Plate 14.)

(iii) The pellet is comprised of larger organoids of both epithelial and vascular origin.

(e) Resuspend the pellet in 10 mL of DMEM/F-12. Within 30 s the larger epithelial organoids will sediment, leaving blood vessel fragments still suspended in the medium. These should be separated from the epithelial organoids as they will eventually settle to the bottom.

Usually, two additional rounds of resuspending the epithelial organoids in 10 mL of fresh medium followed by sedimentation are sufficient to isolate relatively pure fractions of epithelial organoids from the large blood vessels.

(f) Centrifuge the supernate from Step (d) at 125 *g* for 5 min. The pellet will contain small blood vessels. Remove the supernate and centrifuge at 500 *g* for 10 min. The pellet will contain resident fibroblasts.

(g) Seed the epithelial organoids, and other fractions if required, onto Vitrogen-coated 25-cm^2 flasks.

(h) Maintain the epithelial organoids in CDM3 medium (Section 12.3.1) at 37°C, 5% CO_2, changing the medium three times per week.

As culture models for human primary cells have improved, some potentially interesting mechanistic differences as to how homeostasis in the human mammary gland is maintained have also emerged, namely, hierarchical differentiation vs. interconversion. With flow cytometry, sialomucin-positive, CD10-negative, epithelial-specific antigen-positive ($Muc^+/CD10^-/ESA^+$), $Muc^-/CD10^+/ESA^+$, and $Muc^-/CD10^-/ESA^-$ cellular subpopulations were isolated and their functional potential was analyzed in culture assays [Stingl et al., 1998, 2001]. Two-dimensional cultures of $Muc^-/CD10^+/ESA^+$ primary cells, with calf serum-containing media, yielded mixed colonies with ESA^+/Muc^+ luminal cells at the center surrounded by $K14^+$ teardrop-shaped myoepithelial-like cells at the periphery. Conversely, $Muc^+/CD10^-/ESA^+$ cells gave rise to alveolar-like, $K18^+$ luminal cells, and $Muc^-/CD10^+/ESA^-$ cells gave rise to myoepithelial cells in 2D. When cultured in 3D collagen gels, $Muc^+/CD10^-/ESA^+$ cells gave rise to $K18^+/19^+$ spheroids, whereas $Muc^-/CD10^-/ESA^+$ cells generated spheroids with a $K14^+/\alpha$-SMA^- outer layer and a Muc^+ interior layer when cultured in the absence of EGF [Stingl et al., 1998]. In the presence of EGF, the $Muc^-/CD10^-/ESA^+$ cells generated branching terminal ductal lobular unit (TDLU)-like structures [Stingl et al., 2001]. These data suggest a hierarchical organization in that the $Muc^-/CD10^+/ESA^+$ cells contained a putative stem cell that can give rise to the other epithelial populations in the mammary gland.

In addition to a lineage hierarchy, as the previous experiment suggests, the maintenance of two lineages such as the myoepithelial and luminal epithelial lineages in a dynamic tissue characterized by cellular turnover can occur by conversion of one lineage into the other via undifferentiated intermediates [Wagers and Weissman, 2004]. The fact that both luminal epithelial-like cells and myoepithelial-like cells can become clonal in culture opens the possibility of replenishment of cells by simple self-duplication [Petersen and van Deurs, 1988]. Evidence for the notion of conversion of cells within the luminal epithelial lineage into cells of the myoepithelial lineage via intermediates was provided in 2D culture with high levels of cholera toxin [Kao *et al., 1* 995; Pechoux et al., 1999]. Magnetic beads were used to enrich for Muc-expressing cells or CD10-expressing cells from mammoplasty samples, followed by culture in myoepithelial-specific medium (CDM4) or luminal-specific medium (CDM6) (*see* Sections 12.3.2, 12.3.3 for media formulations) [Pechoux et al., 1999]. Using 2D gel electrophoresis, the authors showed that the CD10-enriched cells grown in CDM4 or CDM6 had myoepithelial characteristics. Whereas Muc-enriched cells cultured in CDM6 were primarily luminal epithelial, they would acquire myoepithelial characteristics when switched into CDM4 medium,. In situ, the putative cellular intermediates capable of the interconversion were then identified as $vimentin^+, \alpha$-SMA^-, $K19^+$ cells that appeared to reside in the suprabasal/luminal position. These data concur with those of others using cultured human breast epithelial cells, albeit purified by means of innate differences in attachment and serum dependence [for review *see also* Chang, 2006]. These observations, however, do not exclude the possibility that adult stem cells mature into the lineages as well, and they may even represent a cautionary tale as to the selective power of cell culture. Which of the different mechanisms, hierarchical differentiation or interconversion, are operating in vivo

is currently unknown. Thus, surprisingly in another organ with two lineages, pancreas, it was shown that terminally differentiated beta cells were replenished exclusively by self-duplication despite numerous reports of a vertical connection to exocrine ductal cells under experimental conditions [Dor et al., 2004]. Because the cellular dynamics are quite different during times when the rudimentary mammary gland is formed versus the massive proliferation-involution cycles experienced during pregnancy, both hierarchical and interconversion mechanisms might be at work in the mammary gland, and it will likely require 3D culture models that mimic the physiological conditions to determine what happens in human breast. In either case, because of their limited growth in primary culture, other methods were developed to explore the full potential of the putative mammary stem cells.

Work from the Band laboratory [Wazer et al., 1995] had demonstrated that whereas multiple genetic alterations are needed to cause malignant transformation, expression of human papilloma virus proteins E6 and E7 causes only immortalization [reviewed in Dimri et al., 2005]. Therefore to bypass the problem of senescence and permit more detailed studies of Muc^-/ESA^+ human breast stem cells, we used infection with the HPV proteins E6 and E7 to facilitate immortalization [Gudjonsson et al., 2002b]. Before transduction with E6/E7, the primary cells were cultured in CDM3 medium, a formulation that supports proliferation of both myoepithelial and luminal epithelial primary cells. The Muc^-/ESA^+ cell line, designated D920, was maintained in the non-cholera toxin-containing H14 medium (*see* Section 12.3.4) and was shown to give rise to both Muc^-/ESA^+ and Muc^+/ESA^+ cells. The D920 cell line was isolated from normal mammary tissue; it does not display a transformed phenotype in culture and does not cause tumors in nude mice. Not only do D920 express K19, thought to be a marker for mammary stem cells, but clones were shown to give rise to cells expressing all combinations of K14 and K19 in 2D culture, and to generate discretely bilayered TDLU-like structures with obvious lumina in lrECM (for example, *see* Fig. 12.4 and Plate 15; *see* Protocol 12.2 for 3D culture in lrECM). The cells were shown to repopulate cleared fat pads in nude mice, confirming the presence of a stem cell among Muc^-/ESA^+ cells [Gudjonsson et al., 2002]. These data support a hierarchical model of mammary development, but also demonstrate that 3D lrECM cultures are an appropriate surrogate microenvironment for testing human mammary stem cells.

12.4.2.2. Generation of Mammospheres. Following the example of methods that were used to perpetuate primary neural stem cells in culture as low-attachment structures called neurospheres [Reynolds and Weiss, 1996], nonimmortalized mammary stem cells were shown to form "mammospheres" [Dontu et al., 2003]. These structures are established on low-attachment plates in serum-free medium that does not contain cholera toxin (*see* Section 12.3.5). Mammospheres that are generated from primary cells derived from reduction mammoplasty specimens can be dispersed on collagen-coated surfaces and observed in 2D cultures to give rise to luminal, myoepithelial, and mixed-phenotype colonies based on their keratin staining profiles. When cultured in 3D lrECM, the mixed colonies generated TDLU-like structures as well as bilayered acini (with both luminal and myoepithelial layers). The acini produced β-casein, a milk protein, indicating the functional differentiation of human breast cells. Regeneration of secondary mammospheres from dispersed primary mammospheres that were also capable of forming TDLU-like structures in 3D lrECM demonstrated self-renewal and stem cell characteristics. The cellular composition of mammospheres was shown to be heterogeneous, suggesting that the

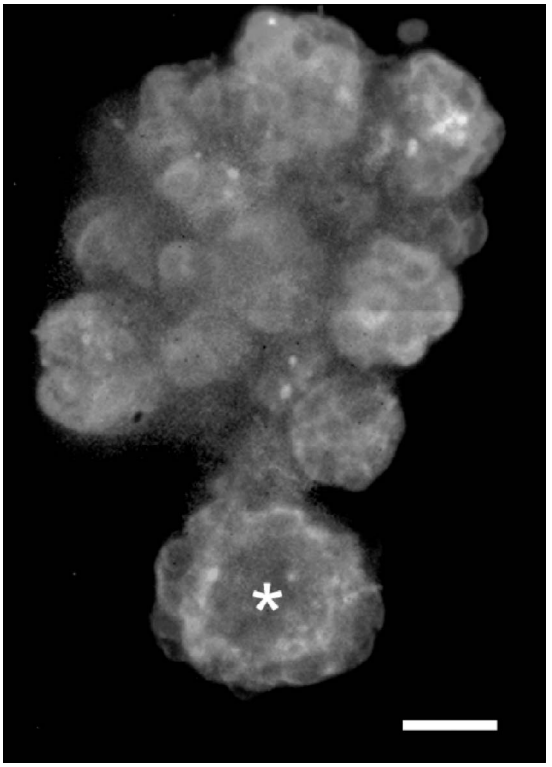


FIGURE 12.4. A TDLU-like structure derived from a Muc^{-}/ESA+/$CD29^{hi}$ D920 cell grown in 3D lrECM culture. The structure was stained in lrECM for K14 (red) and K8 (green) expression and imaged with a confocal microscope. The presented image is a reconstruction of several optical slices to give to appearance of three dimensions. There is a lumen indicated by a star. Scale bar = 50 μm. (*See also* Plate 15.)

mammosphere-initiating cells create a microenvironmental niche appropriate for perpetuating the stem cell.

Protocol 12.2 is for TDLU-forming assays performed with the D920 cell line. However, this basic 3D assay protocol should work for most mammary cells [Petersen et al., 1992].

Protocol 12.2. Three-Dimensional Cultures of Putative Mammary Stem Cells in lrECM

Reagents and Materials

Sterile or aseptically prepared

- ❑ D920 cells or equivalent
- ❑ H14 medium (*see* Section 12.3.4)
- ❑ DMEM/0.5FB/EDTA: DMEM with 0.5% FBS and 0.1 mM EDTA

- ❑ Serum-free DMEM
- ❑ Matrigel
- ❑ Anti-mouse IgG magnetic beads
- ❑ MACS column

Nonsterile

- ❑ Ice bucket with ice

Procedure

(a) Grow D920 cells to 70–80% confluence on collagen I-coated tissue culture flasks in H14 medium.
(b) After trypsinization, resuspend the cells at 1×10^7 cells/mL in DMEM/0.5FB/EDTA.
(c) Incubate with a 1:50 dilution of anti-ESA (VU-1D) for 30 min on ice.
(d) Wash once with 10 mL of serum-free DMEM, then resuspend the cells at 1×10^7 cells/mL in DMEM/0.5FB/EDTA.
(e) Incubate with anti-mouse IgG magnetic beads at the manufacturer's recommended concentration for 30 min on ice.
(f) Wash once with 10 mL of serum-free DMEM then, resuspend the cells at 1×10^7 cells/mL in DMEM/0.5FB/EDTA.
(g) Apply the cell suspension to a MACS column of a size appropriate to your cell number.
(h) Wash the column with DMEM/0.5FB/EDTA medium, using 3× the volume used to apply the sample.
(i) Remove from the magnet and elute with H14 medium.
(j) Keeping your Matrigel on ice, coat the bottom of a 24-well plate with 50 μL per well.
(k) Place at 37°C for 10 min to polymerize.
(l) While the plates are incubating, resuspend 1×10^3–1×10^4 D920 cells in 300 μL of ice-cold Matrigel.
(m) Add to coated wells and incubate for 10 min at 37°C to allow polymerization.
(n) Add H14 medium and change every 2 days for the duration of the assay.

For stem cells in particular, growing structures from single clones is imperative, but not always possible, so some time may be required to work out conditions for each experiment. Some successful approaches have included using low-volume culture vessels such as 384-well plates, conditioned medium, and fibroblast feeder layers [Amit et al., 2004].

Identification of the human mammary stem cell has required specialized culture methods gleaned from multiple fields, and the result is evidence that the human breast is the product of a developmental hierarchy, which can be recapitulated using multiple culture and implantation models. That interconversion can occur in the mammary gland was demonstrated also in 2D cultures, but the evidence is not yet as strong as that for a hierarchical differentiation scheme. The biochemical identity of the human mammary stem cells is most likely $Muc^-/ESA^+/CD10^{+/-}/K19^+/K14^+/\alpha\text{-}SMA^-/vimentin^+$. Based on the observation that human mammary glands do not have terminal end-buds and that the

alveoli are not particularly numerous until pregnancy, it would also seem likely that the stem cells are located in the ducts. At the time of writing, there were not strong data to indicate that CD29, CD24, and CD49f mark human mammary stem cells, as they do in mice.

12.5. CONFLICTING RESULTS OR INFORMATIVE DIFFERENCES?

The combination of multiple 2D and 3D culture models can be powerful tools to study human stem cell populations; however, there is always some cause for caution when interpreting the data. Comparing outcomes in culture experiments of human cells either with outcomes from transgenic and knockout experiments or with primary uncultured human cells has yielded some stark differences. Although confusing, if taken at face value, the differences are also informative, and some of these are described below.

12.5.1. The Side Population

The side population (SP) was first identified in hematopoietic tissues as a subpopulation of cells that could efflux Hoechst 33342 dye efficiently, and efflux was sensitive to verapamil [Goodell et al., 1996]. SP cells isolated from the bone marrow are highly enriched for hematopoietic stem cell activity, and in the absence of surrogate markers, this method has been used to isolate cells from other adult tissues that demonstrate stem cell activity. Accordingly, an SP isolated from mouse mammary epithelial cells that also stained with Sca-1 was shown capable of repopulating a cleared mammary fat pad after 3 days in culture [Welm *et al., 2*002]. In contrast, two other laboratories demonstrated that the SP isolated from fresh, uncultured, mouse mammary epithelial cells did not contain cells with mammary stem cell activity [Shackleton et al., 2006; Stingl et al., 2006]. In cells from human tissue, mammosphere-forming ability is a property attributed to the stem cell population, and SP cells from tissues that did not undergo any intervening culture were enriched for mammosphere-forming ability [Dontu et al., 2003]. The difference between the mouse experiments could be explained by differences in mouse strains or staining protocols, and the difference between uncultured mouse and human cells could be species related. To be sure, comparative experiments need to be performed simultaneously with similar techniques. Nevertheless, these observations may suggest that the SP does not enrich for the most primitive stem cell in the mammary gland, but instead for a vertically related short-term multipotent progenitor that is possibly orthologous to the hematopoietic short-term repopulating cells. If so, then culture conditions would favor the propagation of the earliest transit amplifying progenitor and not the primitive stem cell. Thus the SP may enrich for multipotent progenitors within cultured mammary epithelia, or for multipotent progenitors that can be cultured as mammospheres from primary tissue.

12.5.2. Three-Dimensional Culture Substrata

It is known that gene expression can be changed by microenvironmental determinants [for a mammary-centric review *see* Bissell et al., 2005]. It is therefore not surprising that different 3D microenvironments might elicit different effects from putative mammary stem cells. Multiple investigators have shown that 3D lrECM gels composed either of laminin-1 added to collagen I gels or of Matrigel generate a suitable environment for both human and mouse mammary epithelial cell differentiation [Barcellos-Hoff et al., 1989;

Petersen et al., 1992]. The concept has now been extended to primary and immortalized mammary stem cells isolated from mice [Deugnier et al., 2006; Shackleton et al., 2006; Stingl et al., 2006] and humans [Gudjonsson et al., 2002b; Dontuh et al., 2003]. In this microenvironment, the cells were capable of forming complex TDLU-like structures that showed discrete bilayers of luminal and myoepithelial cells as well as milk production as discussed above. With the collagen gels, putative human mammary stem cells, isolated in a similar manner to those above, formed branching structures that were described as having a $K14^+$ outer layer of myoepithelial cells surrounding an interior of sporadically located $K18/19^+$ luminal cells, but these authors did not report the presence of a lumen or α-SMA expressing myoepithelial cells [Stingl et al., 2001]. Previously we showed that human luminal epithelial cells cultured in collagen I gels have "reverse" polarity that could be corrected with addition of either laminin-1 (but not laminin-5 or 10/11) or myoepithelial cells [Gudjonsson et al., 2002a]. Together, these experiments underscore the important differences between 3D-laminin-rich and collagen-gel cultures. Thus the signaling response to lrECM is not simply growth arrest, but also establishment of correct tissue polarity. These results suggest that collagen gels do not allow complete differentiation of TDLU-like structures from human mammary stem cells, and that lrECM more closely approximates the mammary microenvironment. Note that this outcome of ECM/cell interaction may be tissue specific since, at least in MDCK cells, collagen I generates polarity [O'Brien et al., 2001].

12.5.3. Signaling in Stem Cells

The bulk of our understanding of signal transduction in stem cells has been modeled upon studies performed in other cell types, such as fibroblasts, and on retrospective studies of transgenic and knockout animals. The recent advances described above are facilitating the ability to test the outcomes of modulating signal transduction in mammary stem cells. By the use of mammospheres in combination with cDNA microarray analysis, the Notch pathway was implicated as playing an important role in directing mammary stem cell function [Dontu et al., 2003]. It was shown that activation of the Notch pathway increased the number of secondary mammospheres and favored differentiation of the stem cells toward the myoepithelial lineage when the mammospheres were dispersed onto collagen-coated 2D culture substrata [Dontu et al., 2004]. Conversely, blockade of the Notch-4 receptor with an antibody or blockade of all the Notch pathways with gamma secretase inhibitor resulted in decreased secondary mammosphere formation. The conclusion drawn was that stimulation of the Notch pathway promotes self-renewal of mammosphere-forming cells and differentiation into myoepithelial cells [Dontu et al., 2004]. In contrast, a mouse knockout model of the RBP-Jκ gene, which acts as a key signaling intermediate for all four Notch receptors in mammals, showed that the virgin glands of the mice appeared to be normal, but when the gland differentiated during pregnancy they lacked luminal cells. These authors concluded that Notch pathway activation is required for luminal maintenance [Buono et al., 2006]. The difference between human and mouse cells, or in vivo versus in culture, are possible explanations. However, that the mammary microenvironment is largely composed of laminins and that the mammospheres were cultured on collagen could also explain the difference. As noted above with respect to 3D culture environments, laminin-1 determinants are most likely necessary cofactors for mammary cells to integrate differentiation signals appropriately (*see also* Weaver et al. [2002] for the importance of correct tissue polarity in mammary function).

12.6. CONCLUSIONS

It is neither possible nor ethical to perform genetic engineering experiments in humans; thus development of human mammary cell culture techniques has been crucial and indeed necessary to locate, isolate, and characterize putative human mammary stem cells. Clearly, the models will need to be improved to even more accurately reflect the microenvironment so that existing and yet-to-be developed powerful culture techniques can be brought to bear on a number of new questions:

How do stem cells integrate signals that control their functions and behavior?

What role does the microenvironment (other cells, hormones, growth factors, ECM molecules—in short the niche) play in normal mammary gland morphogenesis and in the initiation and propagation of tumorigenesis?

How do stem cells relate to cancer?

What is the cellular etiology of different types of breast cancers?

It has been observed that histological subtypes in cancers emerge that appear lineage restricted, suggesting that stem and progenitor cells might lie at the cancer's origin. This scenario has been documented in the evolution of two distinct leukemias: Chronic myeloid leukemia is derived from hematopoietic stem cells, and acute myeloid leukemia is derived from committed granulocyte-macrophage progenitors [Daley, 2004; Jamieson et al., 2004]. Stemlike cells, isolated from a breast tumor, could be serially transplanted as a tumor in nude mice, but whether these were directly related to normal mammary stem or progenitor cells is not known [Al-Hajj et al., 2003]. It was additionally hypothesized that the stem cell microenvironment may play an important initiating or promoting role in tumorigenesis [reviewed in Bissell and Labarge, 2005]. Now that a hierarchy among mammary stem and progenitor cells has been identified for rodents and, more recently, for humans [Villadsen et al., 2007], as well as their respective locations within the mammary gland, a detailed analysis of their transformed phenotypes and what roles their microenvironments play in fostering specific behaviors should take place. One particular challenge will be providing unequivocal proof that one normal stem cell does give rise to a mammary gland, and that the same cell, if transformed, gives rise to a tumor in vivo. Because 3D culture environments can model form and functions as complex as TDLU formation from single cells, they may also provide a malleable proving ground to demonstrate such a relationship.

12.7. SOURCES OF MATERIALS

Item	Supplier
Anti-mouse IgG magnetic beads	Miltenyi Biotech
Ascorbic acid	Sigma
B27 supplement	Invitrogen (GIBCO)
Bovine serum albumin (fraction V)	Sigma
Cholera toxin	Sigma
Collagenase	Sigma
Culture flasks, 25 cm^2	Nunc
Dibutyryl cAMP	Sigma

Item	Supplier
DMEM	Invitrogen (GIBCO)
Epidermal growth factor (EGF)	BD Biosciences
17β-Estradiol	Koch-Light Ltd., Suffolk, UK
ESA (VU-1D9)	NovoCastra
Ethanolamine	Merck
Fetuin	Sigma
bFGF (FGF-2)	Sigma
Fibronectin	Sigma
Glutamine	Sigma
Ham's F-12	Invitrogen (GIBCO)
Heparin	Sigma
Hepatocyte growth factor, human recombinant	Sigma
HEPES buffer	Invitrogen (GIBCO)
Horse serum	Atlanta Biologicals
Hydrocortisone	BD Biosciences
Insulin	Sigma
MACS column	Miltenyi Biotech
Mammary epithelial growth medium, MEGM	Cambrex (previously BioWhittaker)
Matrigel	BD Biosciences
Phosphoethanolamine	Sigma
Prolactin	Sigma
Sodium selenite	BD Biosciences
Trace element mix, GIBCO	Invitrogen
Transferrin	Sigma
Triiodothyronine	Sigma
Vitrogen-100	Cohesion Technologies

ACKNOWLEDGMENTS

Our work has been supported by the Danish Cancer Society, Dansk Kræftforskningsfond, Danish Research Council, Fru Astrid Thaysens Legat for Lægevidenskabelig Grundforskning, The Meyer Foundation, The Novo Nordic foundation, the European Commission Research Directorates (contract HPRN-CT-2002-00246), Bernhard Rasmussen og hustru Meta Rasmussens fond (to LR-J) and US National Cancer Institute (Grant CA-64786 to M. J. B. and O.W.P.), and by an Innovator award from the US DOD and by a grant from the OBER office of the USDOE (to M. J. B.). M.A.L was supported by a postdoctoral fellowship from the American Cancer Society.

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CHAPTER 13

TISSUE CULTURE OF ADIPOSE-DERIVED STEM CELLS

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Culture of Human Stem Cells, Edited by R.I. Freshney, G.N. Stacey and J.M. Auerbach

13.1. BACKGROUND

13.1.1. Context

Reservoirs of stem and progenitor cells exist in several types of adult tissue, including skin, muscle, bone marrow, and fat [Adachi et al., 2002; Nnodim, 1987; Caplan, 1994; Prockop, 1997; Nicoll et al., 2001; Weissman et al., 2001; Young et al., 2001]. Growing evidence suggests that these cells may retain multilineage potential and are capable of giving rise to cell lineages other than those of the resident tissue. Recently, adult adipose tissue has become recognized as a alternative and rich source of mesenchymal stem cells [Gimble et al., 1992; Halvorsen et al., 2000, 2001a; Zuk et al., 2001; Erickson et al., 2002; Safford et al., 2002; Awad et al., 2003]. These cells have been termed adipose-derived stem cells (ASCs). Under specific culture conditions, ASCs can be induced to differentiate into various mesenchymal and neural lineages.

13.1.2. Objectives

Adipose tissue is the most abundant and accessible source of adult stem cells [Gimble and. Guilak, 2003]. The isolation of a population of progenitor cells from adipose tissue was first described in 1964 by Rodbell, whose work was done in rodents [Rodbell, 1964]. This procedure for cell isolation was adapted in later years to isolate progenitors from human adipose tissue [Hauner et al., 1989; Moore et al., 1995; Lalikos et al., 1997]. In 2001, Halvorsen et al. published a modification of existing isolation methods using liposuction waste as a starting material, demonstrating the potential of this abundant and replenishable tissue source for future clinical therapies [Halvorsen, et al., 2001a; Zuk et al., 2001].

13.1.3. Rationale for Choosing ASCs for Differentiation

Current methods for isolating ASCs from adipose tissue vary slightly among investigators but generally rely on a collagenase digestion followed by centrifugal separation to isolate the stromal/vascular cells from primary adipocytes. Differential centrifugation separates floating mature adipocytes from the pellet of stromal/vascular cells. This pellet contains blood cells, fibroblasts, pericytes, and endothelial cells in addition to ASCs [Gimble and Guilak, 2003; Hauner et al., 1989; Deslex et al., 1987]. This stromal/vascular fraction is plated on plastic tissue culture dishes. Stromal cells adhere to plastic, and during further culture nonadherent hematopoietic and other contaminating cells can be depleted. The

final population of ASCs can be maintained in an undifferentiated state for extended periods.

ASCs display a fibroblast-like morphology and lack intracellular lipid droplets seen in adipocytes. After expansion in culture, ASCs display a distinct phenotype based on cell surface protein expression and cytokine expression [Gronthos et al., 2001]. This phenotype is similar to that described for marrow-derived stromal cells and skeletal muscle-derived stem cells [Zuk et al., 2001, 2002; Gronthos et al., 2001; Williams et al., 1994; Young et al., 1999]. Adipose tissue is a rich source of stem cells, as the frequency of stem cells within adipose tissue range from 1:100 to 1:1500 cells, which far exceeds the frequency of MSCs in bone marrow [De Ugarte et al., 2003; Kral and Crandall, 1999].

13.2. PREPARATION OF MEDIA AND REAGENTS

13.2.1. Media

13.2.1.1. Adipose-Derived Stem Cell Culture Medium (ASC Medium). ASC medium is Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin, 100 U/mL and streptomycin, 100 μg/mL.

13.2.1.2. Neural Induction Medium.

(i) DMEM — 1×
(ii) Butylated hydroxyanisole in EtOH — 36 μg/mL (0.2 mM)
(iii) KCl — 5 mM
(iv) Valproic acid in water — 2 mM
(v) Forskolin in DMSO — 10 μM
(vi) Hydrocortisone in EtOH — 1 μM
(vii) Insulin in water — 5 μg/mL
(viii) Penicillin — 100 U/mL
(ix) Streptomycin — 100 μg/mL

13.2.1.3. Adipocytic Induction Medium.

(i) DMEM/F12 — 1×
(ii) FBS — 3%
(iii) Biotin — 33 μM
(iv) Pantothenate — 17 μM
(v) Bovine insulin — 1 μM
(vi) Dexamethasone — 1 μM
(vii) Isobutylmethylxanthine — 0.25 mM
(viii) Rosiglitazone — 2 μM
(ix) Penicillin/streptomycin — 100 U/mL, 100 μg/mL

13.2.1.4. Adipocyte Maintenance Medium. Identical to the induction medium (*see* Section 13.2.1.3) but without isobutylmethylxanthine and rosiglitazone

(i) DMEM/F12 — 1×
(ii) FBS — 3%
(iii) Biotin — 33 μM
(iv) Pantothenate — 17 μM
(v) Bovine insulin — 1 μM
(vi) Dexamethasone — 1 μM
(vii) Penicillin/streptomycin — 100 U/mL, 100 μg/mL

13.2.1.5. Osteogenic Induction Medium.

(i) DMEM (high glucose) — 1×
(ii) FBS — 10%
(iii) β-Glycerophosphate — 10 mM
(iv) Ascorbate-2-phosphate — 0.2 mM (50 μg/mL)
(v) 1,25-$(OH)_2$ vitamin D_3 — 10 nM
(vi) Dexamethasone — 10 nM
(vii) Penicillin/streptomycin — 100 U/mL, 100 μg/mL

13.2.1.6. Chondrogenic Induction Medium.

(i) DMEM (low glucose) — 1×
(ii) Sodium pyruvate — 110 mg/L
(iii) ITS+ — 1%
(iv) Ascorbate-2-phosphate — 0.15 mM
(v) TGF-β1 — 10 ng/mL
(vi) Dexamethasone — 100 nM
(vii) Penicillin/streptomycin — 100 U/mL, 100 μg/mL

13.2.2. Trypsin

Dilute trypsin stock (0.25%) 1:10 with Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBSA) to give final concentration of 0.025%.

13.2.3. Collagenase Type II

Add 1 g of bovine serum albumin (BSA) to 0.1 g of collagenase type II per 100 mL of Hanks' buffered salt solution (HBSS).

13.3. TISSUE HARVEST AND CELL ISOLATION

Human and murine adipose tissues can both serve as sources for ASC isolation, and both yield substantial numbers of ASCs. Human adipose tissue is generally obtained as liposuction waste. Murine adipose tissue is harvested from the inguinal fat pads.

13.3.1. Harvest of Murine ASCs from Inguinal Fat Pads

Protocol 13.1. Harvest of Murine Adipose Tissue

Reagents and Materials

Sterile

- ❑ Hanks' buffered salt solution (HBSS)
- ❑ ASC medium (*see* Section 13.2.1)
- ❑ Collagenase solution (*see* Section 13.2.3)
- ❑ Betadine iodine solution
- ❑ Petri dishes, 9 cm
- ❑ Centrifuge tube, 50 mL
- ❑ Tissue culture flask, 25 cm^2
- ❑ Scissors (2 pairs)
- ❑ Forceps (2 pairs)
- ❑ PBSA

Nonsterile

- ❑ Mice, 4–6
- ❑ Anesthetic: TBE (tribromoethylene)
- ❑ Water bath at 37°C

Procedure

(a) Warm HBSS and ASC medium in 37°C water bath.
(b) Anesthetize and euthanize animals.
(c) Transfer anesthetized animals to laminar flow hood.
(d) Swab abdomen with 70% alcohol.
(e) Open abdomen through low transverse incision.
(f) Using forceps, remove gonadal/epididymal and inguinal fat pads.
(g) Place fat in Petri dish with HBSS until all animals are sacrificed.
(h) Transfer fat to new Petri dish.
(i) Add HBSS + 5% Betadine iodine solution for 2–3 min.
(j) Rinse tissue by aspirating Betadine solution and washing fat with HBSS.
(k) Place fat in 50-mL centrifuge tube with sterile forceps.
(l) Finely mince fat with sterile scissors.
(m) Add one tissue volume of collagenase solution and mix.
(n) Place tube in 37° water bath for 60 min, swirling occasionally.
(o) Centrifuge sample at 50–100 *g* for 5 min.
(p) Remove tube from centrifuge, shake vigorously (to complete separation of stromal cells from primary adipocytes), and centrifuge again for 5 min.
(q) Carefully aspirate the oil on top, which includes primary adipocytes. Do not disturb the stromal-vascular fraction at the bottom.

(r) Add 5–10 ml of PBSA; resuspend pellet; centrifuge again for 5 min.
(s) Wash and spin 3 more times, being careful not to aspirate stromal-vascular fraction.
(t) After last wash, resuspend pellet in 8 ml of ASC medium and plate in 1 T-25 flask; place at 37°C under 5% CO_2.
(u) Allow cells to attach and grow for 2–4 days before changing medium.
(v) Change medium, removing nonadherent cells with spent culture medium.
(w) Change the medium twice weekly thereafter.

13.4. ADIPOSE STEM CELL CULTIVATION AND EXPANSION

13.4.1. ASC Culture

Once isolated, ASCs can be grown and expanded by serial passage. Within 2–3 passages of the initial plating of the primary culture, ASCs cells appear as a monolayer of large, flat cells, 25–30 μm in diameter (Fig. 13.1). As the cells approach confluence, they assume a more spindle-shaped, fibroblastic morphology.

Protocol 13.2. Primary Culture of Adipose Stem Cells

Reagents and Materials

- ❑ ASC medium (*see* Section 13.2.1.1)
- ❑ Tissue culture flasks, 25 cm^2

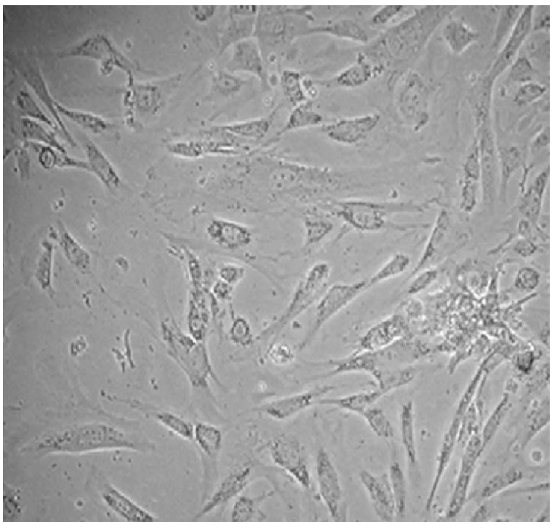

FIGURE 13.1. ASCs grown under control conditions grow as a monolayer of large, flat cells. Magnification 200×.

Procedure

(a) Allow cells to attach and grow for 2–4 days before changing medium.
(b) Change medium, removing nonadherent cells with spent medium.
(c) Change medium twice weekly thereafter.

13.4.2. ASC Expansion

ASCs can be expanded by serial passage. Primary cultures of stromal cells isolated from adipose tissue include a small number of hematopoietic cells, pericytes, endothelial cells, and smooth muscle cells [Zuk et al., 2001]. The frequency of these other cells appears to diminish quickly through serial passages in culture, and in particular, cells expressing either hematopoietic or epithelial markers are not present by 2–3 passages of culture [Safford et al., 2002; Gronthos et al., 2001].

Protocol 13.3. Subculture of Adipose Stem Cells

Reagents and Materials

- ❑ ASC medium
- ❑ PBSA
- ❑ Tissue culture flasks, 25 cm^2 (T-25)
- ❑ Trypsin (*see* Section 13.2.2)
- ❑ Cell scraper

Procedure

(a) Subculture cells once they reach approximately 80% confluence.
(b) Remove medium and rinse adherent cells with PBSA.
(c) Add enough trypsin to cover cells; place flasks at 37°C under 5% CO_2.
(d) After cells have been incubated with trypsin for 3 min at 37°C, scrape flask with cell scraper.
(e) Inactivate trypsin with ASC medium.
(f) Reseed all the cells into 2 T-25 flasks, giving a split ratio of 1:2.

13.5. CHARACTERIZATION AND DIFFERENTIATION OF ASCS

Once in culture, ASCs can be maintained in an undifferentiated state for extended periods. ASCs display a fibroblast-like morphology, lacking the intracellular lipid droplets seen in adipocytes. After expansion in culture, ASCs display a distinct phenotype based on their cytokine and cell surface protein expression [Gronthos et al., 2001]. This phenotype is similar to that described for marrow-derived and skeletal muscle-derived stem cells [Zuk et al., 2001, 2002; Gronthos et al., 2001; Williams et al., 1994b; Young et al., 1999].

13.5.1. Neural Differentiation of Adipose Stromal Cells

Similar to findings seen with bone marrow-derived stromal cells, recent studies have reported the differentiation of ASCs into neuronlike cells [Safford et al., 2002, 2004; Zuk et al., 2002; Kang et al., 2003]. Neuronal differentiation of ASCs can be achieved by several different techniques but generally involves exposing ASCs to a cocktail of induction agents. The induction media used by Safford et al. [2002] and Zuk et al. [2001, 2002] are similar, including the use of butylated hydroxyanisole, valproic acid, and forskolin. A recent study by Kang et al. employs a different induction protocol [Kang et al., 2003]. These researchers first expose the ASCs to 5-azacytidine, a demethylating agent capable of affecting gene expression [Holiday, 1996]. Further differentiation is achieved by maintaining the cells in neurobasal medium containing B27 supplement [Brewer et al., 1993] (*see* Section 13.6). In another study, Ashjian et al. treated ASCs with indomethacin, isobutylmethylxanthine, and insulin to induce neural differentiation [Ashjian et al., 2003]. Protocol 13.4 below follows the method used by [Safford et al., 2002] and [Zuk et al., 2001].

Within several hours of exposure of ASCs to neural induction medium, many cells display changes in cellular morphology, with retraction of cytoplasm toward the nucleus and formation of compact cell bodies with cytoplasmic extensions. The majority of ASCs exposed to neural induction medium become increasingly spherical and refractile, with a perikaryon suggestive of a primitive neuronal/glial phenotype. These morphological changes can be seen as early as 5 h after exposure to neural induction medium [Safford et al., 2002].

As determined by immunohistochemistry, neuronal markers expressed by neuronally induced ASCs include NeuN, MAP2, tau, β-III tubulin, and NSE. Expression of glial markers including glial fibrillary acidic protein (GFAP), vimentin, and S100 are also demonstrated by immunocytochemistry. Expression of the oligodendrocyte marker O4 has not been seen on neurally induced ASCs in vitro. The percentage of ASCs expressing neuronal phenotypic markers in vitro is relatively high, with some markers such as NeuN seen in approximately 80% of ASCs exposed to neuronal induction media. The high percentage of cells expressing protein markers suggests that the majority of the cells in this heterogeneous population are undergoing at least a degree of neural differentiation. Further, the coexpression of NeuN and GFAP in some cells suggests that at least in short-term culture, some ASCs may retain the potential for neuronal as well as glial development.

Protocol 13.4. Neural Differentiation of Adipose Stem Cells

Reagents and Materials

- ❑ Neural induction medium (*see* Section 13.2.2.2)
- ❑ PBSA

Procedure

(a) Aspirate medium.
(b) Rinse adherent cells with PBSA.
(c) Add neural induction medium and return flasks to incubator.

13.5.2. Adipogenic Differentiation of ASCs

With a technique originally developed by Halvorsen et al., ASCs can be induced to express adipogenic markers [Halvorsen et al., 2001a; Guilak et al., 2006]. ASCs are exposed to adipogenic induction medium for three days, at which point the medium is changed to adipocyte maintenance medium [Guilak et al., 2006]. Under adipogenic conditions, ASCs demonstrate perinuclear lipid droplets and expression of differentiation-specific genes including aP2, PPAR2, and C/EBP [Erickson et al., 2002; Halvorsen et al., 2001a,b].

Protocol 13.5. Adipocytic Differentiation of Adipose Stem Cells

Reagents and Materials

- ❑ Adipocytic induction medium (*see* Section 13.2.1.3)
- ❑ Adipocyte maintenance medium (*see* Section 13.2.1.4)
- ❑ PBSA

Procedure

(a) Aspirate medium.
(b) Rinse adherent cells with PBSA.
(c) Add adipocytic induction medium and return flasks to incubator.
(d) After 3 days, change medium to adipocyte maintenance medium.

13.5.3. Osteogenic Differentiation of ASCs

With a technique originally developed by Halvorsen et al., ASCs can be induced to express osteogenic markers, demonstrating potential for tissue engineering applications [Halvorsen et al., 2000; Halvorsen, 2001a; Guilak et al., 2006].

Protocol 13.6. Osteogenic Differentiation of Adipose Stem Cells

Reagents and Materials

- ❑ Osteogenic induction medium (*see* Section 13.2.1.5)
- ❑ PBSA

Procedure

(a) Aspirate medium.
(b) Rinse adherent cells with PBSA.
(c) Add osteogenic induction medium and return flasks to incubator.

13.5.4. Chondrogenic Differentiation of ASCs

ASCs have also been shown to differentiate in vitro into cells with a chondrogenic phenotype. After exposure to chondrogenic induction medium, ASCs express lineage-specific proteins, transcription factors, and genes that are not seen on ASCs before exposure [Zuk et al., 2003; Gimble and Guilak, 2003; Gimble, 2003].

Protocol 13.7. Chondrogenic Differentiation of Adipose Stem Cells

Reagents and Materials

- ❑ Chondrogenic induction medium (*see* Section 13.2.1.6)
- ❑ PBSA

Procedure

(a) Aspirate medium.
(b) Rinse adherent cells with PBSA.
(c) Add chondrogenic induction medium and return flasks to incubator.

13.6. SOURCES OF MATERIALS

Item	Supplier
Ascorbate-2-phosphate	Sigma
B27 supplement	Invitrogen
Biotin	Sigma
Bovine insulin	Sigma
Butylated hydroxyanisole	Sigma
Collagenase type I	Sigma
Dexamethasone	Sigma
DMEM/F-12	Invitrogen
Fetal bovine serum	Invitrogen
Forskolin	Sigma
β-Glycerophosphate	Sigma
Hanks' buffered salt solution (HBSS)	Invitrogen (GIBCO)
Hydrocortisone	Sigma
Insulin	Invitrogen
Isobutylmethylxanthine	Sigma
ITS+	Sigma
KCl	Sigma
Pantothenate	Sigma
Penicillin/streptomycin	Invitrogen
Phosphate-buffered saline (PBSA)	Invitrogen
Rosiglitazone (Avandia)	GlaxoSmithKline
Sodium pyruvate	Sigma

Item	Supplier
TGF-β1	Invitrogen (GIBCO)
Trypsin	Invitrogen
Valproic acid	Sigma
1,25-$(OH)_2$ Vitamin D_3	Sigma

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SUPPLIERS

Supplier	*Web address*	*Products cited*
Abcam	www.abcam.com	Antibody to p63
Ambion	http://www.ambion.com/	DNAse I, RNase free
American Type Culture Collection (ATCC)	www.atcc.org	STO cells (CRL-1503); NIH/3T3 fibroblasts
Ansys Diagnostics	http://www.varianinc.com	SPEC, 3-m NH2 columns
Applied Biosystems Inc	http://www.appliedbio-systems.com/	GeneAmp RNA-PCR kit; SYBR Green RT-PCR reagents.
ATCC *see* American Type Culture Collection		
Autogen Bioclear	www.autogenbioclear .com	Antibodies: mouse monoclonal anti-human CD45, CD33, CD7 anti-glycophorin-A; fetal bovine serum (FBS); ES-grade FBS (ESFBS)
Barloworld Scientific	www.barloworld-scientific.com/	Universal containers, 30 mL
Barnstead/Thermolyne	www.barnsteadthermo-lyne.com	Mixer, variable-speed tilting
Baxter	www.baxter.com	Acid citrate dextrose formula-A (ACD-A)
BD Biosciences	www.bdbiosciences.com/	bFGF; Cell Strainer; centrifuge tubes; ITS (insulin, transferrin, and selenium); Matrigel; nylon mesh; penicillin/streptomycin; tissue culture dishes and flasks
Bibby Sterilin *see* Barloworld Scientific		
Biogenesis	http://www.biogenesis .co.uk	Antibody to K3
Bio-Rad Laboratories	http://www.bio-rad.com	Acrylamide/Bis 30% solution (37.5:1); TBE (10×)
Biosource (*see* Invitrogen)		
Boehringer Mannheim (*see* Roche Diagnostics)		
BRB Industrial Services	www.brb-industrial.co.uk/	Chain mail gloves
Calbiochem-Novabiochem	www.emdbiosciences.com	Cholera toxin; Fluorosave

Cambrex (now part of Lonza Group)	www.cambrex.com/bio-products	Sodium pyruvate; ultraglutamine
Cell Sciences	www.cygenics.com/cellsciences	Cytomatrix; fibroblast growth factor 2 (FGF-2)
Chemicon	www.chemicon.com	Antibodies: ABCG2, clone BXP-2; ABCG2, FITC-conjugated, clone 5D3; mouse IgG2b isotype; GFAP; β-tubulin; human nuclear; TRA-1-60; TRA-1-81; troponin I; leukemia inhibitory factor (LIF), human recombinant; ESGRO murine LIF
Codman	www.codman.com	Iris scissors, curved, 11 cm
Cohesion	www.cohesiontech.com/	Vitrogen
Corning	www.corning.com	Centrifuge tubes, 50 ml; Cryovial, 4.5 ml; dishes, 3.5 cm; flasks, 175 cm^2; flasks, 25 cm^2; flasks, 75 cm^2; PES membrane filter system; plates, 12 well; plates, 6 well; ultra-low attachment Petri dishes
Covance	http://www.covance.com/	Antibody to PAX6
Cytomatrix	www.cytomatrix.com	Fibronectin-coated scaffold
Dako	www.dakocytomation.com	Antibodies: mouse monoclonal anti-human CD45, CD33, CD, glycophorin-A
DSHB (Developmental Studies Hybridoma Bank, University of Iowa)	www.uiowa.edu/~dshb-www/	Antibodies: myosin, PGC surface marker; SSEA1, SSEA3, SSEA4
Dynal (*see* Invitrogen)		
eBioscience	www.ebioscience	Antibody to transferrin receptor, OKT9/CD-71
Electron Microscopy Sciences (EMS)	www.emsdiasum.com/microscopy	Paraformaldehyde, 16% solution
European Collection of Cell Cultures (ECACC)	www.ecacc.org.uk/	STO cells
Falcon (*see* BD Biosciences)		
Fisher Scientific	https://www1.fishersci.com	Plastic spatula, "Cell Lifter"; TE (100×); Triton X-100
GIBCO (*see* Invitrogen)		
GlaxoSmithKline	www.gsk.com/	Rosiglitazone (Avandia)
Green Cross Engineering Maintenance	www.gcem.co.kr	Blood collection bag and syringe
HyClone	www.hyclone.com	Fetal bovine serum (FBS); PBS (PBSA)
Innovative Chemistry, Inc.	www.innovativechem.com	AuthentiKit
Invitrogen	www.invitrogen.com	α-MEM; antibiotics; antimycotics; B27 supplement; Colcemid (KaryoMAX); collagenase IV;

		DMEM; DMEM/F-12; Dynabeads; Dynal magnetic particle concentrator; Dulbecco's modified Eagle's medium (DMEM); Eagle's basal medium (EBM); fetal bovine serum (FBS); Ham's F-12; Hanks' buffered salt solution (HBSS); HEPES; fungizone; gentamicin; Glutamax; L-glutamine; heat-inactivated newborn calf serum; insulin; Iscove's modified Dulbecco's medium (IMDM); ITS (insulin/transferrin/selenium); knockout DMEM (KO-DMEM); knockout serum replacement (KO-SR); β-mercaptoethanol; neurobasal medium A; nonessential amino acids; penicillin/streptomycin; phosphate-buffered saline without calcium and magnesium (PBSA); TGF-β 1; Trypan blue; trypsin; trypsin/EDTA
Iwaki	www.barloworld-scientific.com/	Tissue culture flasks and plates
LEC Instruments Pty	www.lecinstruments.com/	Vitrification straws
Life Sciences International *see* Thermo Electron Corp.		
Marienfeld GmbH	www.superior.de	Cell counting chamber (hemocytometer)
Merck Biosciences	www.merckbiosciences.co.uk	Glass beads
Millipore Corp.	www.millipore.com/	Millicell microporous membrane tissue culture insert
Miltenyi Biotec	www.MiltenyiBiotec.com	Cell sorting columns: MS+/RS+, LS+/VS+; microbead anti-CD34; FcR blocking agent; magnetic cell separator
Mundipharma	www.mundipharma.co.uk	Betadine
Nalge Nunc (now part of ThermoFisher)	www.nalgenunc.com/	Cryotubes; multiwell plates; tissue culture flasks and plates
Novocastra	www.novocastra.co.uk	hTERT antibody
Nunc, *see* Nalge Nunc		
PAA	www.paa.at	DMEM; ESC-tested fetal bovine serum
Peprotech	www.peprotech.com/	FGF-2
Perbio	www.perbio.com/	Filter unit, Stericup-GV
Perkin Elmer	www.perkinelmer.com/	Plate reader
Pierce Biotechnology	http://www.piercenet.com/	EZ-Link Sulfo-NHS-LC-Biotin; TEMED

Polysciences	http://www.polysciences.com	BioMag particles; anti-mouse IgM magnetic particle (IgM isotype used to recognize SSEA-3)
Promega	www.promega.com/	Cytotoxicity assay kit; dNTPs, 2 mM; loading dye, 5×; nucleotides: dNTPs, 2 mM; primers: oligo$(dT)_{18}$; oligo$(dN)_{10L}$; ribonuclease inhibitor: RNAsin; *Taq* buffer, 10×; *Taq* DNA polymerase
Purdue	www.pharma.com	Betadine
Q-BIOgene	www.qbiogene.com/	Geneclean gel cleaning kit; RNeasy RNA extraction kit
Qiagen	www.qiagen.com/	*Taq* polymerase; RNA Later; RNase-free water; RNeasy Mini kit
R & D Systems	www.rndsystems.com	Cytokines; Flt-3 ligand; granulocyte-macrophage colony-stimulating factor (GM-CSF); interleukin-6; methylcellulose; Nanog antibody; stem cell factor; TGF-β 1; TPOFLK cytokines mix; thrombopoietin
Roche Applied Science	www.roche-applied-science.com	5-Bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (BCIP); collagenase A; Dispase II. 2.4 U/mL; fibronectin; Nitro-Blue tetrazolium chloride (NTB); pronase
Santa Cruz Biotechnology	www.scbt.com/	OCT-4 antibody
Seikagaku	http://www.acciusa.com/	Endo-β-galactosidase; keratanase II
Shandon *see* ThermoShandon		
Sigma-Aldrich	www.sigmaaldrich.com	Acetic acid, glacial; α-actinin, mouse monoclonal antibody; agar gel powder; agarose, low-melting point (LMP); all-*trans* retinoic acid; amphotericin B; antibody to N-CAM, anti-human antibody; ascorbate-2-phosphate; ascorbic acid; biotin; bovine insulin; bovine serum albumin (BSA); butylated hydroxyanisole; chambered glass culture slides; cloning rings; Colibri suturing forceps, 0.1 mm; collagenase IV; collagenase I; corneal scissors, Wescott; cytosine arabinoside; DAPI (4′,6-diamidino-2-phenylindole); dexamethasone;

		dimethyl sulfoxide (DMSO); DMEM/F-12; DNase I; Dulbecco's modified Eagle's medium (DMEM); EGF, human recombinant; ethidium bromide; FGF-2 (bFGF); fibronectin; Ficoll-Hypaque; fluorodeoxyuridine; formaldehyde; forskolin; gelatin; gentamicin; glucose; glutamine; β-glycerophosphate; guinea pig complement; Hanks' balanced salt solution (HBSS); HMBA; Hoechst 33342; hydrocortisone; Immu-mount; indomethacin; insulin; isobutylmethylxanthine; ITS+; kanamycin; KCl; laminin, human, placental; linoleic acid-bovine serum albumin; MCDB-201; 1-mercaptoethanol; mitomycin C; monopotassium phosphate; nonessential amino acids; pantothenate; paraformaldehyde; penicillin/streptomycin solution; phosphate-buffered saline tablets; picric acid; Platelet-derived growth factor (PDGF); poly-D-lysine; poly-L-ornithine; pronase; propidium iodide; sodium pyruvate; TGF-β 1; TRI reagent; triiodothyronine; tropomyosin, rabbit polyclonal antibody; uridine; valproic acid; verapamil; 1,25-$(OH)_2$ vitamin D_3
StemCell Technologies Inc.	www.stemcell.com/	Gelatin
Sterilin *see* Barloworld Scientific		
Thermo Electron Corp.	www.thermo.com	Chemicals, equipment, plastics
ThermoFisher	www.thermofisher.com	Media, plastics, equipment
ThermoShandon	www.thermoshandon.com	Chambered glass culture slides; Immu-Mount
Vector	www.vectorlabs.com	DAPI (4′,6-diamidino-2-phenylindole)
Wako Chemicals	www.wakousa.com/	L-Ascorbic acid phosphate
World Precision Instruments	http://store.wpiinc.com	Colibri suturing forceps, 0.1 mm; corneal scissors, Wescott, 19 mm blades, sharp tip
Worthington	www.worthington-biochem.com/	Collagenase type I

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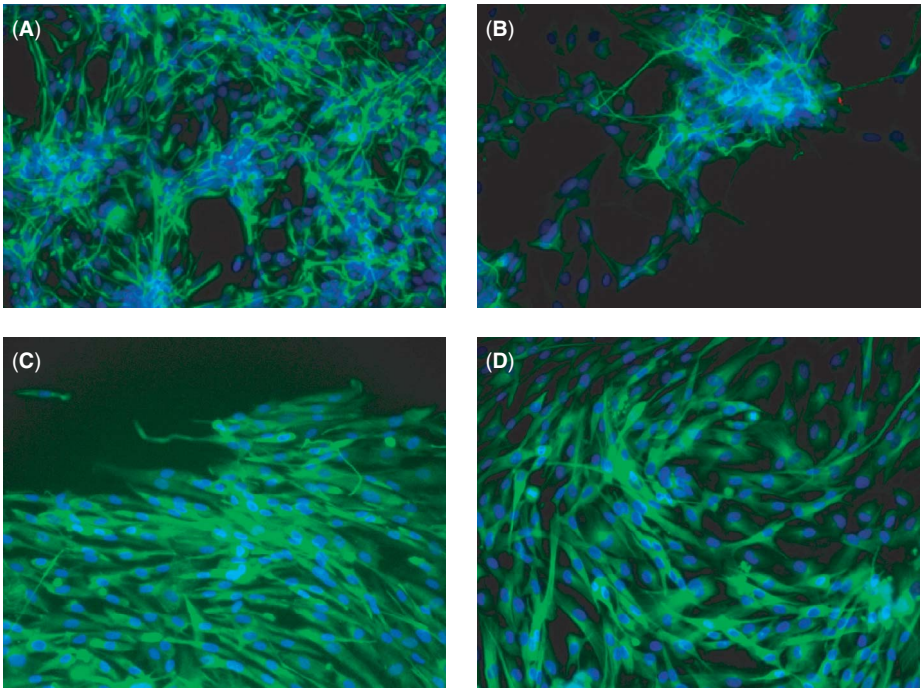

PLATE 1. HUES-1 NS cells. Immediately after replating: (**A**) TUJ-1, (**B**) nestin. After 3 passages: (**C**) TUJ-1, (**D**) nestin. (*See also* Fig. 3.2.)

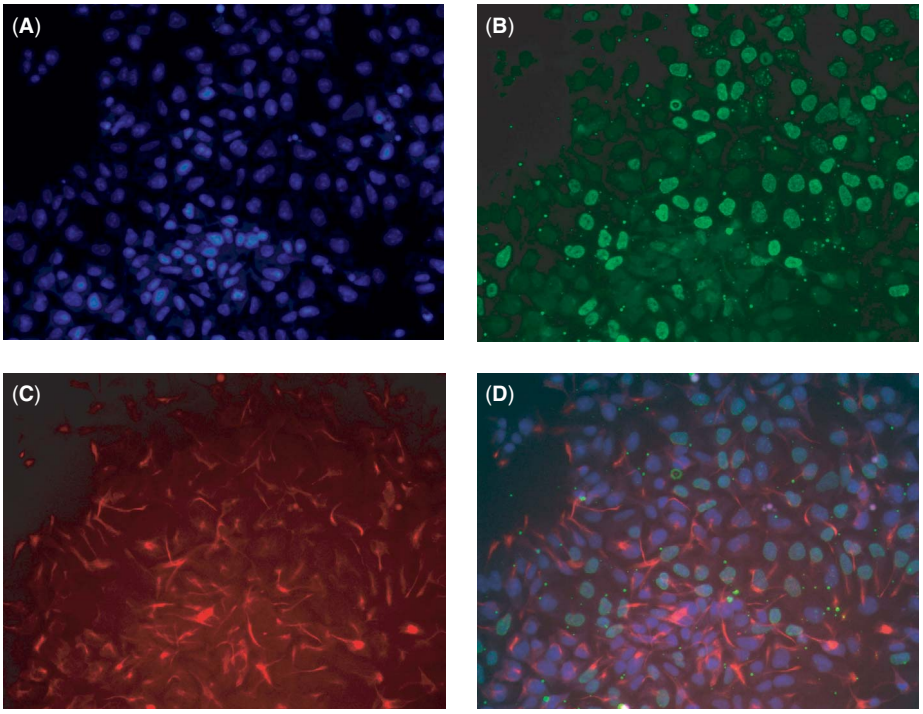

PLATE 2. Undifferentiated EC cells. Exposed to BrdU for 1 h, the cells were fixed and stained for BrdU incorporation and nestin expression. (**A**) Blue, Hoechst 33342. (**B**) Green, BrdU. (**C**) Red, nestin. (**D**) Overlay of all three. (*See also* Fig. 3.3.)

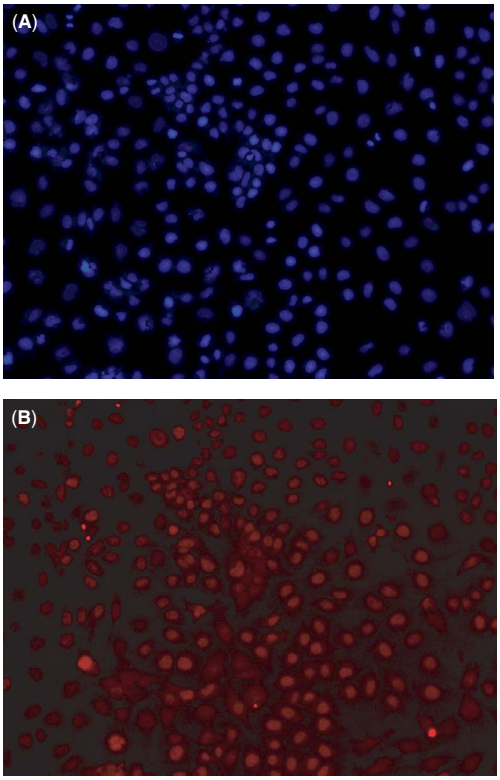

PLATE 3. Undifferentiated NTERA2 cells, stained for the expression of Sox2. (**A**) Hoechst 33342. (**B**) Sox2. (*See also* Fig. 3.4.)

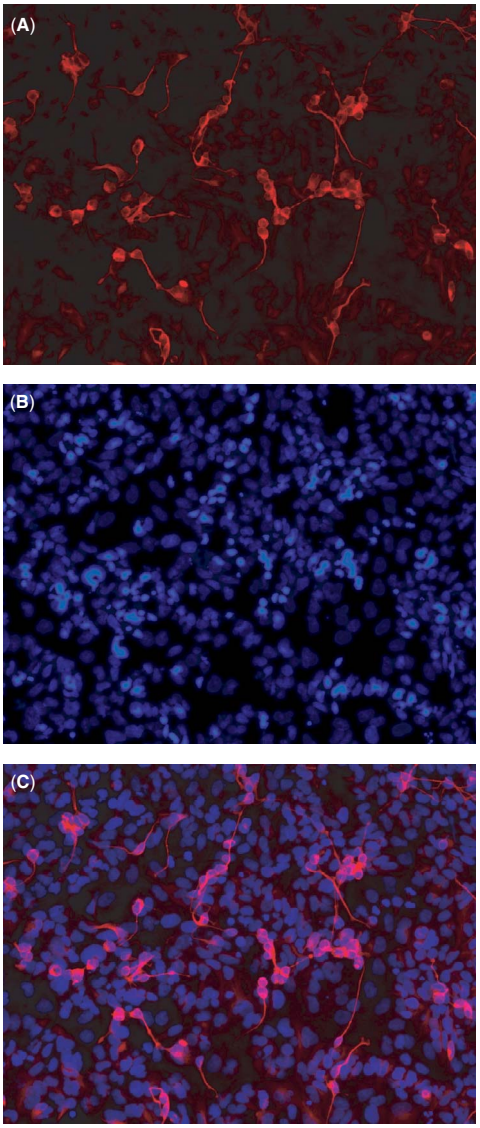

PLATE 4. A2B5-positive NTERA2 cells were FACS purified after 18-day RA exposure. Cells were then replated and stained 48 h later. (**A**) TUJ-1. (**B**) Hoechst 33342. (**C**) Overlay. (*See also* Fig. 3.5.)

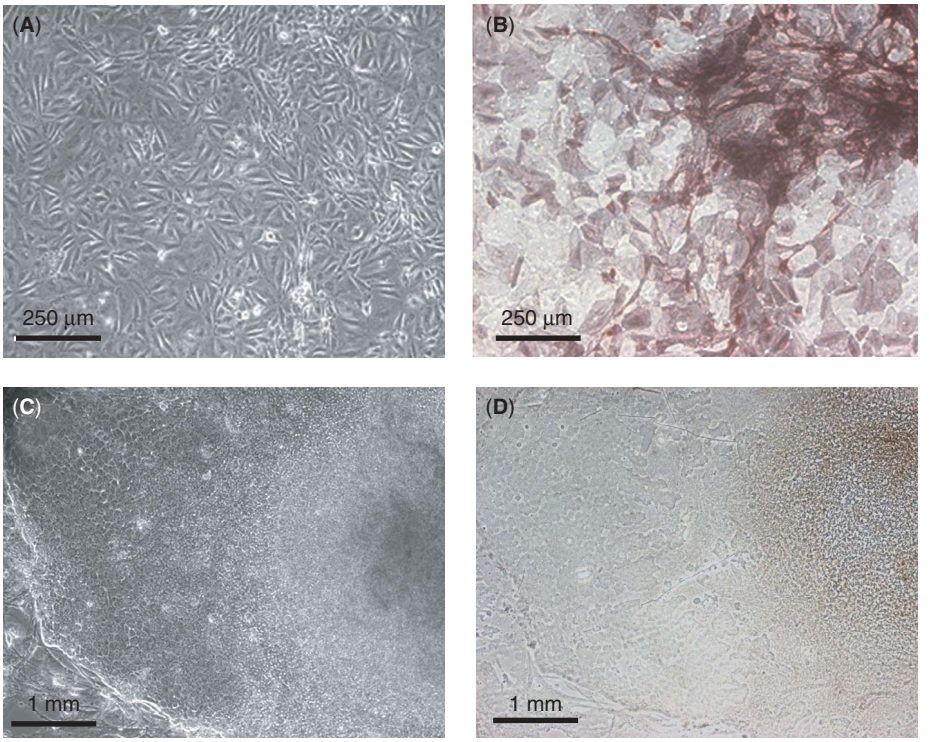

PLATE 5. Colony morphology. Colony morphology does not necessarily predict staining pattern for alkaline phosphatase. (**A**) Bright field image in which it is difficult to discern a discrete colony. (**B**) Alkaline phosphatase (AP) staining of the same colony reveals vigorous germ cell growth. In contrast, the colony easily visible in (**C**) lacks appreciable AP staining (**D**). Size bar = 250 µm (**A, B**) and 1 mm (**C, D**). (*See also* Fig. 5.3.)

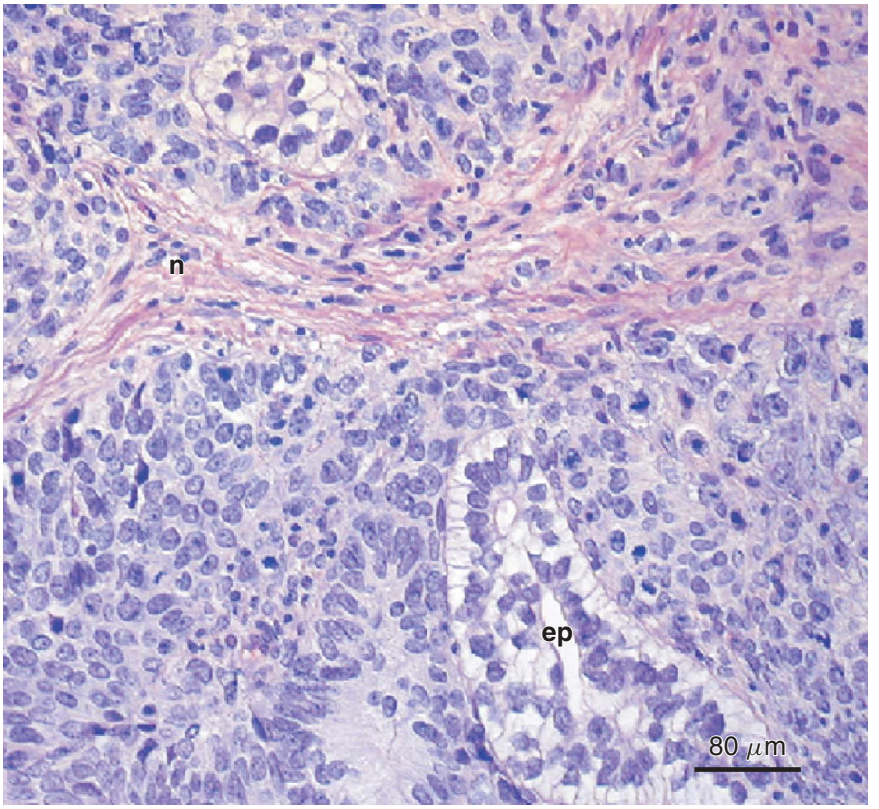

PLATE 6. Formation of teratomas by human EC stem cells. Neural tissue (n) and primitive epithelia (ep). Scale bar: 80 μm. (*See* Fig. 6.4 for further details.)

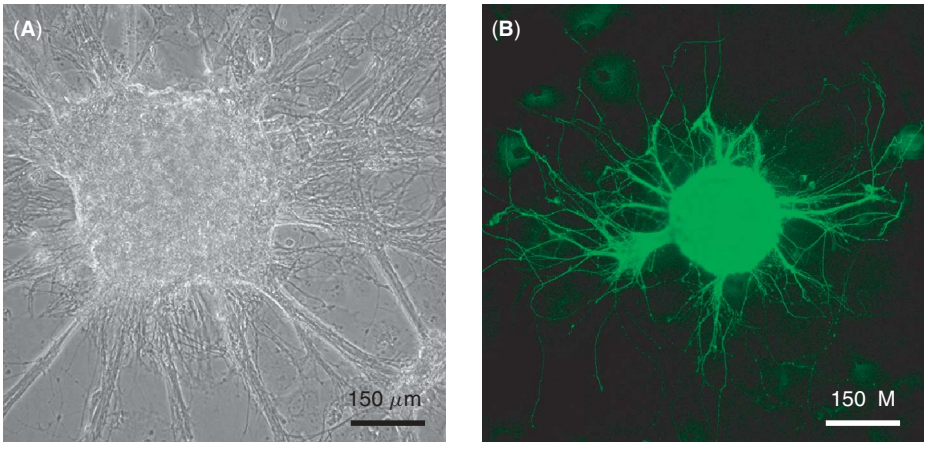

PLATE 7. Suspended aggregates of differentiating human EC cells plated onto to poly-L-ornithine and laminin coated surfaces often form elaborate neurite outgrowths as seen (**A**) by phase microscopy and (**B**) by immunofluorescent staining for the neural marker TUJ-1. Scale bars: 150 μm. (*See also* Fig. 6.10.)

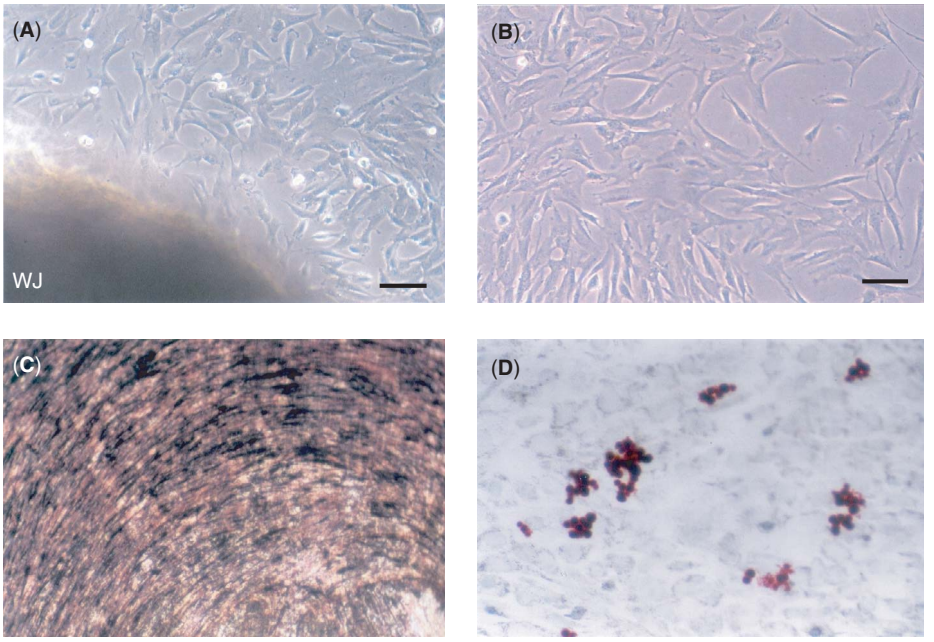

PLATE 8. Photomicrographs and cell surface markers of stem cells isolated from Wharton jelly (WJ) by the explant method. Photomicrographs of (**A**) primary WJ-derived stem cells and (**B**) subcultured cells at second passage. Bar is 100 µm. (**C**) Osteogenic differentiation evaluated by von Kossa and (**D**) adipogenic differentiation evaluated by Oil Red O staining. (*See also* Fig. 7.1.)

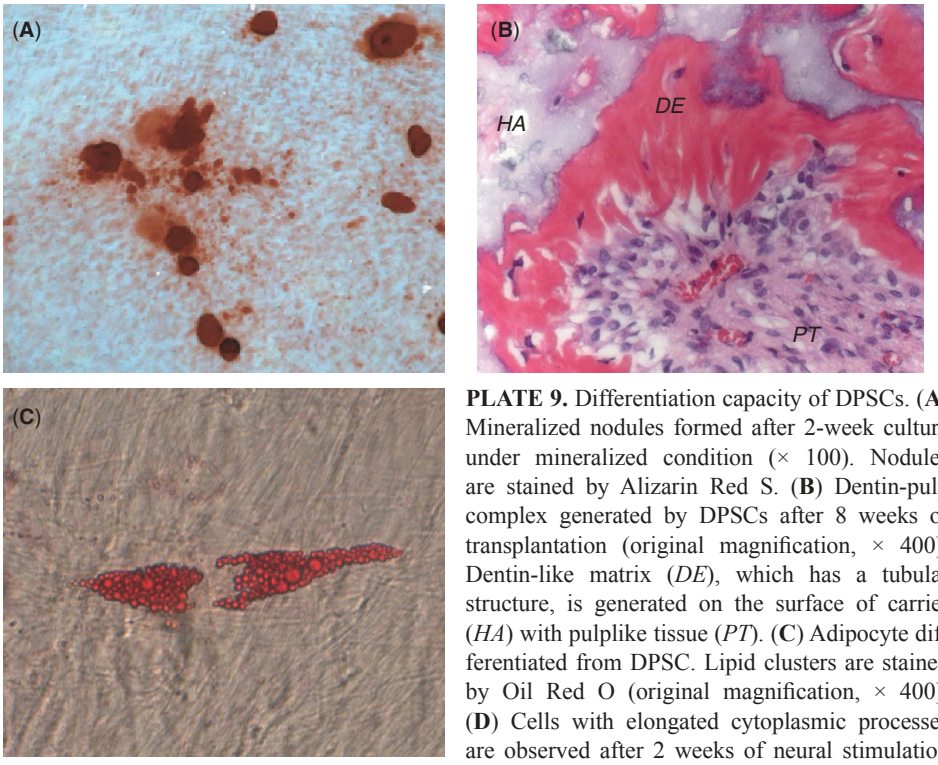

PLATE 9. Differentiation capacity of DPSCs. (**A**) Mineralized nodules formed after 2-week culture under mineralized condition (× 100). Nodules are stained by Alizarin Red S. (**B**) Dentin-pulp complex generated by DPSCs after 8 weeks of transplantation (original magnification, × 400). Dentin-like matrix (*DE*), which has a tubular structure, is generated on the surface of carrier (*HA*) with pulplike tissue (*PT*). (**C**) Adipocyte differentiated from DPSC. Lipid clusters are stained by Oil Red O (original magnification, × 400). (**D**) Cells with elongated cytoplasmic processes are observed after 2 weeks of neural stimulation (original magnification, × 400). (*See also* Fig. 8.3.)

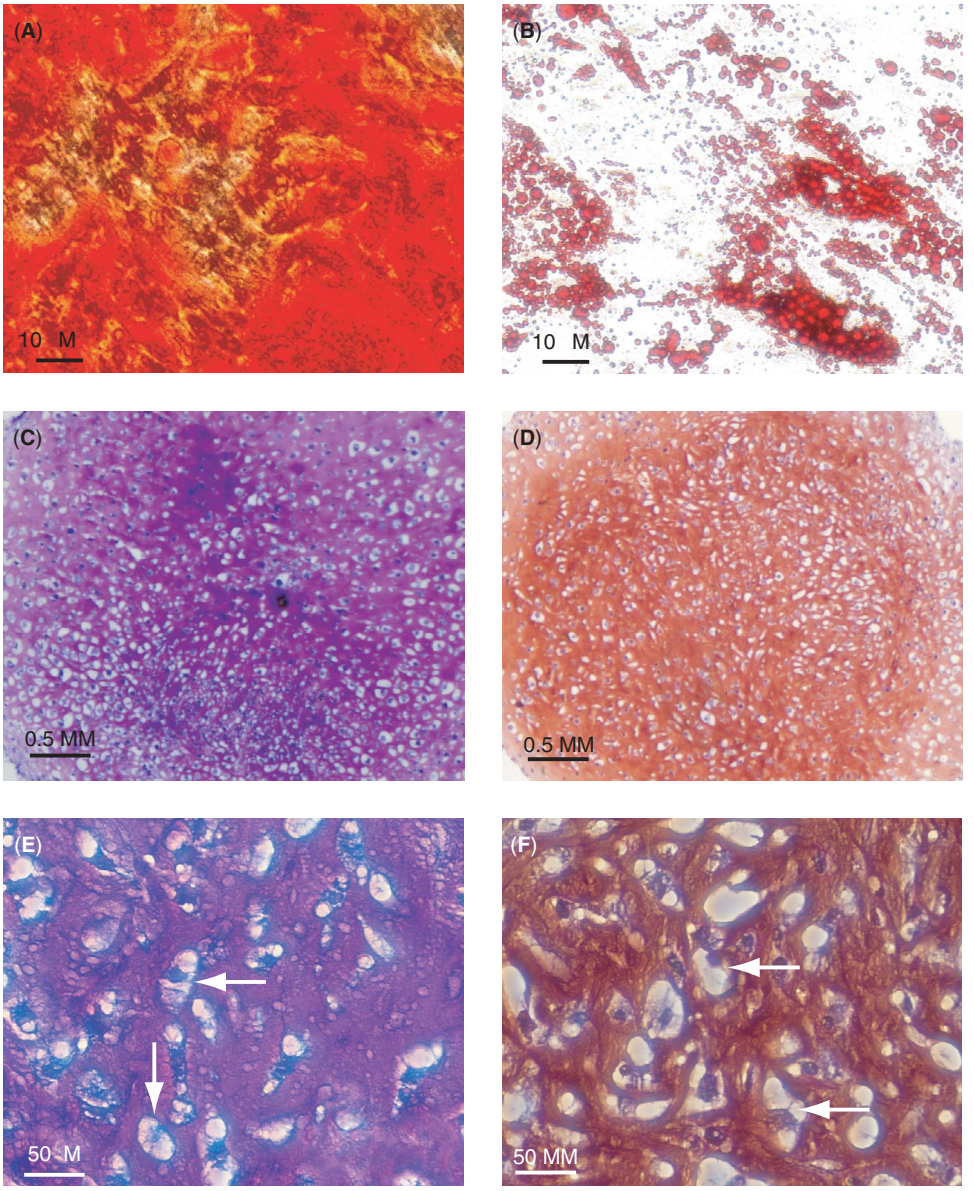

PLATE 10. Results of differentiation assays. (**A**) Alizarin Red S-stained monolayer of osteogenic MSCs. (**B**) Oil Red O-stained monolayer of adipogenic MSCs. (**C–F**) Stained 10-µm sections of chondrogenic pellets: (**C, E**) Toluidine Blue, (**D, F**) Safranin O. High-power images (**E, F**) show the morphology of the cartilage and the lacunae (arrowed) populated by chondrocytes. (*See also* Fig. 9.4.)

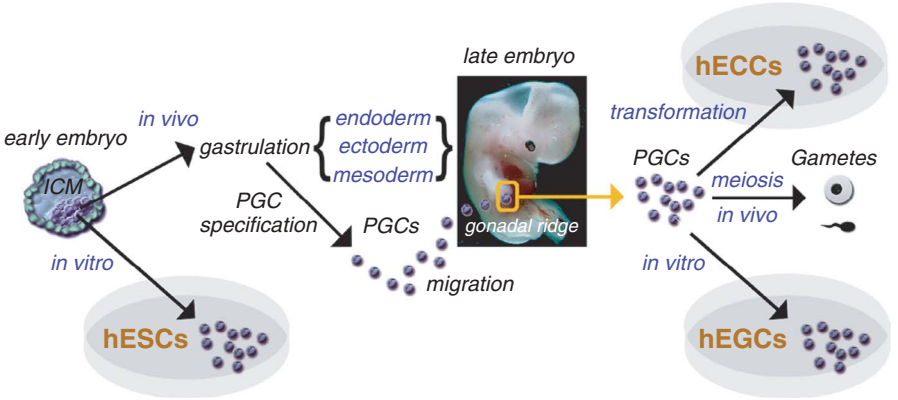

PLATE 11. Schematic of human pluripotent stem cell sources and their respective cells of origin. (*See also* Fig. 5.1.)

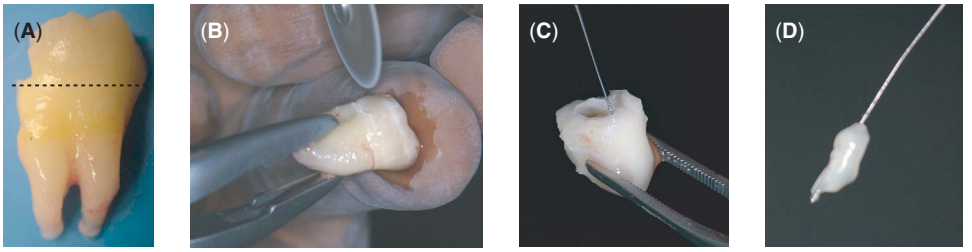

PLATE 12. Isolation of pulp tissue from an extracted third molar. (**A**) Gross view of an extracted tooth. The dotted line shows the cementum–enamel junction. (**B–D**) The tooth is cut along with the cementum–enamel junction, and then pulp tissue is isolated. All these procedures should be carried out aseptically. (*See also* Fig. 8.1.)

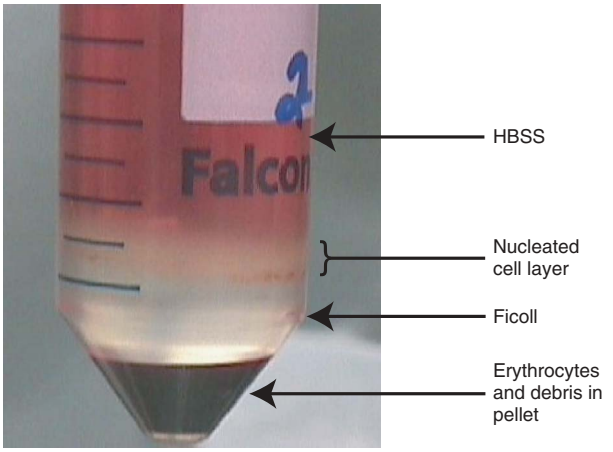

PLATE 13. Processing of rodent and human bone marrow. Photograph after centrifugation of the discontinuous Ficoll gradient used to separate the mononuclear fraction of human bone marrow. The mononuclear layer is recovered from the white band at the interface between the two layers.
Note: The volumes here are lower than in Protocol 9.1 and in Fig. 9.1.

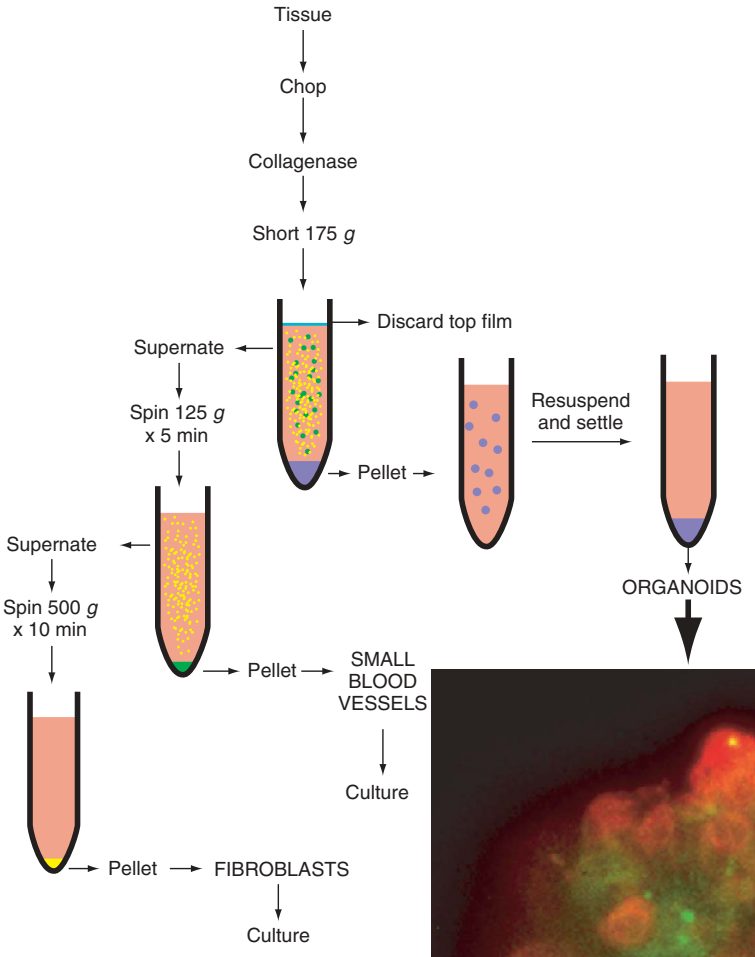

PLATE 14. Selection of organoids. Flow chart of isolation of different fractions by selective sedimentation and centrifugation following collagenase digestion. (*See also* Fig. 12.3.)

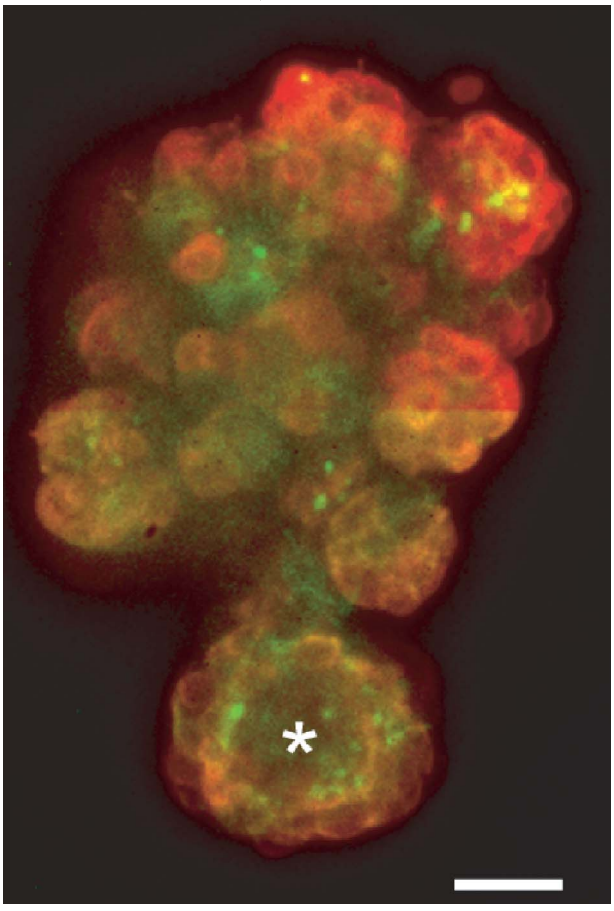

PLATE 15. A TDLU-like structure derived from a $Muc^{-}/ESA^{+}/CD29^{hi}$ D920 cell grown in 3D lrECM culture. The structure was stained in lrECM for K14 (red) and K8 (green) expression and imaged with a confocal microscope. The presented image is a reconstruction of several optical slices to give the appearance of three dimensions. There is a lumen indicated by a star. Scale bar = 50 µm. (*See also* Fig. 12.4.)