EMERGING TECHNOLOGY PLATFORMS FOR STEM CELLS

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FOREWORD

The successful isolation of human embryonic stem cells (hESCs)by Dr Jamie Thomson in 1998 has lead to subsequent derivation of over a hundred additional lines. The field has advanced quite rapidly ever since and significant improvements and variations in techniques in all aspects of ESC methodology, including derivation, propagation, differentiation, and genetic manipulation have been reported.

These include isolation of parthenogenetic lines, isolation from the morula stage embryo, as well as embryos at later stages of embryonic development including the epiblast stage, isolation of PGD lines, and lines from parthenogenetic embryos. Equally important, there have been reports of successful reprogramming of adult cells using as few as four genes and isolation of germ cell lines from fetal and adult tissue that appear pluripotent. Thus, there are possibilities of obtaining cells that may not make teratomas or contribute to the germ line or be classified as living embryos and may even bypass the ethical issues raised by oocyte donation while still retaining many of the characteristics of a pluripotent stem cell.

Equally important advances have been made in developing serum-free animal origin-free, feeder-free, methods of propagating ESC on a large scale as well as methods to rapidly profile the epigenetic and genetic status of ESC. These advances coupled with development of methods to direct differentiation of cells into specific lineages and techniques to isolate specific cell types have made the possibility of clinical therapy that much closer to reality. These methods which can be applied to any differentiated cell types irrespective of the cell of origin has emboldened companies to consider investing in clinical trials. Indeed, in the United States, the Food and Drug Administration (FDA) has approved an Investigational New Drug application for using human central nervous system stem cells, isolated from fetal brain tissue, in clinical trials testing a treatment for Batten disease, a fatal inherited

disorder of the nervous system. Furthermore, unconfirmed reports indicate that the FDA is reviewing an application from Geron to use hESC-derived oligodendrocytes to treat spinal cord injury and there are press reports from India, China, and other countries of ESC-derived cell transplants.

Researchers have also made important advances in modifying the tools and reagents that have worked well in mouse ESC work and in bioproduction and cell engineering to develop technology to insert genes and modulate gene expression in ESC cell and adult stem cells, and several companies have announced plans to consider primary cell screening as a more realistic model of normal development than the currently used cell lines.

These advances have expanded the potential utility of stem cells and highlighted an important need to have collated information available. Although several attempts are ongoing, I believe this textbook authored by my colleagues is the first effort to see the light of print. It is truly comprehensive in covering all major aspects of the field. Most of the chapters have been written by luminaries in the field and the editing has ensured a consistent and direct style. It seems to this reader that every effort has been made to include the latest results and identify the latest references.

I was impressed by the book and I hope others will find it as informative and useful as I do.

I congratulate the editors on a job well done.

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PREFACE

The exponential increase in the number of researchers exploring the intricacies of stem cell biology make this an exciting time. The ability of stem cells to differentiate into multiple lineages allows them to be used in ways that have the potential to dramatically improve the human condition. The recent explosion in stem cell research can be attributed to the isolation of human embryonic stem cells. However, the increased interest in these cells has also highlighted the limitations of current technology. Therefore, as the field progresses toward its ultimate applications in drug discovery and regenerative medicine, several hurdles need to be overcome. The future pace of progress in stem cell research will therefore largely depend on the development of new technologies that can either overcome or minimize current constraints.

The idea for this book was conceived as a result of our unending obsession in working with stem cells. Most techniques and protocols used in stem cell research continue to be more of an art than science, and this has been a source of constant frustration for stem cell researchers across the world. In this book, we present some of the elegant studies that have translated technical skill into a scientific process. We have attempted to bring to the forefront some of the emerging technology platforms and their applications in stem cell research.

Above all, this book is an ode to stem cell researchers who are involved in painstakingly assembling the small pieces to create a larger picture. We are sincerely thankful to all the contributors for sharing their pioneering work and scientific perspectives in this book. Our hope is that this book will ignite a passion in people who are new to the field while also engaging researchers currently working on different aspects of stem cell biology. Our belief is that the technologies described in this book will have far-reaching effects in the

field of stem cell research, and will help accelerate the path to the clinic. The purpose of this book is to provide a realistic picture of the potential and challenges of stem cell research as it continues its trailblazing journey toward the quest for a better human life.

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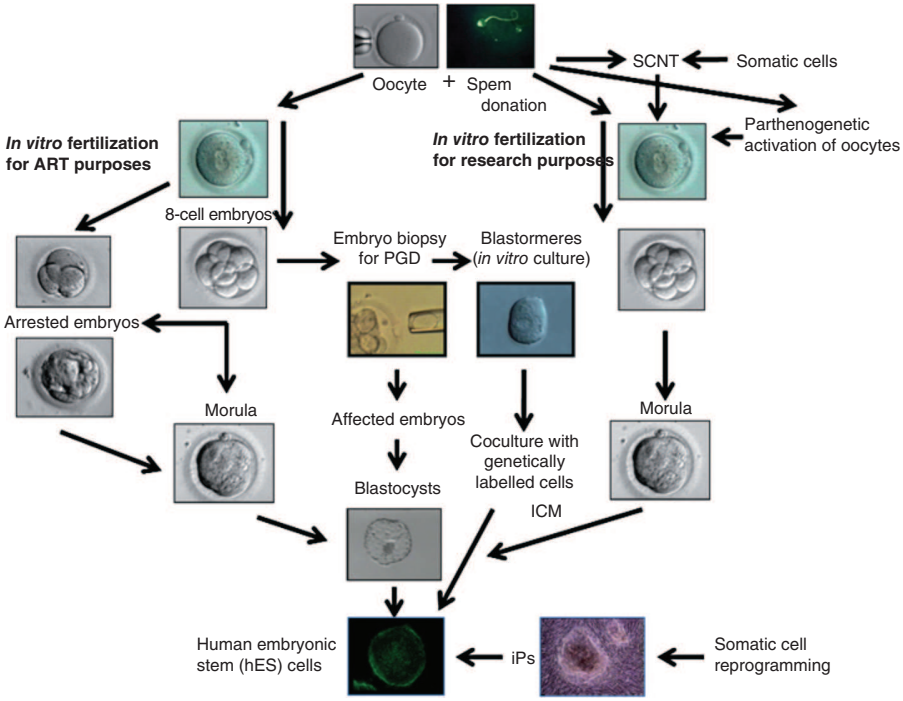


Figure 1.1. A schematic representation of possible hES cell derivation strategies that have so far been practiced worldwide.

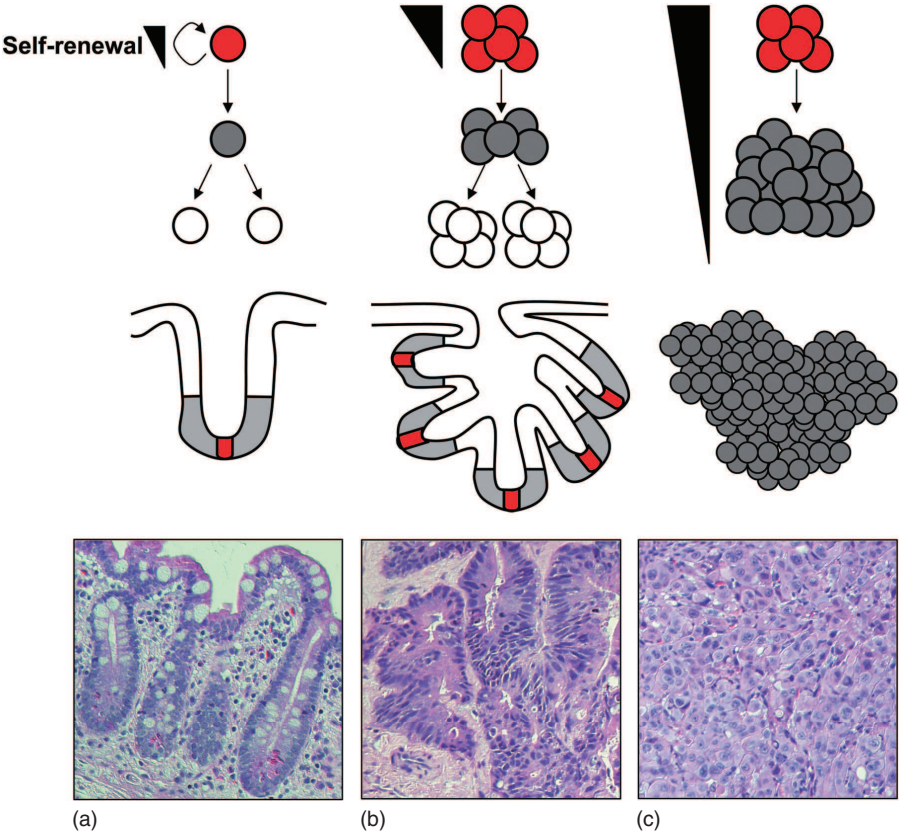


Figure 8.1. Colorectal-centric view of tumor progression. (a) Normal tissue-resident stem cell populations, such as the colon stem cell (red), which is localized at the base of the crypt, have both the unique ability to self-renew (relative ability indicated by a black triangle) and to generate the differentiated progeny that comprise this tissue. Progeny of the colon stem cell include highly proliferative transit amplifying cells (progenitor; gray) and terminally differentiated enteroendocrine or goblet cells (white). (b) In tumors where the CSC lies at the level of the tissue-resident stem cell, increased symmetric division fates result in expanded stem cell numbers and thus, a similar expansion of their resulting progeny. This sudden increase in cells vying for space within a tissue may manifest as an adenocarcinoma. (c) Aggressive, end-stage carcinomas might result from the gain of self-renewal capacity by an already proliferative progenitor cell pool with limited to no differentiation capacity, often resulting in amorphous, largely undifferentiated tumors. Hematoxylin and Eosin stains show histopathology of (a) normal colon crypts, (b) adenocarcinomas, and (c) aggressive, poorly differentiated carcinomas of the colon, each of which might reflect the cellular identity and self-renewal capacity of the stem cell population.

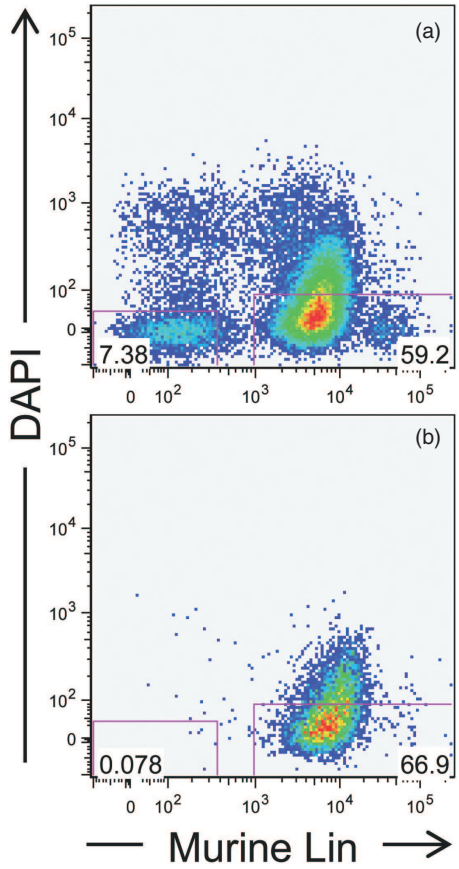
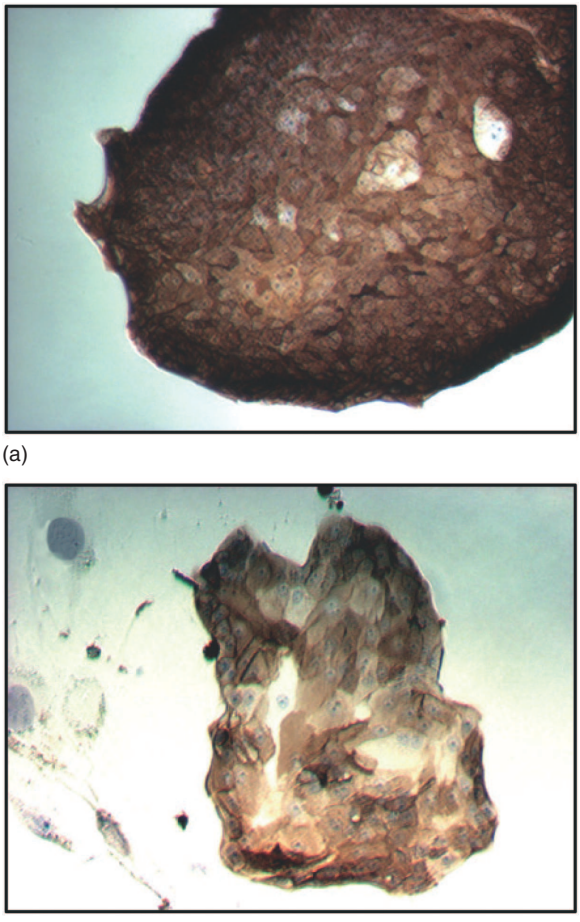


Figure 8.2. Palpable tumor generation should not alone be equated with CSC. Prospective colorectal tumor cells were isolated and transplanted into mice as described previously (29). Three months later, tumors were removed and analyzed by flow cytometry for live human and murine lineage (Lin) cells. Whereas tissue mass (a) was likely generated by a colorectal CSC because it contained live human cells resembling the parental tumor, tissue mass (b) consisted of only murine stromal elements that likely proliferated for a finite time period alongside human non-CSCs.



(a)

(b)

Figure 8.3. An example of colorectal colony morphology differences in vitro. Highly enriched colorectal CSCs were plated *in vitro* in either (a) serum-free medium or (b) medium containing fetal calf serum. After 16 d of culture, colonies were fixed and stained for human CD44 (magnification = 100×) and counterstained with Hematoxylin.

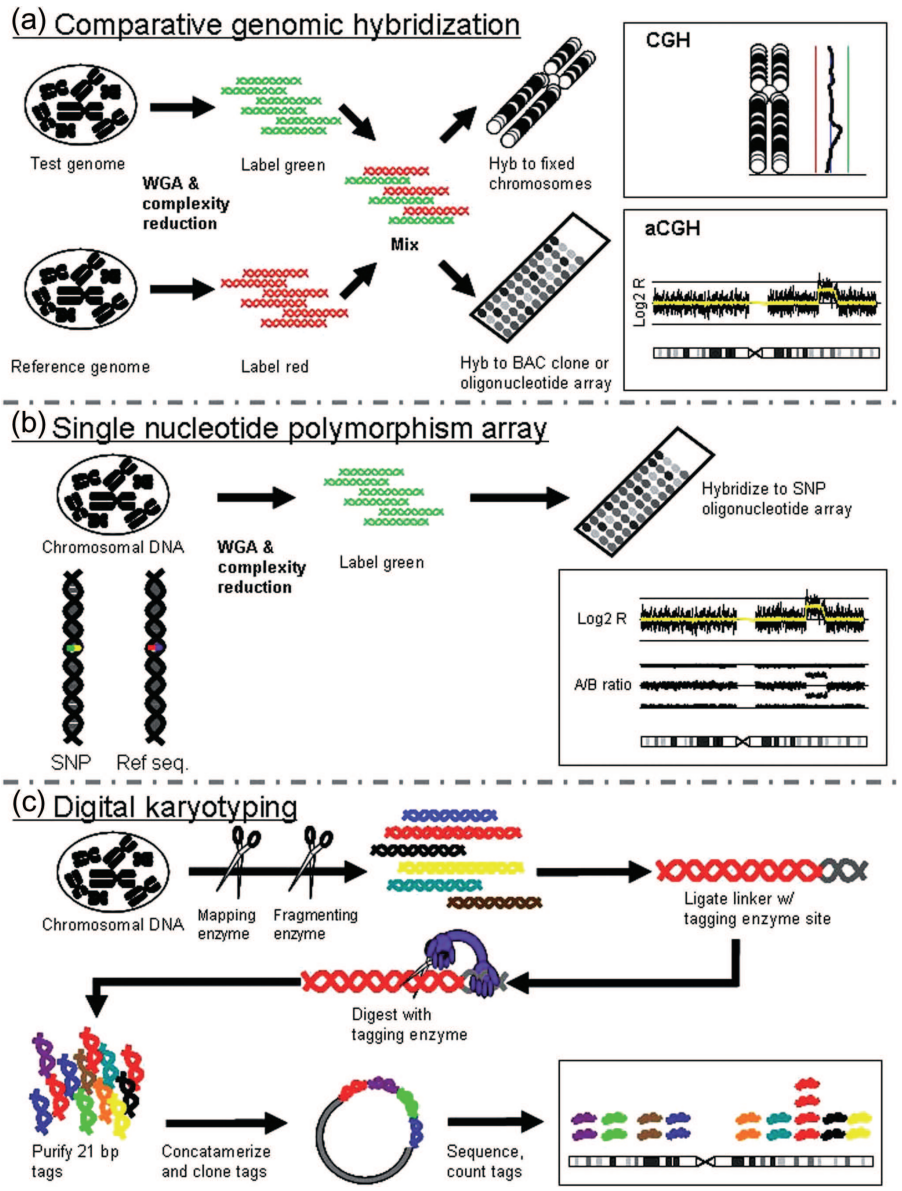


Figure 10.1. Schematic diagram of methods for molecular cytogenetics. Three molecular biological approaches for assaying DNA copy number are presented in cartoon form; many details of each procedure have been omitted for clarity. The mapping of results across one chromosome is illustrated in boxes at the right of each figure. (See text for full caption.)

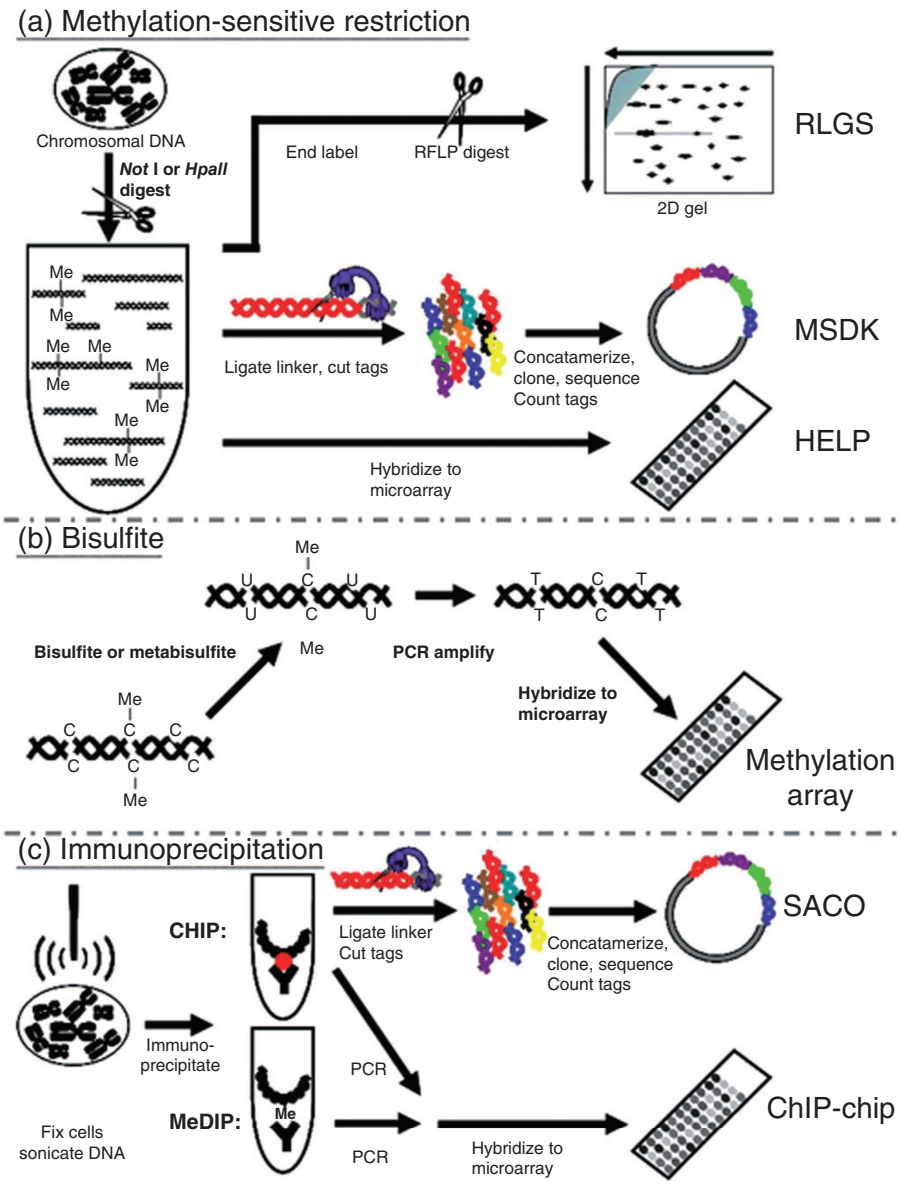


Figure 10.2. Schematic diagram of epigenomics methods. Three types of single-locus assays are typically used to assay methylation or histone modification at a site on the DNA. (See text for full caption.)

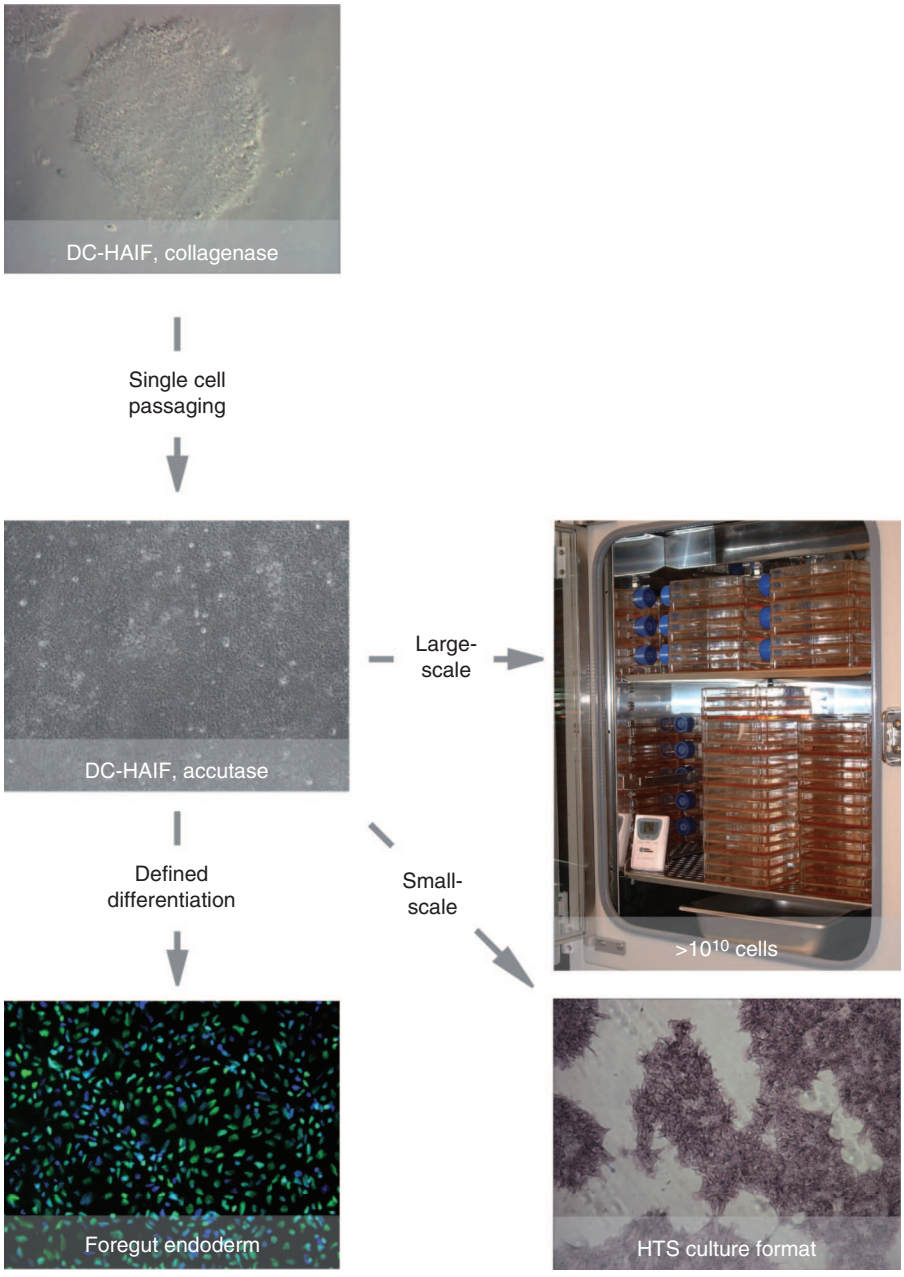
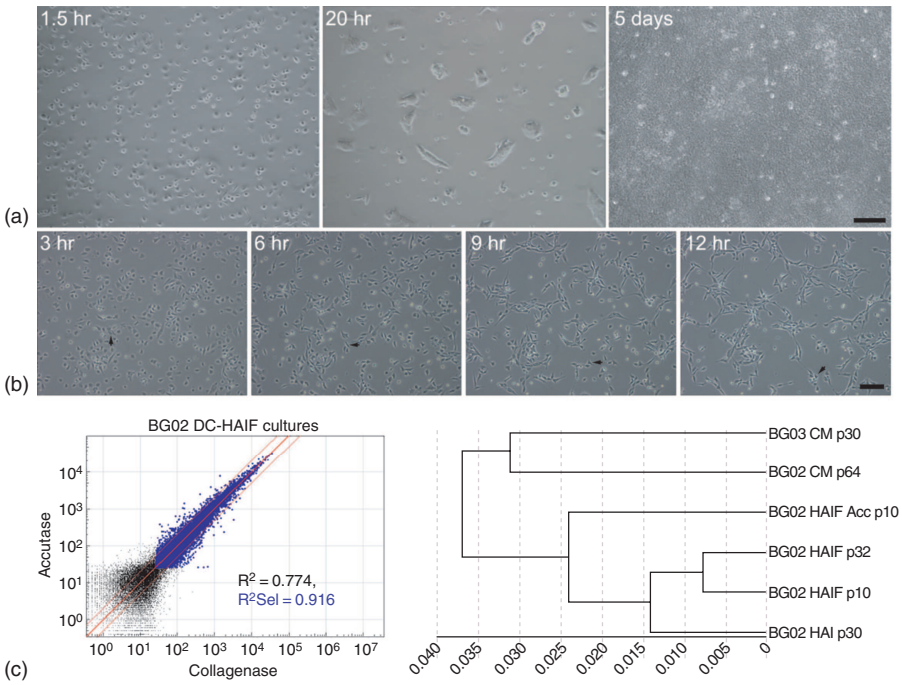


Figure 13.1. An advanced culture system for hESCs. DC-HAIF medium supported the propagation of undifferentiated euploid hESCs, including cultures that were split to single cells at passaging. Single cell suspensions could be plated at defined densities with high viability and maintained without manual selection. This enabled the directed differentiation of hESCs using defined media (HNF1β$^+$ foregut endoderm is shown in green), reliable culture in high throughput screening culture formats (alkaline phosphatase$^+$ hESCs in a 384-well tray are shown), and scaled expansion to >10^{10} cells.



(d)

G-banding

BG01	HAIF	Acc p11	46,XY [19/20]
	HAIF	Acc p17	46,XY [19/20]
BG02	HAIF	Acc p5	46,XY [20/20]
	HAIF	Acc p5	46,XY [20/20]
	HAIF	Acc p5	46,XY [19/20]
	HAIF	Acc p18	46,XY [20/20]

(e)

FISH

	% Normal hChr (n=)			
	12	17	X	Y
BG01 HAIF Acc p5	97 (512)	97 (922)	98 (516)	NA
BG02 HAIF Acc p49	96 (521)	96 (667)	98 (597)	97 (822)
CyT49 HAIF Acc p7	98 (602)	96 (542)	97 (572)	97 (533)

Figure 13.2. Robust single cell passaging of hESCs. (See text for full caption.)

(a)

FISH

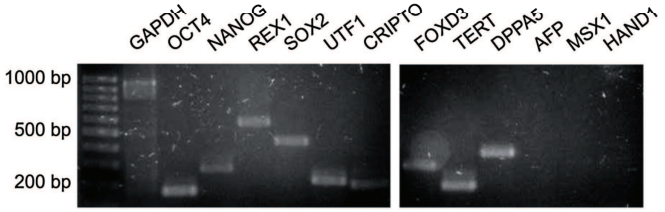
	% Normal nuclei (n=)			
hChr	12	17	X	Y
Pre-batch	98 (523)	98 (577)	98 (578)	97 (605)
1x Triple T175	95 (1108)	98 (1063)	93 (1222)	99 (1049)
52x Triple T175	98 (1076)	98 (1151)	95 (1188)	98 (1120)

(b)

G-banding

	Primary analysis	Secondary analysis
Pre-batch	46,XY [19/19]	46,XY [31/32]; 46,XY del(11)(q13q21) [1/32]
LOT1 +1	46,XY [20/20]	46,XY [50/52]; 46,XY del(11)(q13q21) [2/52]
LOT2 +1	46,XY [19/19]	46,XY [45/47]; 46,XY del(11)(q13q21) [2/47]
LOT3 +1	46,XY [15/19] 44~46,XY del(11)(q13q21) [4/19]	

(c)



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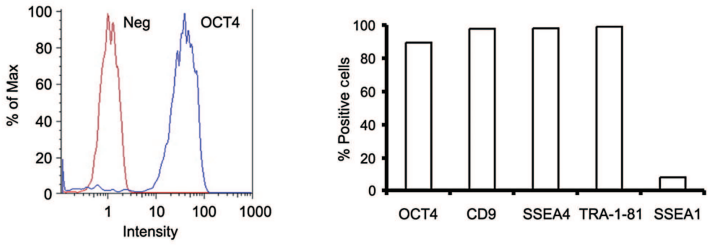


(e)

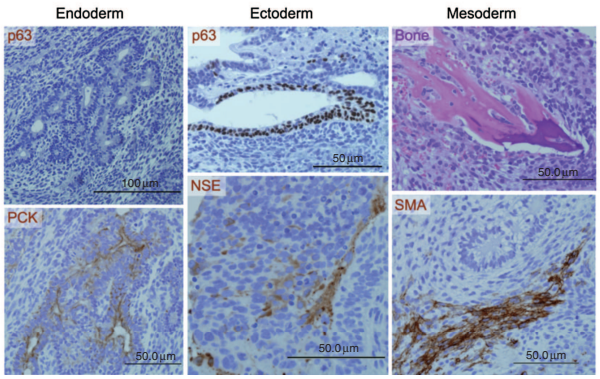


Figure 13.4. Characterization of the scaled batch of BG02 cells. (See text for full caption.)

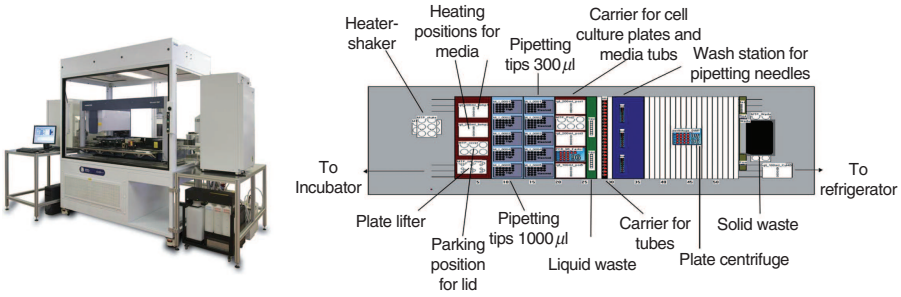


Figure 14.1. The Cell[host] system consists of a pipetting workstation contained in a laminar airflow cabinet. The cabinet is connected to two robot-accessible incubators for the storage of cell cultures and media (a). For efficient performance of cell culture tasks, a plate lifter for the complete removal of media from cell culture plates, heating positions for cell culture media, a centrifuge, and a heater-shaker module for enzyme-treatment of cell cultures have been integrated (b). Adapted from Ref. (5) with permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.

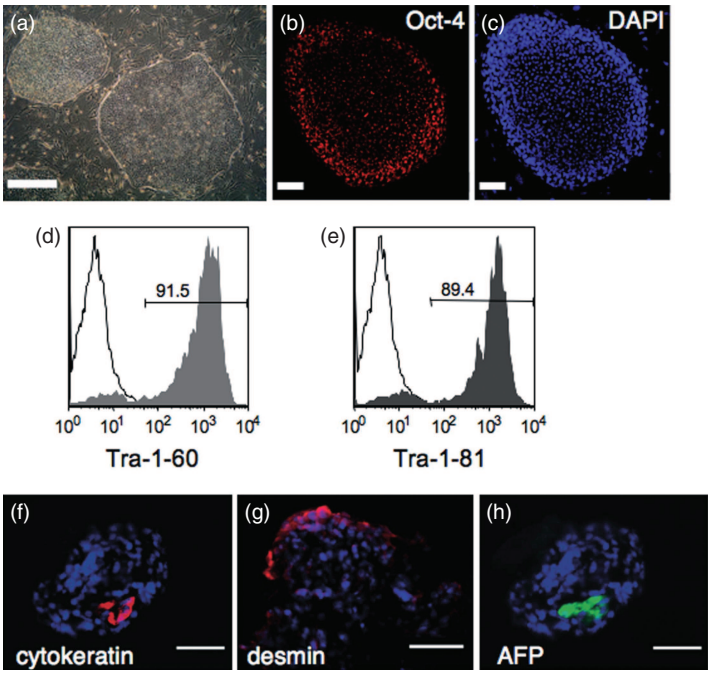


Figure 14.3. hESC cultured by the automated system display typical morphology (a) and maintain expression of pluripotency-associated markers Oct-4, Tra-1-60, and Tra-1-81 (b–e). *In vitro* differentiation potential is conserved as hESC readily form embryoid bodies expressing the germ layer-associated markers cytokeratin (f, ectoderm), desmin (g, mesoderm) and α-fetoprotein (AFP, H, endoderm). Nuclei are counterstained with DAPI (blue). Open histograms correspond to negative controls and filled histograms represent staining of the indicated pluripotency-associated markers. Scale bar = 500 µm (a)/100 µm (b, c)/50 µm (f–h). Adapted from Ref. (5) with permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.

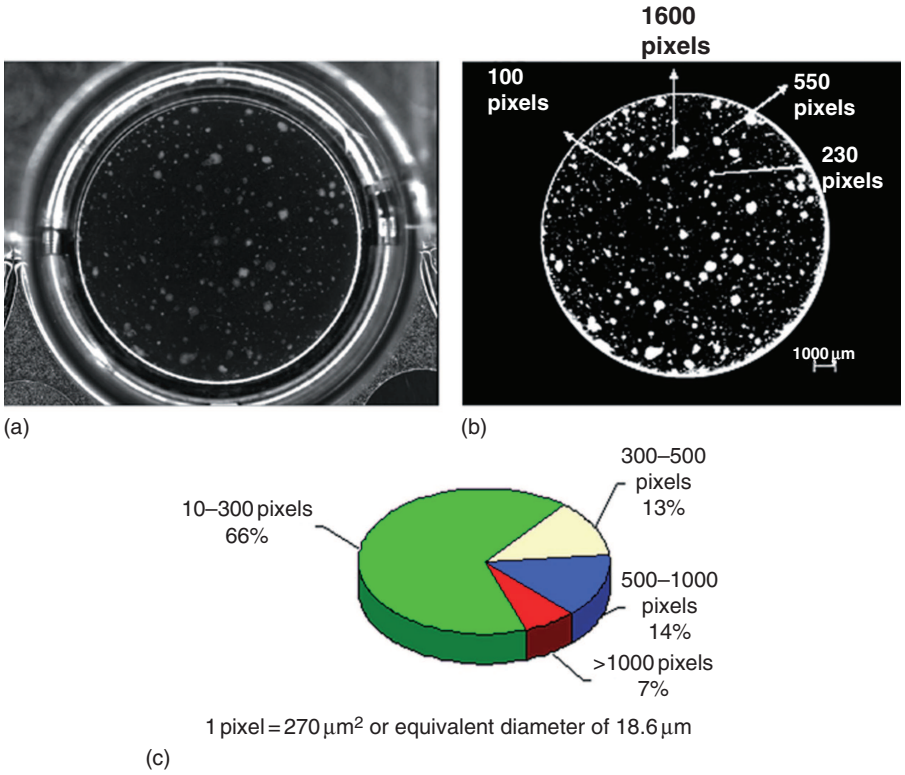


Figure 15.2. Images and size distributions of hESC clusters. (a) A typical example of a captured image in a 24-well plate 1 d after seeding hESC clusters. (b) An image of the hESC clusters is segmented into areas represented by pixels and presented for data analysis. (c) The size/area distribution of clusters are presented in a pie chart showing the percentages of the seeded clusters in different size ranges as represented in pixels. Note that 1 pixel is equivalent to an area of 270 µm^2 or an equivalent diameter of 18.6 µm.

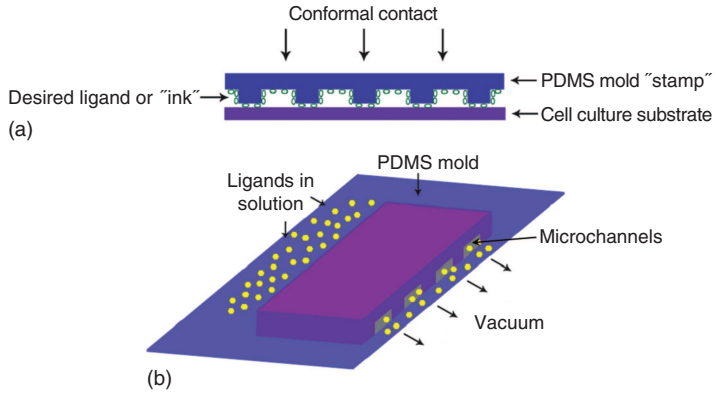


Figure 17.1. Micropatterning techniques. (a) Microstamping patterning. A PDMS mold is created to form a "stamp" and subsequently coated with the desired molecules for the cell culture substrate. The molecules are then transferred to the cell culture substrate through conformal contact. Following removal of the PDMS stamp, the cell culture substrate is often blocked with a molecule such as polyethylene glycol. (b) Microfluidic patterning. The predesigned mold is placed on top of the cell culture substrate. Microchannels formed between the mold and the substrate are flushed with a ligand solution through vacuum pressure, which deposits ligands in a specific micropattern. The mold is then removed and blocking molecules are added as necessary.

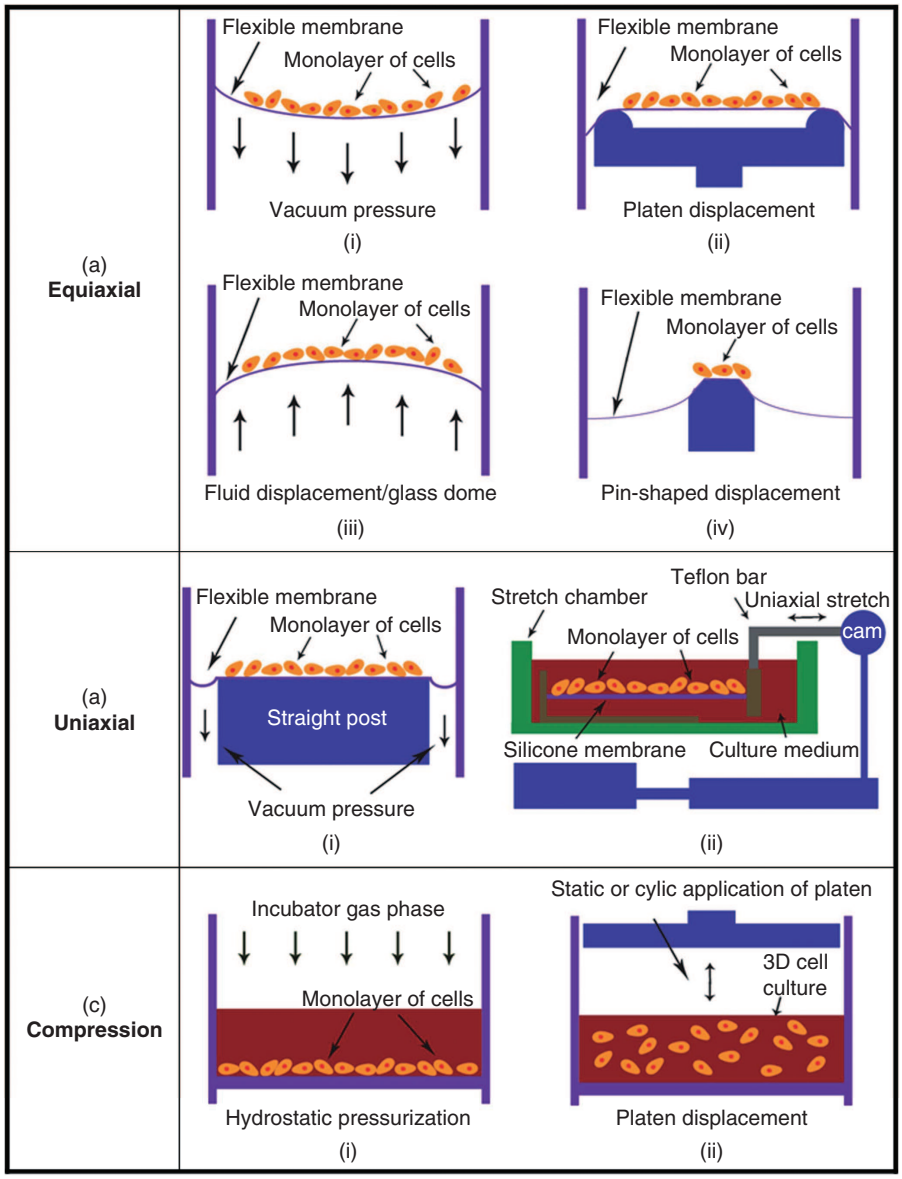


Figure 17.3. Various techniques used to exert dynamic loading. (a) Equiaxial strain. (i) Flexercell® system. Cells are cultured on a flexible-bottomed plate that deforms under vacuum pressure, creating either static or cyclic strain. (ii) Equiaxial strain system where cells are cultured on a flexible membrane and strain is applied through a platen. (iii) Equiaxial strain system where strain is applied through either a glass dome or fluid displacement. (iv) Equiaxial strain system where strain is applied through a pin-shaped displacement. (b) Uniaxial strain. (i) Generating uniaxial strain using vacuum and a rectangular straight post. (ii) A custom-made uniaxial stretch machine. A Teflon bar is attached to a silicone cell culture membrane and controlled by a motor and cam to apply cyclic stretch. (c) Compression. (i) System where compression is applied through hydrostatic pressurization to a monolayer of cells. (ii) System where compressive forces are applied through direct contact with matrix embedded with cells.

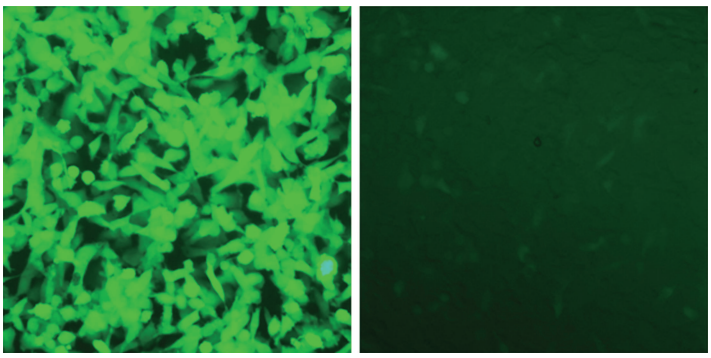


Figure 18.4. HiPerform™ viral constructs generate higher protein expression levels than constructs lacking the new elements. HT1080 cells were transduced with EmGFP constructs in either the non-HiPerform™ vector, pLenti6 (left panel) or pLenti6.3 HiPerform™ (right panel). The HiPerform™ construct displays high efficiency transduction of the cell population and significantly higher EmGFP expression.

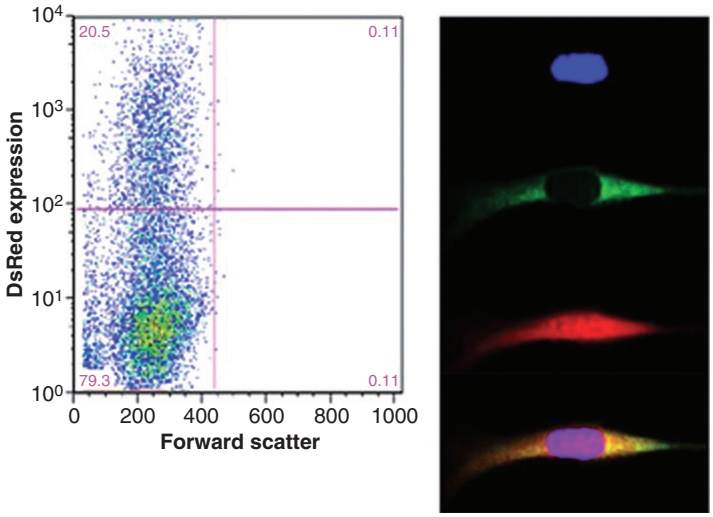


Figure 19.2. Generation of mouse MAPC exhibiting stable expression of DsRed and luciferase. Mouse MAPC were mixed with two independent transposons encoding DsRed or Luciferase and nucleofected along with pCMV-HSB2 as a source of transposase. Gene transfer was demonstrated by flow cytometry of DsRed expressing cells (dot plot). Individual cells exhibiting the highest levels of DsRed expression were sorted into new cultures and expanded. Immunohistochemical analysis of clonal DsRed positive cells was performed using chamber slides to demonstrate coexpression of luciferase (panel). From top to bottom; DAPI (blue), FITC conjugated antibodies specific for luciferase (green), DsRed (red). Cells coexpressing both DsRed and luciferase appear yellow (merge). Adapted from reference 52, with permission.

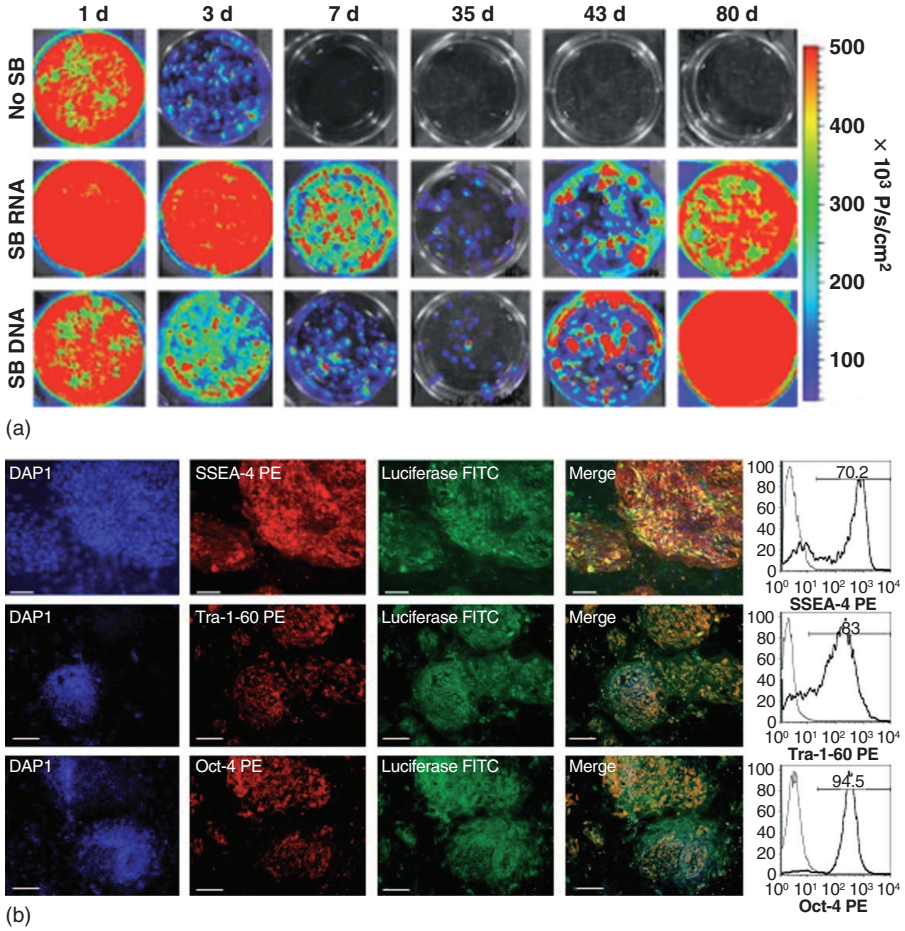


Figure 19.3. Generation of human ES cells exhibiting stable expression of luciferase. (a) Stable expression of luciferase. Human ES cells were nucleofected with SB transposons encoding luciferase along with either no SB transposase (top row, negative control), rU-SB11-U (+SB RNA, middle row), or pPGK-SB11 (+SB DNA, bottom row) as a source of transposase. Luciferase expression was monitored as evidence for transfected cells by bioluminescence imaging. After 1 month of continuous culture, colonies demonstrating the highest levels of luciferase activity (spots pseudo-colored red) were manually transferred to new cultures and expanded. Examples of bioluminescence images obtained from human ES cells maintained in culture for 2–3 months (8–12 passages) under these conditions are depicted. (b) Luciferase positive colonies remain undifferentiated. Immunohistochemical analysis of luciferase positive colonies cultured with MEFs using chamber slides and stained with DAPI (blue; panel 1), PE conjugated antibodies specific for SSEA-4, Tra-1, or Oct-4 (red; panel 2) also demonstrated by flow cytometry (histograms; far right), and FITC-conjugated antibodies against luciferase (green; panel 3). Cells coexpressing both SSEA-4 and luciferase appear yellow (merge; panel 4). Scale bars, 100 µm. Adapted from reference 70, with permission.

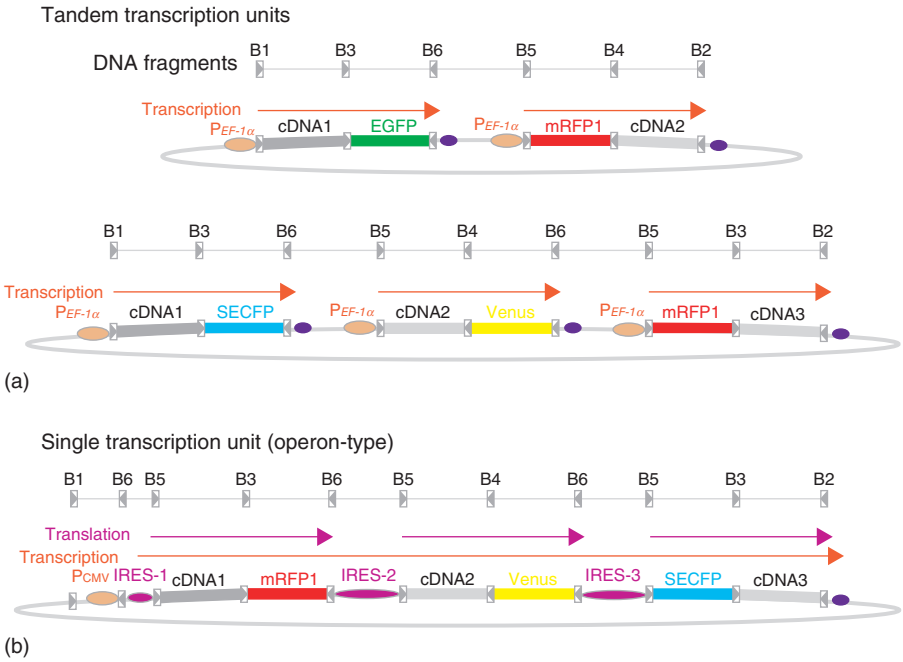


Figure 21.2. Multiple cDNA expression clones with multicolored fluorescent protein tags.

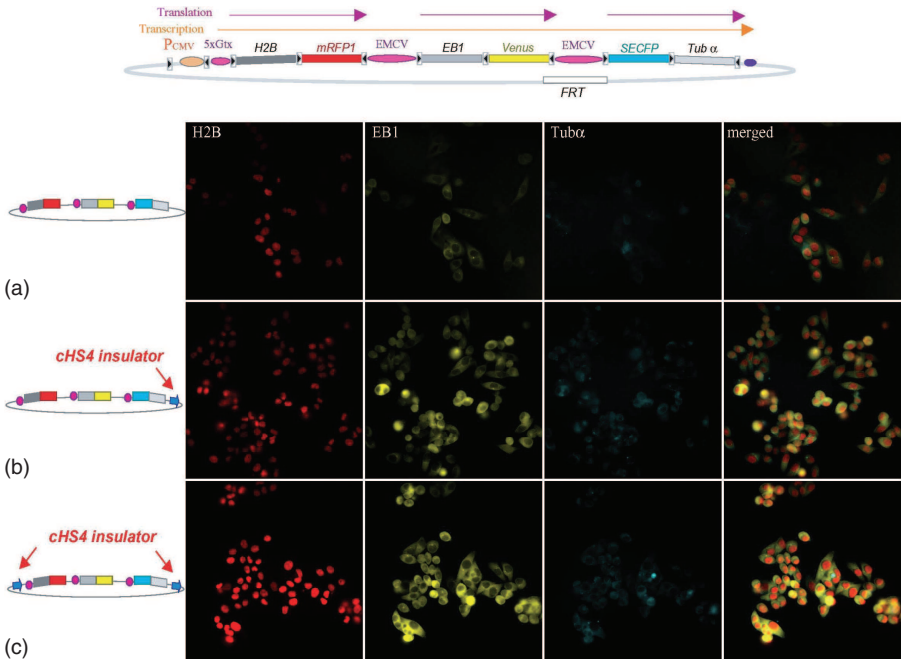


Figure 21.3. Expression levels of an operon type 3-cDNA clone with and without a cHS4 insulator element. Operon-type three fused cDNAs expression constructs were integrated into three *FRT* sites on the chromosomes of HeLa cells. The other interpretations are in the text.

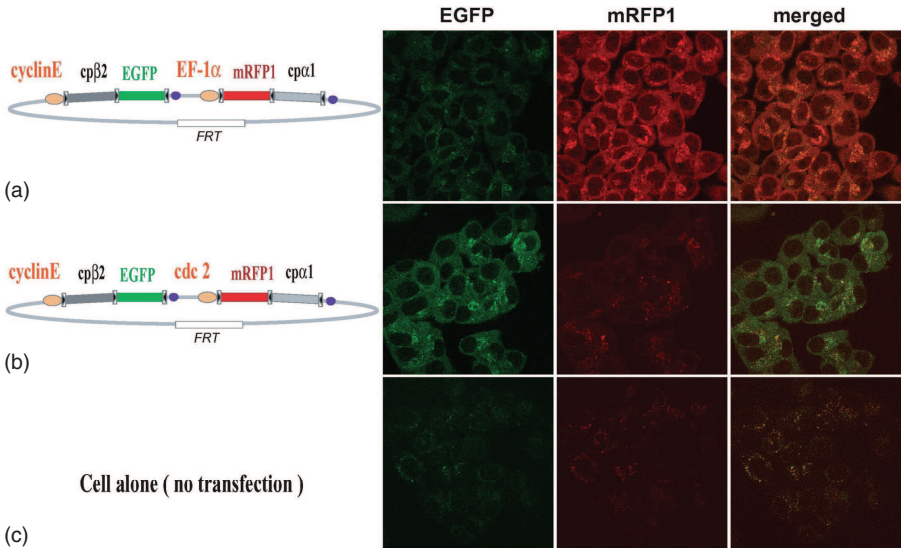


Figure 21.4. Differential expression levels of two tandem cDNA constructs with weak promoters. Differential expression levels were observed by confocal laser microscopy with two fluorescent tagged transgenes of *cpβ2* and *cpα1* introduced into three *FRT* sites on the chromosomes of HeLa cells.

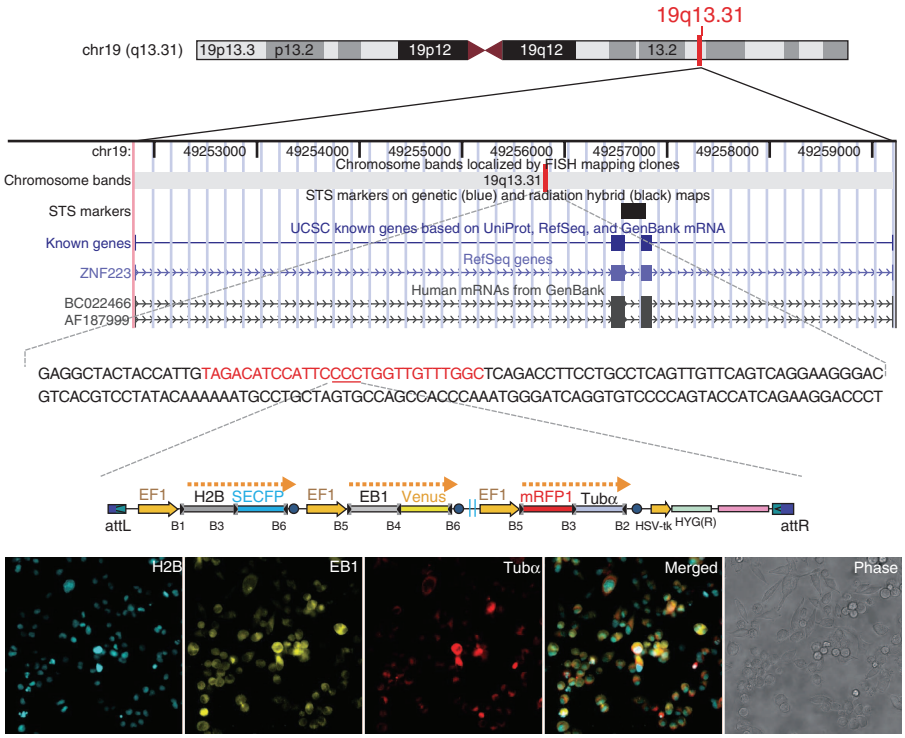


Figure 21.5. Transduction of msGW clone onto chromosome by ΦC31 recombination system. Tandem three fused cDNA expression constructs were introduced by ΦC31 integrase into 19q13.31 pseudo *att*P sites in the HeLa cell genome. The other interpretations are in the text.

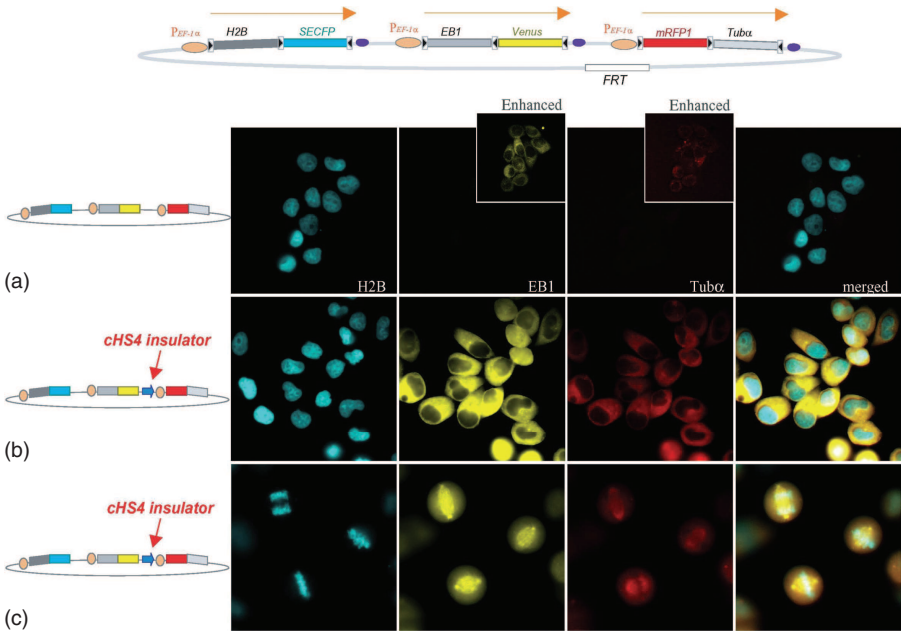


Figure 21.6. Alleviation of transcriptional interference by cHS4 on 3 cDNAs in tandem. Tandem three fused cDNA expression constructs were integrated into three *FRT* sites on the chromosomes of HeLa cells. The other interpretations are in the text.

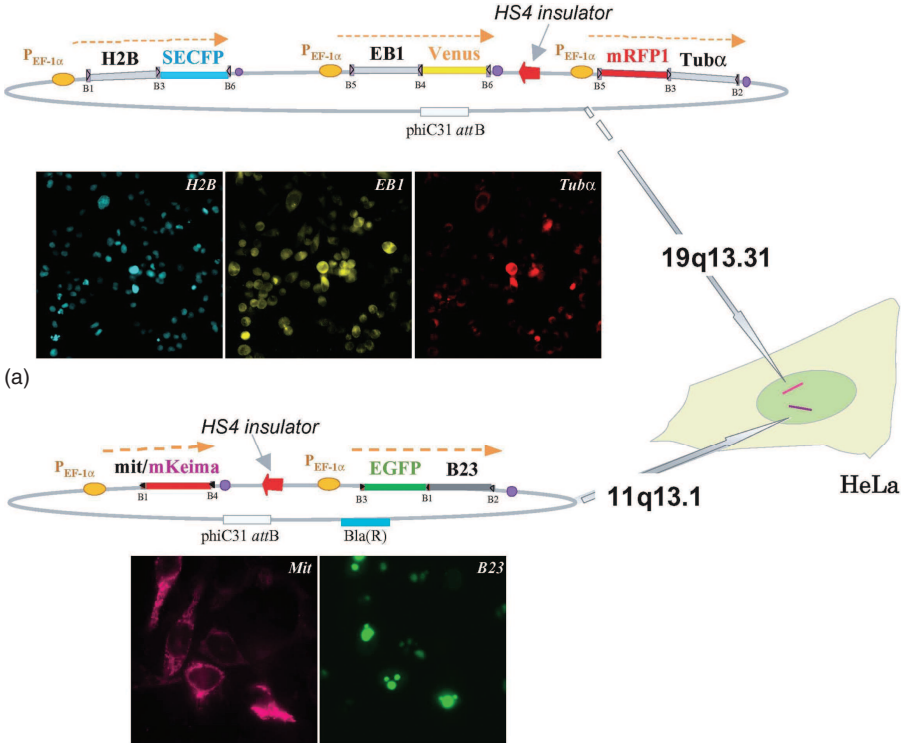
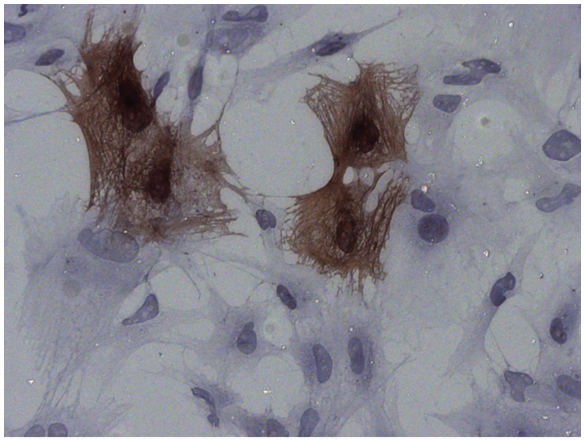
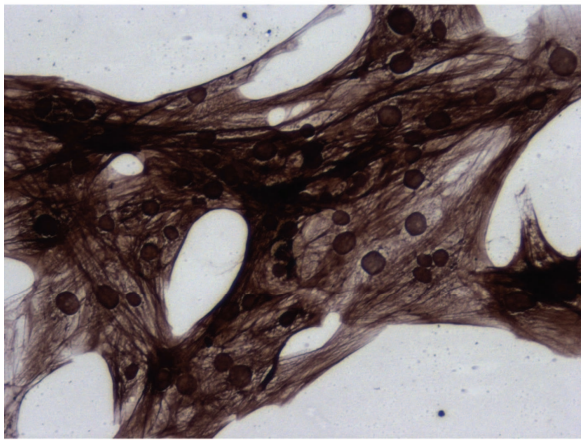


Figure 21.7. Successive introduction of multi-cDNA expression constructs into chromosomes. A tandem three fused cDNA expression constructs and a tandem two fused cDNA expression clones were successively introduced by ΦC31 integrase into pseudo *att*P sites at genomic loci 19q13.31 and 11q13.1, respectively. The other interpretations are in the text.



(a)



(b)

Figure 22.1. Lineage selection to generate a pure population of cardiomyocytes. A transgenic construct comprising the murine a-MHC promoter driving the neomycin resistance gene was introduced into the HESC line hES3. The transgenic line was differentiated into cardiomyocytes by EB formation in END-2 conditioned medium and then subjected to G418 antibiotic selection. In the absence of G418 selection (a), about 10% of the cells were positively stained for α-MHC. Upon antibiotic selection with G418 (b), greater than 99% of the cells were positively labeled for the same cardiomyocyte marker.

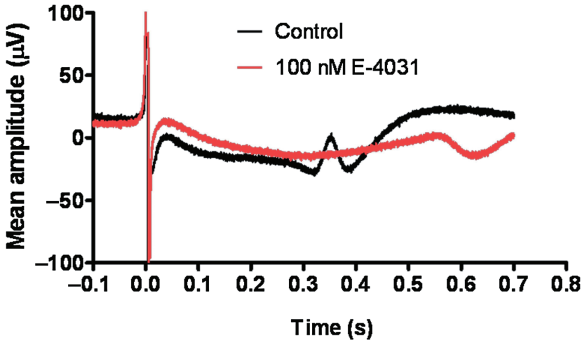


Figure 22.2. A cellular assay for assessing drug-induced QT prolongation. Transgenic cardiomyocytes after G418 selection were dissociated and plated onto a microelectrode array. Extracellular field potentials recorded under control conditions showed spontaneous cardiac action potentials with a duration, or QT interval, of 350 ms. Addition of the hERG channel blocker E-4031 prolonged the QT interval to 580 ms.

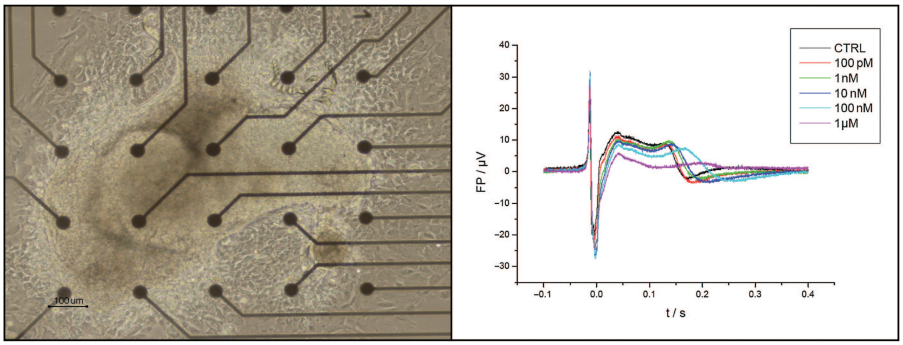


Figure 23.2. Spontaneously contracting cardiomyocyte clusters were derived and isolated from human ES cells, and subsequently plated on microelectrode arrays (MEA) (a). After adhesion of the clusters to the MEA surface, the electrical activity was recorded as the extracellular field potential by the substrate integrated electrodes of the MEA. The addition of the QT-prolonging drug, E-4031, causing hERG channel blockade was measured as a delayed repolarization of the cardiac field potentials (b). Increasing drug concentrations were added to obtain cumulative dose response curves. Notably, E-4031 did not affect the beating frequency over the experiments but caused a sustained prolongation in the low nanomolar concentrations of the cardiac field potential. This is indicative for a hERG channel block known to be caused by this compound. The data were recorded in collaboration with Dr Thomas Meyer (Multichannel Systems, Germany).

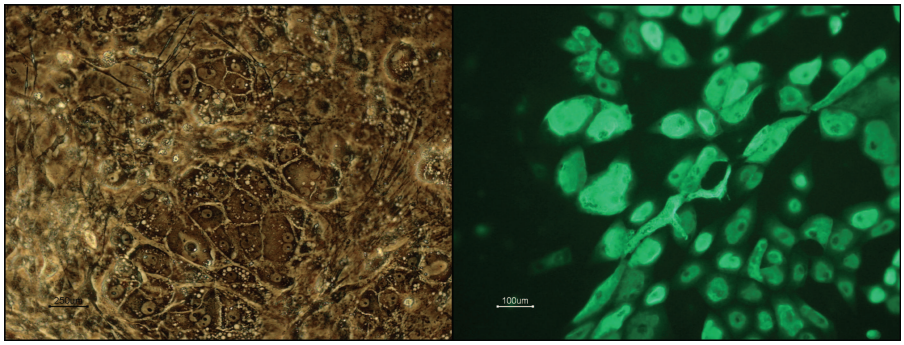


Figure 23.3. Phase contrast image showing hepatocyte-like cells derived from hES cell line SA002 via definitive endoderm (DE-Hep) after 21 d in culture. The cells exhibit a hepatocyte-like morphology; they are big, rhombic, granulated and often bi-nucleated. Immunofluorescent labelling of CYP3A4/7 in DE-Hep derived from SA002 after 38 d in culture.

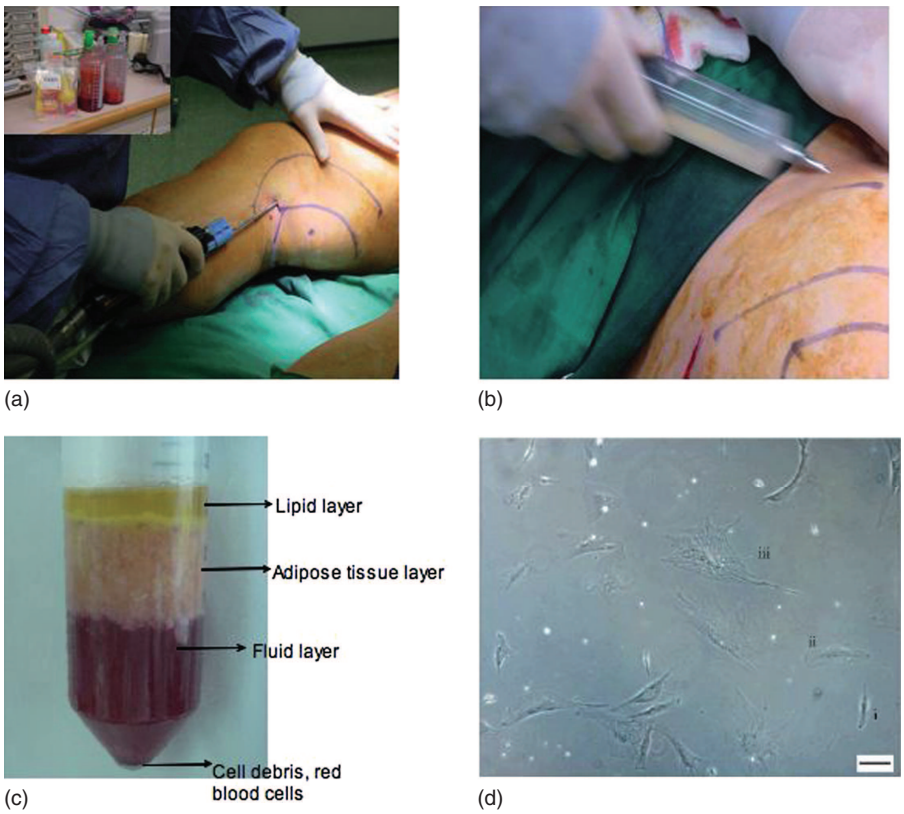


Figure 24.2. In a general liposuction protocol, epinephrine in normal saline solution (1:1 000 000) cooled to 4oC was used (Asken S 1988). This fluid was first infiltrated into the site to be aspirated, via stab incisions. The epinephrine/saline solution infused vasoconstricted the blood vessels to minimize blood loss. After 15 to 20 min, the liposuction cannula was inserted through the previous stab incisions in the abdomen. The pump assisted liposuction procedures were performed with a power-assisted Lipoplasty device, PAL-200® (Microaire, VA, USA) fitted with a size 4 Triport III tip cannula. The maximum negative pressure of –100 kPa (–30 in Hg) was created with a Hercules® liposuction machine (Well Johnson, AZ, USA). A sterile 600 mL drain bottle (Primed®, Halberstadt, Germany) was interposed between the PAL-200 device and the liposuction machine to collect the fat aspirated. (See text for full caption.)

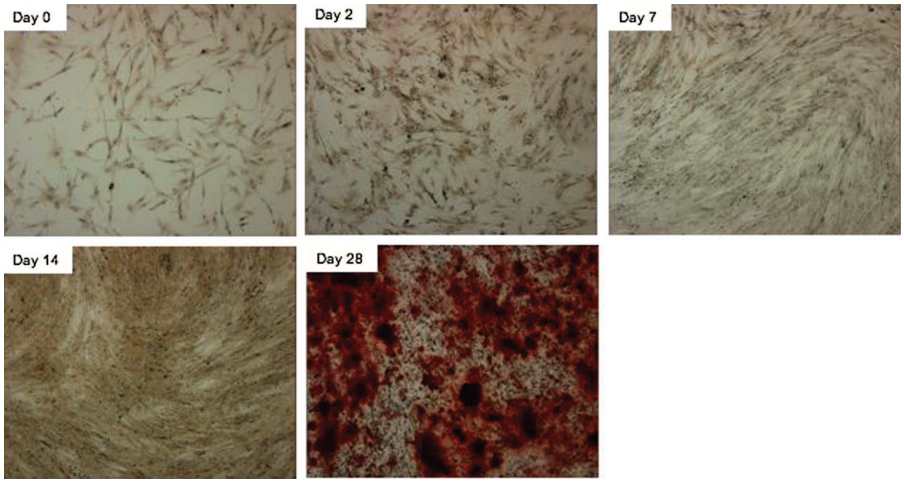


Figure 24.5. The Alizarin Red staining of ADSCs cultured for 4 weeks under osteogenic conditions.

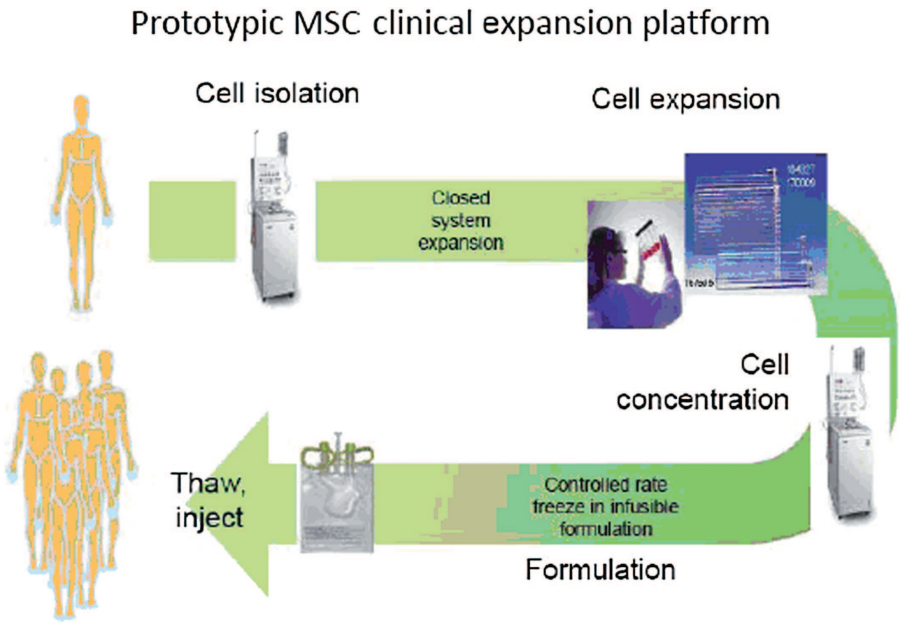


Figure 25.1. Prototype MSC clinical expansion platform. Proposed workflow for the use of MSC in a clinical setting.

PART I

1

DERIVATION METHODS FOR HUMAN EMBRYONIC STEM CELLS: PAST, PRESENT, AND FUTURE

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HUMAN EMBRYO CULTURE FOR HUMAN EMBRYONIC STEM (hES) CELL PRODUCTION

The first hES cell lines were derived from blastocyst-stage embryos nearly 10 years ago (1, 2). So far, more than 500 successful hES cell line isolation from human fresh or frozen morula and/or blastocyst-stage human embryos have been reported by numerous groups worldwide (3, 4). However, in nearly all of these reports, the possible correlations between embryo development parameters and successful derivation were poorly defined, making a true efficiency for hES cell derivation impossible to ascertain from embryology aspects (5).

Irrespective of the isolation methodology used to derive hES cell lines, embryos that are used for derivation should, in principle, be produced by assisted reproductive technologies (ARTs). Therefore, the material to be used is a product of a complex process involving controlled ovarian hyperstimulation, *in vitro* fertilization (IVF) procedures, and intense ethical clearance protocols. These techniques have been in clinical use for nearly three decades, and

Emerging Technology Platforms for Stem Cells, Edited by Uma Lakshmipathy, Jonathan D. Chesnut, and Bhaskar Thyagarajan

are still continuously being improved to increase pregnancy rates and healthy delivery rates worldwide.

Despite acceptable take-home baby rates that have been obtained, results of human ART have shown that during *in vitro* culture, only 30%–40% of the fertilized human oocytes can in fact have the capacity to develop successfully to blastocyst stage. That is, approximately 70%–75% of these zygotes/embryos exhibit varying degrees of developmental abnormalities, including unequal cleavage and excessive fragmentation leading to developmental arrest. Even for those that can become expanded blastocysts, some eventually lose viability before or shortly after implantation, possibly due to induced apoptosis in both inner cell mass (ICM) and trophectoderm lineages.

In most cases, the etiology behind this extensive embryonic loss is unknown, and several studies have so far pointed to a variety of possible causes, including inadequate oocyte maturation, suboptimal culture conditions, and chromosomal/nuclear abnormalities during early cleavage stages, pointing out the need for improved techniques/protocols that can minimize these negative factors *in vitro* (6–13). At different stages of development during which embryonic stem cells have been derived—that is, morula, late blastocyst, and epiblast—at every stage, there is possible embryonic loss and hence there is scope to refine the methodology.

Current research that is aimed at optimizing human embryo development and minimizing this embryonic loss thereby increasing the success rate in ART is in turn expected also to improve the hES cell derivation process. Compared to the late 1990s, today's human embryo culture systems provide more standardized and optimal ingredients and protocols that are more beneficial for human embryo as well as hES cell survival. Recent experience has shown that improvements in human embryo culture conditions help hES culture derivation in two major ways: improvements in embryo quality that may lead to better ICM development and improvements in culture conditions that can provide culture environment that can be closer to *in vivo* (14). It recently has been reported that low O_2 concentration increases the viability of preimplantation embryos, assists their normal development, and forms healthy blastocysts with well-formed ICMs with greater cell number (15). Although current studies on human embryos have not shown relatively increased pregnancy rates with low oxygen culture, numerous authors have indicated a beneficial role of low O_2 on embryo quality, even in early cleavage stages (16).

Although *in vitro* culture systems are continuously being perfected to improve the quality of the materials cultured, intrinsic (paternal) factors that are inherited from infertile couples can also affect the human embryo quality and outcome of hES cell derivation. That is, in many cases, the nature of infertility resides on the quality and quantity of oocytes/spermatozoa to be retrieved, and embryos that are produced from inferior quality oocytes and/or spermatozoa can carry numerous metabolic and/or genetic problems that may affect their development and implantation. Excess embryos from these couples, if used in hES cell derivation, may reach blastocyst stage but still carry abnormal

developmental patterns that can affect the derivation as well as differentiation profile.

Increased rate of imprinting and other epigenetic abnormalities in human *in vitro*-produced preimplantation embryos (17) is a clear example of such consequence. Furthermore, the significantly higher incidence of Beckwith–Wiedemann Syndrome in IVF babies, an imprinting disorder caused by loss of imprinting (LOI) of insulin-like growth factor (IGF2) and other imprinted genes, raises the prospect that the brief *in vitro* culture of human embryos as part of the infertility treatment may cause epigenetic abnormalities (18). This issue may indicate that initial hES cell culture may create relatively diverse epigenetic profiles that can lead to different developmental and differentiation profiles in extended *in vitro* culture. Whether this finding can explain the differences in cultural behaviors of hES cell line in similar *in vitro* settings remains to be seen in the near future.

Although direct association of these problems with a specific ART technique or culture characteristics has not yet been specifically defined, a possible use of human embryos obtained from fertile couples can in theory be a superior source for hES cell derivation.

Nowadays, the allocation of human embryos for hES cell isolation from fertile couples can be realized in two different ways: First, in order to obtain a healthy and human leukocyte antigen (HLA)-compatible child with his/her sibling, fertile couples carrying a specific single gene disorder can undergo IVF and preimplantation genetic diagnosis (PGD) combined with (HLA)-typing procedures. Second, fertile couples can undergo IVF and PGD in order to obtain a child with a desired gender. Although the latter approach is strictly banned in many countries worldwide, in some it can be practiced for family balancing purposes. Therefore, healthy but HLA-incompatible embryos or embryos carrying undesired sex chromosome constitution can be donated for hES cell derivation purposes, thereby allowing the production of hES cells that can be obtained from fertile couples (19).

HUMAN EMBRYOS AS hES CELL SOURCES

So far, nearly all hES cell derivation studies have used either inferior-quality or excess fresh or frozen/thawed human embryos that are donated for research after ART treatments, or donated human ova and sperm cells to produce embryos to be used for this purpose. Several recent studies have also utilized embryos that have shown developmental arrests and discarded from routine IVF treatments, embryos that are produced and donated after PGD, as well as embryos that are parthenogenetically created from donated human oocytes. Very recently, successful generation of hES cell lines from biopsied human blastomeres was also reported. These alternative sources of hES cell lines are depicted in Figure 1.1.

In the majority of hES cell isolation studies, fresh or frozen/thawed spare human embryos were used as a source material. Very few of the fresh spare

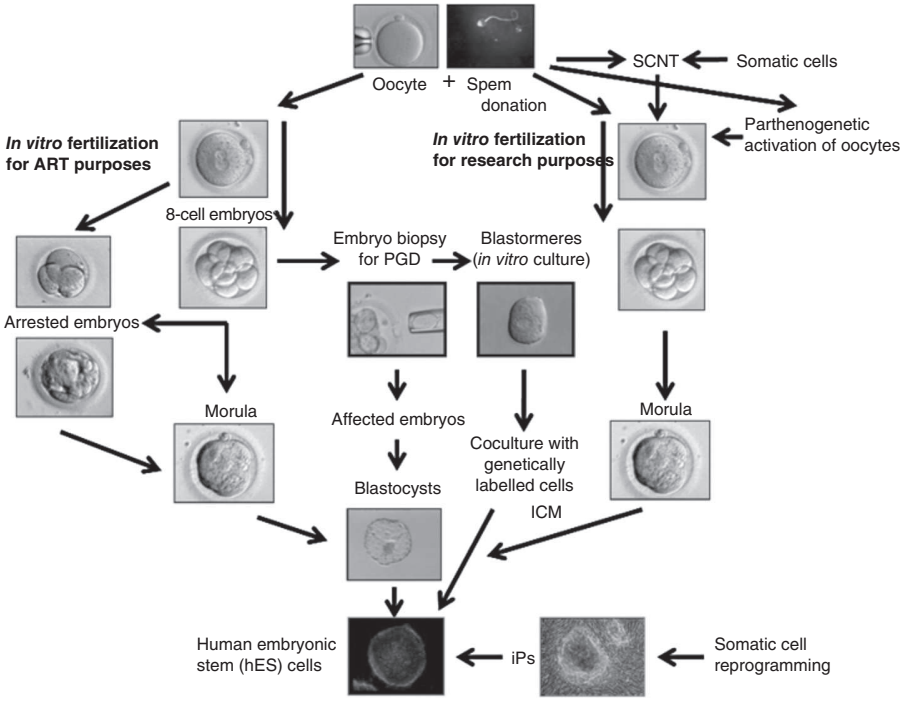

Figure 1.1. A schematic representation of possible hES cell derivation strategies that have so far been practiced worldwide. (See color insert.)

embryos can survive and form blastocysts in extended culture, which results in lower rate of embryonic stem cell derivation compared to frozen/thawed counterparts (20).

hES cell isolation from embryos that were specifically created for this purpose from donated human sperm and oocytes was reported by Lanzendorf et al. (21). In their study, insemination of 142 donated oocytes gave 68% fertilization and 50% blastocyst development rates, respectively. Forty blastocysts were used for immunosurgery, resulting in 18 distinct ICM structures and eventually 3 hES cell lines. Since embryos were produced from gametes of healthy donors, it remains to be argued whether better embryo development and hES cell derivation rates can be obtained with current technology.

Utilization of arrested human embryos for hES cell isolation has recently been reported by Zhang and his colleagues (22). This approach corroborates with several proposals and alternative ways to derive new hES cells without destruction of viable human embryos (23). Especially in countries with a non-flexible policy, arrested embryos can provide a more ethical source for hES cell derivation and hence can resolve some of the political issues surrounding research using human embryos.

PGD technique, in its simplest terms, involves the screening of preimplantation embryos for chromosomal abnormalities or for single gene defects. In this context, it becomes an alternative option to traditional prenatal diagnosis. Besides its diagnostic value and expanding indications such as cancer predisposition, dynamic mutations, and late onset disorders, a new feature, namely PGD combined with HLA typing, also demonstrates its novel therapeutic role in contemporary medicine (24, 25).

The application of PGD has not only helped couples carrying specific genetic problems to have disease-free children, but has also created a novel source for hES cell research. After biopsy and genetic analysis, embryos that are diagnosed as chromosomally abnormal or carrying a specific monogenic disease can be donated for research, thereby resulting in hES cell lines that can be the earliest *in vitro* models for that particular genetic anomaly. On the other hand, a recent report by Munne and his colleagues has further proposed that embryos that were diagnosed as abnormal after PGD could in part have a self-correction ability, that is, revert to a normal karyotype after prolonged culture, resulting in hES cell lines with normal and stable karyotypes (26). However, this finding still remains to be confirmed by other independent studies.

To date, PGD is the only technique that allows the removal of a cell from a developing embryo, without impairing its potential to create pregnancy (27). If in theory, removed cells could be cultured and expanded for several days, they would be an ideal and ethically acceptable hES cell sources for future research and therapy. Retrieval of individual human blastomeres through PGD and expanding them directly in culture mainly for detection of chromosomal aneuploidy have been documented by some groups, and proof-of-concept study has been performed on mice (28–31). Several studies initially aimed to culture individual human blastomeres *in vitro*. However, the results were hampered by the fact that culture conditions and techniques were inefficient to successfully culture and expand diploid human blastomeres *in vitro*.

The fact that cleavage during preimplantation stage as well as in hES cell cultures largely depends on the presence of adjacent neighboring cells as well as several unknown factors produced by them requires further attention, and technical improvements and alternatives should be sought to clarify the feasibility of this approach. In mice, whether a blastomere is to become either a trophoblast or an ICM cell appears to be specified by its position during first cleavage (32). It was also demonstrated that asymmetrical distribution of Cdx2 gene product in mouse oocytes and embryos defines the lineage of trophectoderm (33). In humans, this process happens to occur during compaction stage. This observation is in correlation with recent studies in that a signal for ICM or trophectoderm lineage can be present in some blastomeres far more earlier than the phenotypical characteristics would have emerged (34, 35).

Despite all the limitations and initial reports indicating unsuccessful attempts to derive stem cells from biopsied human blastomeres, Klimanskaya

et al. were first to report the derivation of new hES cell lines from isolated human blastomeres (36, 37). Although their technique was later extensively argued that the approach was not the same as human embryo biopsy for PGD purposes, the same group has very recently announced the extension of their initial reports on embryos obtained from routine PGD cases (38). These results can indicate that if one or two biopsied blastomeres would be enough to expand the line, the same embryo would also be implanted in the uterus, creating a viable offspring and comparable pregnancy rates as well as an unlimited stem cell source for that sibling, hence opening a new era in both clinical applications and ethically permissive hES cell isolation and derivation studies.

Unless derived from the same embryos as mentioned above, creating clinical grade hES cells was considered to be of limited use unless there existed a similar HLA match between the cells and the potential recipient. For this reason, recently, somatic-cell nuclear transfer (SCNT) technology has been applied in the creation of patient-specific human and primate embryonic stem cell lines by several groups (39, 40).

Compared to SCNT, parthenogenetic activation of oocytes is a relatively simple method to create histocompatible stem cells since the technique does not require complex instruments and micromanipulation experience. In mice and primates, parthenogenetic embryonic stem cell lines have been derived and their pluripotency has been demonstrated by different groups (41, 42). Patient-specific stem cell lines from human parthenogenetic blastocysts have recently been reported by different groups (43, 44).

As a valid alternative to patient-specific hES cell line derivation by SCNT, it was proposed that a minimum of 40–50 homozygous hES cell lines would be necessary to cover 50% of the HLA isotypes in the American population and 150 cell lines to cover the UK population, thus minimizing the immune rejection of hES cell-derived transplants (45, 46). However, although these numbers may be underestimated due to the ethnically diverse nature of the populations in question, the creation of master hES cell banks for future therapeutic as well as research applications seems to be a realistic approach which will be helpful to the standardization of hES cell cultures and the reduction of the cost and unnecessary derivation of new hES cell lines in the near future (47–49).

Apart from human gametes and embryos as hES cell sources, several groups have recently announced that they have produced induced pluripotent (iP) cells from human adult somatic cells (50, 51). Although they have used slightly different protocols, their approaches commonly involved overexpression of a group of four genes (Oct-3/4, Sox2, Klf4, and c-Myc) that are known to be actively expressed in hES cells. The results have shown that these induced cells show common pluripotency and differentiation characteristics of hES cells, although they are not identical as shown by DNA microarray analyses. However, if this “reprogramming of differentiated cells” is possible, there would be no need for blastocysts to be disintegrated to extract ICMs, hence

the technology would be ethically permissive and there becomes no need for therapeutic cloning.

Besides many important issues such as epigenetic status, safety issues related to gene modification and tumorigenic potential of these cells remain to be explored; the approach could at least in theory be an alternative way to produce hES cells from human embryos and hence could be more acceptable from an ethical point of view.

ISOLATION METHODS

So far, the derivation methodology of the reported hES cell lines included isolation of ICM cells from trophectoderm (TE) cells by immunosurgery, mechanical, chemical, or laser-assisted removal of ICM, and direct plating of intact blastocysts on feeder cells/dishes coated with extracellular matrix proteins without prior ICM dissection and proper staging of blastocysts for hES cell derivation (1, 2, 52–55).

Immunosurgery method utilized the exposure of embryos to pronase enzyme, animal-derived complement system reagents, and antibodies raised against human cells (Figure 1.2). Recent experiences, however, have shown that this technique is not the optimal derivation method when poor-quality, spare embryos with small or nearly visible ICMs are used. Moreover, hES cells that have been isolated with this method will eventually not be suitable for therapeutic use due to their risk of carrying animal pathogens (56).

Compared to immunosurgery, the application of "whole culture method" or "direct culture method" was also reported to be equally effective in the derivation process in several recent reports (20, 57–59). This method has been mentioned to be superior to immunosurgery since it not only shows better success rates, but it also clearly avoids the use of animal-derived antibodies, hence making the culture system—one step—more suitable for therapeutic use. Several recent publications also reported novel isolation strategies, which indicate that isolation technique can be optimized according to the quality of the embryo used in the derivation process; therefore, a combined strategy that utilizes varying culture and isolation methods could be the method of choice (60).

Chemically removing zona pellucida by means of acid Tyrode's approach is generally thought to be superior to expose the embryo to pronase, an enzyme that is extracted from bacteria. However, exposure of embryos to the acidic solution (pH of 2.5–3) can be hazardous for ICM cells unless the incubation time is carefully optimized and the procedure itself is done by an experienced staff. Laser is widely used in contemporary ARTs for artificial opening of zona pellucida before intracytoplasmic sperm injection (61), embryo transfer (assisted hatching) (62), or embryo biopsy during PGD applications (63). The use of laser technology in ICM isolation for hES cell derivation has recently been reported by Turetsky and his colleagues (55). Their study indicates that

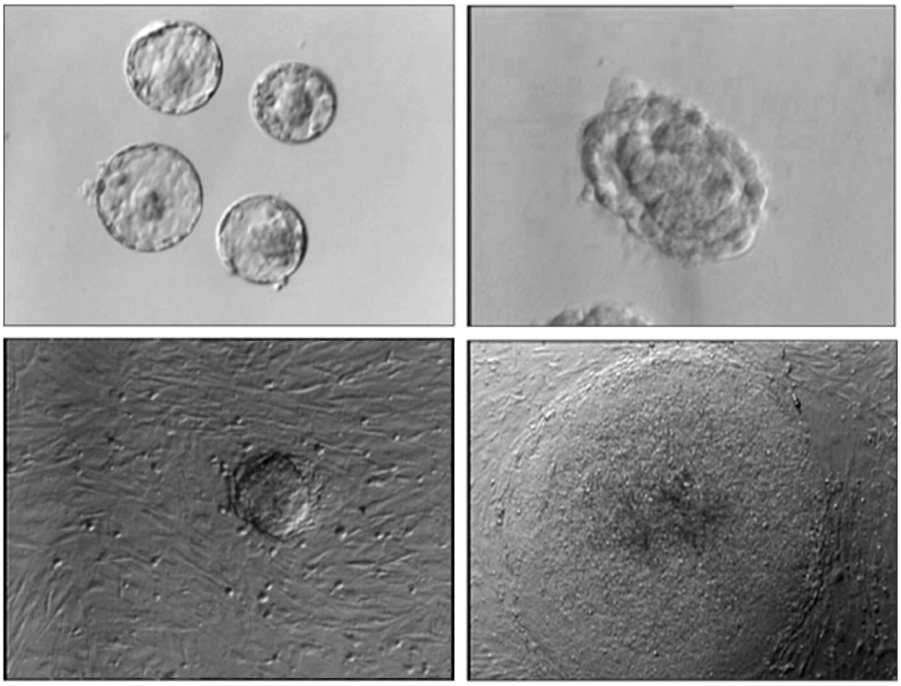

Figure 1.2. Establishment of an hES cell line by immunosurgery. (a) Good quality human blastocysts that are suitable for immunosurgery; (b) A zona-free human blastocyst in culture media containing complement proteins. Note the degenerating trophoblast cells due to complement action; (c) A picture of growing ICM cells attached on feeder surface after immunosurgery; (d) A good quality sixth-day human embryonic stem cell colony.

this approach can potentially be useful in xeno-free hES cell derivation since it avoids the use of animal-derived enzymes of immunological substances that have traditionally been used for this purpose. As another potentially advantageous isolation method, Strom et al. developed a mechanical isolation protocol that utilized two metal needles with sharpened tips that can cut both zona pellucida and ICM, therefore avoiding exposure of embryos to either acidic environments or animal-derived substances (54). Patented technologies for laser ablation and removal of trohoectoderm by mechanical means or transferring the ICM to a second plate are developed recently.

CULTURE, MAINTENANCE, AND UPSCALING OF hES CELLS FOR THERAPEUTIC USE

The proper maintenance and expansion of hES cells is one of the most important issues in hES cells biology. Although it has been nearly 10 years aince the

first successful report on hES cell isolation and expansion, *in vitro* culture of these cells still need direct exposure to one or several undefined culture ingredients of nonhuman origin. The use of blood-borne complement system to isolate ICM from an expanded human blastocyst has already been discussed above. However, current hES cell culture still needs other materials such as serum, mitotically inactivated mouse embryonic fibroblasts (MEF), or MEF-derived extracts.

The feeder layer provides certain currently unknown factors, which support undifferentiated growth of hES cells. Although hES cell lines were initially cultured on mouse-derived fetal fibroblasts, recently, these feeders have been replaced with human counterparts that are isolated from various tissues (64). Another recent approach involved the culture of whole blastocysts on defined or purified cell extracts or matrix proteins such as collagen VI, fibronectin, laminin, and vitronectin of human origin (65).

Previous studies that were performed to develop feeder-free culture environments to support established hES cell lines have indicated that three factors, namely transforming growth factor (TGFβ1), leukemia inhibitory factor, and bone morphogenic protein antagonist Noggin act together to suppress hES cell differentiation and promote self-renewal (66, 67). In order to develop chemically defined media that sustain hES cell self-renewal, it is very important that signals and mechanisms controlling hES cell fate choices (such as choosing to differentiate into a particular lineage or continue to proliferate as undifferentiated progeny) should be understood in detail. Several recent studies have found out that in hES cell self-renewal, a major role is played by members of the Wnt and TGFβ superfamily of signaling molecules (68, 69). TGFβ family members seem to stimulate hES cell self-renewal by inducing phosphorylation of the intracellular mediators Smad2 and/or Smad3. On the other hand, bone morphogenic proteins induce hES cell differentiation to extraembryonic lineages or to germ cells by phosphorylation of Smad1/5/8 (70–72). β Fibroblast growth factor (βFGF) and IGF-II also play important roles in hES cell self-renewal by inducing expression of TGFβ family molecules such as TGFβ/Activin/Nodal (73).

In a very recent study, albumin-associated lipids, which are essential ingredients in knockout serum replacement, have also been found to have strong positive effects on the self-renewal of hES cells, indicating that deeper understanding of the mechanisms will eventually lead us to produce xeno-free chemically defined culture media for hES cell self-renewal and differentiation (74). Clearly, lack of xeno-free reagents during the process of derivation of hES cell lines is a huge gap that needs to be addressed and availability of reagents such as knockout serum replacement that is made completely with xeno-free materials or with human materials would enable researchers to derive next generation hES cell lines with greater potential for human clinical cell therapy relevance.

Upscaling process involves the disaggregation of undifferentiated hES cell colony pieces from their original culture and transferring them into new

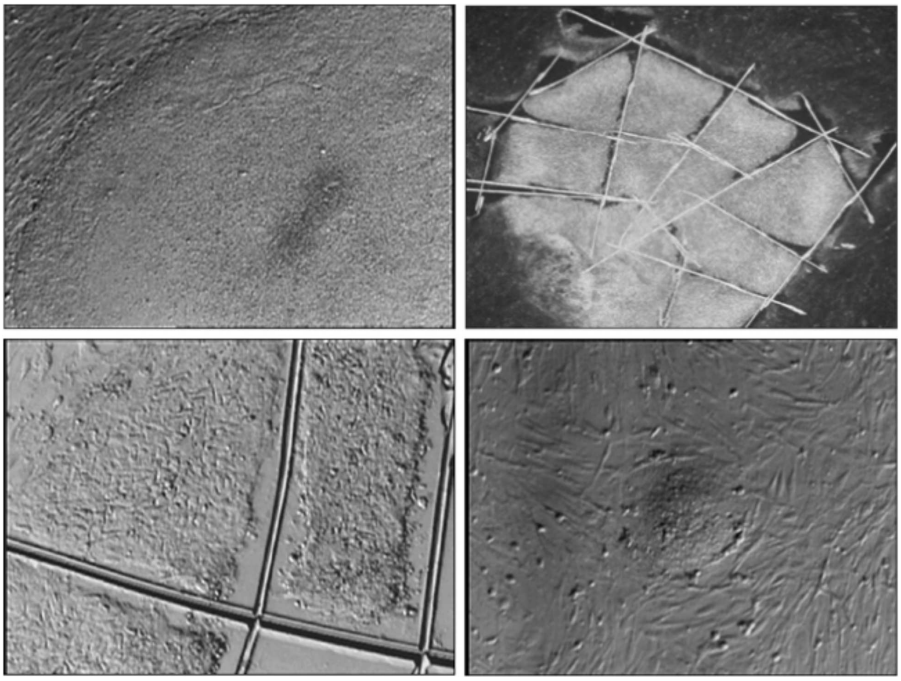

Figure 1.3. Mechanical passaging of an hES cell colony. (a) A well-expanded and undifferentiated hES cell colony that is ready for passaging; (b) Uniform cutting of a suitable colony by finely drawn glass Pasteur pipette; (c) A closer look at the picture in B: At this stage, colony pieces start rounding from the edges and pieces are dissociated from the culture plate by scratching them with a polished glass pipette; (d) A growing hES cell colony 1 d after mechanical passage.

culture environment, which again supports undifferentiated growth of hES cells. The most widely used method to maintain undifferentiated "high-quality" hES cells in culture is mechanical passaging (Figure 1.3). In this method, micropipettes or finely drawn Pasteur pipettes are used to cut the proper colonies in pieces and optimum numbers of pieces are transferred into new culture dishes every 4–7d. Although this method seems to be advantageous over enzymatic dissociation in that no animal-derived dissociation enzyme is used, it certainly becomes a limited technique when large-scale hES cell production is required. Passaging and upscaling of hES cells by enzymatic techniques has, on the other hand, recently been questioned by several reports. Compared to the ones that were mechanically passaged, cells that were treated with dissociating enzymes have shown accumulated chromosomal abnormalities, indicating a potential technique-induced genetic instability of the hES cell lines studied (75, 76).

In summary, therapeutic-grade hES cell culture requires three major technological advances:

1) Completely good manufacturing practice (GMP)-grade production of human embryos followed by animal- and pathogen-free ICM isolation.
2) Completely feeder-free (animal or human origin) hES cell culture systems, which only involve purified and screened human extracellular matrix proteins as well as growth factors, cocktails, etc.
3) A newer cGMP products of xeno-free nature that are clear alternatives to extracellular attachment factors such as matrigel, and completely humanized or xeno-free reagents such as knockout serum replacement.
4) Suitable large-scale hES cell culture expansion systems that allow mass production and upscaling of hES cells without any negative effect on cellular proliferation, differentiation, and genetic instability.
5) Well-defined differentiation protocols/systems that allow mass production of certain precursors/ultimate somatic cell types.

CONCLUSIONS

Since 1998, accumulated data on hES cell derivation and culture indicate that hES cell research is exponentially expanding and moving forward to keep its promise in many scientific and medical disciplines, including developmental biology, human embryology, toxicology, pharmacology, genetics, as well as regenerative and reproductive medicine.

Although so far, all reported hES cell lines have been derived by using animal-derived material either during derivation or cultivation and hence are not suitable for clinical use, recent advances in understanding embryonic stem cell self-renewal and expansion mechanisms will no doubt bring the contemporary stem cell research one step closer to xeno-free hES cell lines that can be utilized in medicine in the near future.

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2

ISOLATION OF HUMAN EMBRYONIC STEM CELLS FROM VARIOUS STAGES OF THE HUMAN EMBRYO

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INTRODUCTION

Preimplantation genetic diagnosis (PGD) provides an exciting possibility for obtaining HLA-matched stem cells (SCs) for treatment of siblings with bone marrow disorders (1–6). This involves preselection of HLA-matched embryos in couples with affected siblings requiring the HLA-compatible SC transplantation, obviating the need for therapeutic cloning, which is highly controversial at the present time. However, in emergency situations, it may be too late for awaiting for the birth of the HLA-matched child, so other possible approaches need to be developed. For example, some of the tested embryos may fail to reach the developmental stage to be considered for transfer, so they might be used for establishment of human embryonic SC (hESC) lines. Alternatively, partially matched embryos may be also considered for this purpose with the patients' consent, or one blastomere can be removed from the HLA-matched embryo and be used for the establishment of HLA-compatible hESC (7).

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So PGD, in addition to improving the access to the HLA compatible SC transplantation, also provides a novel source for hESC (8–11). Although hESCs are usually derived from the culture of the inner cell mass of the preimplantation blastocyst, as shown in mice and humans (12, 13), the possibility of the establishment of ESC from morula in mink, and cow, and from embryonic germ cells was also reported (14–16). The current NIH repository of hESC lines contains a few dozens of hESC lines, of which not all have met the NIH scientific criteria, including the presence of L-alkaline phosphatase (TRA-2-39), Oct-4, telomerase, high molecular weight glycoproteins (antibodies TRA-1-60, TRA-1-81), stage-specific embryonic antigens (SSEA-3, SSEA-4), euploid karyotype, and teratoma formation in severe combined immunodeficiency (SCID) mice (17).

We developed an original technique for the establishment of hESC cell lines from the human embryos at the morula stage, described below, and also used the other available methods of the hESC derivation from the blastocyst stage embryos, for the establishment of the world's first and largest repository of hESC lines with normal and abnormal genotypes presented in this chapter.

ESTABLISHMENT OF hESC LINES FROM THE MORULA STAGE EMBRYOS

The isolation of hESC from the embryos at the morula stage was performed as described elsewhere (9). In brief, following PGD, the day 4 morula stage embryos were cultured 72 h in Gardner's G1 and then in human tubal fluid (HTF) medium supplemented with plasmanate. After removal of zona pellucida by pronase, the naked morula was injected under feeder layer, representing the murine primary embryonic fibroblasts, or buffalo rat liver (BRL) cells, mitotically inactivated by mitomycin C for 5–6 hrs. The cells were cultured in Alpha MEM or DMEM medium, supplemented with 10%–20% of fetal bovine serum or knockout serum replacement (SR-1), with the addition of beta-mercathoethanol and human recombinant basic fibroblast growth factor (FGF) at final concentration 5 ng/ml. After cell outgrowth and spreading into layer, observed in approximately 8–14 days, the cells were disaggregated, using EDTA in Hank's or phosphate buffer saline (PBS) Ca^{2+} Mg^{2+} free solutions. The soft loose cell clumps were cut and transferred into a new dish with feeder layer, while loose cells were carefully transferred onto feeder layer and allowed to proliferate. Fast proliferating colonies with ES-like morphology were isolated and propagated further. Within the next two to five passages (four to six cell population doublings per each passage), the uniform proliferating cells were selected, and the colonies of established ESC lines were passaged using collagenase in HTF-HEPES or EDTA, followed by the harvesting with cell lifter, while the undifferentiated ESC population was isolated using EDTA solution.

In comparison to hESC obtained from inner cell mass (ICM), the cell proliferation from the morula stage embryos creates a plate of compacted cells, proliferating further in the culture dish. Following the initial passage of proliferating

cell clusters, the disaggregated cells provide the cell outgrowth in up to 80% of the morula cultures initiated, after they are placed on the top of feeder layer. During the next two to four passages, only the cell colonies with the morphology of ESC are selected for a further culture. It is at this stage that, approximately, a quarter of morula-derived cell lines failed to further proliferate. Overall, only one quarter of morula stage embryos result in the initial outgrowth, with only up to 20% yielding ES cell lines, not significantly different from the outcome of the hESC lines originating from blastocyst stage embryos.

The isolation of ESC from morula was first attempted in rabbit embryos almost 50 years ago, which was also compared to the establishment of ES cells from blastocyst (18). The morula-derived ESC lines were also established from murine embryos and were shown to be similar to the ESC isolated from the blastocyst stage embryos (19). Then this was also reproduced from morula stage mink and cow embryos (14, 15).

However, the basic method for obtaining hESC is still derivation of ESC lines from the ICM of human blastocyst (13). The majority of our hESC lines were also obtained using this method, with a few modifications. In brief, after mechanical removal of the collapsed trophoblast, the isolated ICM (or the whole blastocyst) was placed on the top of feeder layer, with proliferating cells observed within 24–48 h. The initial passage of the blastocyst outgrowth was performed, following 7–10 min exposure to EDTA in PBS or Hank's Ca, Mg-free solution, with further passages performed using collagenase or EDTA, followed by harvesting with the cell lifter. The cell suspension was put through the 100-mkm cell strainer before placing on the fresh feeder layer.

It is therefore of interest that the hESC lines may be produced from the human morula, despite the differences of morula and blastocyst-derived cells in size of adjacent cytoplast and gene expression pattern, including unstable maternal methylation pattern that persists until the morula stage, in contrast to that at the blastocyst stage, characterized by the low methylation pattern independent of the parental origin (20). For example, the bovine embryos display a high sensitivity to ouabain (inhibitor of the Na/K-ATPase), with the enzymatic activity undergoing a ninefold increase from the morula to the blastocyst stage (21). mRNA expression patterns have been also shown to be different in the mouse morula and blastocyst stages embryos (22, 23).

As described, the method of derivation of hESC lines from the morula stage embryos is robust and efficient, comparable to the derivation of hESC lines from the whole blastocyst or ICM. Despite the above-mentioned differences in the cell morphology and gene expression patterns between morula and blastocyst stage embryos, comparative studies of hESC lines obtained from the embryos of different stages of embryonic development revealed no differences either in the cell morphology or the pattern of marker expression, including alkaline phosphatase (AP), TRITC-immunofluorescence of expression of Oct-4, immunofluorescence of tumor recognition antigens (TRA)-2-39 (L-AP), TRA-2-60, and TRA-2-80, detected by monoclonal antibodies labeled by fluorescein isothiocyanate (FITC), antigens stage-specific embryonic antigens (SSEA)-3 and SSEA-4.

No differences between the blastocyst and morula-derived hESC were also observed in the patterns of marker expression, following their culture in the feeder layer free medium, and their ability to differentiate into different type of cells. However, further studies are needed to investigate if the morula-derived hESC would display differential characteristics at later stages of differentiation, depending on a possible partitioning of inside and outside cells of morula on the time of harvesting, from which the hESC were derived. Because the morula inside cells were shown to express Oct-4 (24), the resulted morula-derived hESC may have originated from these cells. The same cells may be involved in the forming of the blastocyst epiblast, from which the hESC have been derived (25).

Thus, it is possible to derive hESC lines from human morula, representing typical hESC morphology and marker expression, which may be used in the embryo research and cell therapy clinical application.

REPOSITORY OF hESC LINES WITH NORMAL AND ABNORMAL GENOTYPES

The above developments have made it possible to initiate the establishment of the repository of hESC lines with different genetic abnormalities. Although the major emphasis of the establishment of hESC lines was initially on the development of the cell-replacement therapies, it is currently also important for the studies of mechanisms of genetic disorders through generating the sources of normal and genetically abnormal cells and tissues. The ability to obtain the hESC lines with specific genetic disorders, which could produce unlimited quantities of the disease tissue where the disease has a genetic basis, makes it realistic to undertake the research on the primary disturbances of the cellular processes in the genetically abnormal cells and to identify the molecular mechanisms that might be blocked to prevent the disease progression. Therefore, there is obvious need for establishment of the hESC lines originating from embryos with genetic and chromosomal abnormalities, to provide the basis for understanding of the mechanisms of phenotype realization of genetic defects and for the development of new approaches for their possible treatment.

As mentioned, the derivation of hESC lines with genetic disorders has become possible with the introduction of PGD, as a result of which the mutation-free embryos are transferred back to the uterus, while the affected ones provide the valuable source of hESC lines with genetic abnormalities, which can be initiated using the above techniques of derivation of hESC lines from various stages of the human preimplantation embryo.

Initially, a poorer outcome of hESC lines from the embryos with autosomal aneuploidies was expected because of a known selective disadvantage of these cells in culture. On the other hand, there were reports of the risk of the *de novo* chromosomal abnormalities in the process of the propagation and maintenance of hESC lines. Incidental chromosomal abnormalities were first

detected in the hESC line approved by the NIH as a source of hESC and distributed to more than hundred research institutions around the world. This hESC line was obtained by the Wisconsin-based stem cell registry WiCell, and was originally shown to have a normal female karyotype, after derivation and maintenance in the culture for several months. This hESC line was also shown to have the ability to differentiate into the neural and beating cardiac muscle cells. The karyotyping changes involving the gain of the chromosome 17q were observed in the three independent hESC lines, on the five different occasions, which also revealed the occasional gain of chromosome 12, suggested to be attributable to a selective advantage of these cells to the propagation of undifferentiated cells (26). This phenomenon was observed also in other studies (27) and thus created a concern over the use of hESC lines for the stem cell-based therapies, because of a possibility that cytogenetic changes may be only a part of genetic abnormalities acquired in the process of the establishment, maintenance, and differentiation of human ES cell lines.

The established hESC lines from our repository were tested for alkaline phospatase, stage-specific antigens SSEA-3 and SSEA-4, high molecular weight glycoproteins or tumor rejection antigens, TRA-1-60 and TRA-1-80, and Oct-4. The hESC lines were maintained *in vitro* for up to over a dozen passages before freezing in sufficient amounts. The list of the hESC lines presently contains 212 hESC lines, including 155 with normal genotype, 46 with different genetic disorders, and 11 with chromosomal abnormalities, which are frozen and available at different passages (Table 2.1).

The repository of hESC line with genetic disorders includes 13 hESC lines derived from the embryos with autosomal recessive disorders, including six with beta-globin mutations (three thalassaemia mutation carriers, two affected with thalassaemia and one with sickle cell disease), one with Fanconi anaemia, complementation group A, three with cystic fibrosis (two homozygous for ΔF-508 mutation, and one double heterozygous R117C/W128X), two with Sandhoff disease, and one with spinal muscular atrophy.

Twelve hESC lines were derived from the embryos with X-linked disorders, including one with adrenoreukodystrophy, two with fragile site mental retardation (one affected male and one carrier female), two with ocular albinism, and seven with Duchenne (two affected and two carriers), Becker, and Emery–Dreifuss (one affected and one carrier) type muscular dystrophy.

Twenty-two hESC lines were derived from the embryos with autosomal dominant conditions, including seven with neurofibromatosis type 1, one with Marfan syndrome, one with torsion dystonia, two with tuberoses sclerosis, one with pterigium syndrome, four with fascioscapular muscular dystrophy 1A, two with muscular dystrophy, and four with Huntington's disease, the latter six representing dynamic mutations, partially described previously (2, 11).

The collection of hESC lines with chromosomal disorders presented in Table 2.1 contains overall 11 lines, including four lines with translocations, one with trisomy 21, one with trisomy 14, one with triploidy, and four with aneuploidy of sex chromosomes, including 45,X, 47, XXX, and two with 47, XXY,

TABLE 2.1. List of Human Embryonic Stem Cell Lines with Genetic and Chromosomal Disorders

Genetic Disorder
Autosomal Dominant
Huntington's disease, affected, expansion ($n = 4$)
Marfan syndrome, affected, G7712A/N
Myotonic dystrophy, affected, expansion ($n = 2$)
Neurofibromatosis, type I affected ($n = 7$)
Torsion dystonia, DYT1, affected, Exon 7 GAG deletion
Tuberosis sclerosis, TSC1, affected ($n = 2$)
Popliteal pterigium syndrome, PPS. affected
Fascioscapular muscular dystrophy 1A; FSHMD1A ($n = 4$)
Subtotal: 22
Autosomal Recessive
Beta-thalassaemia, affected, IVSI-110/Cd39
Beta-thalassaemia, affected, IVSII-1/Unknown
Beta-thalassaemia, carrier, IVSII-1/N
Beta-thalassaemia, carrier, IVSI-110/N ($n = 2$)
Cystic fibrosis, CFTR, affected, R117C/W128X
Cystic fibrosis, CFTR, affected, ΔF-508/ΔF-508 ($n = 2$)
Fanconi anaemia-A, 14 bp deletion, carrier
Sickle cell disease
Spinal muscular atrophy, exon 7 deletion
Sandhoff disease ($n = 2$)
Subtotal: 13
X-Linked
Adrenoleukodystrophy, X-linked, affected
Becker muscular dystrophy, affected, del
Duchenne muscular dystrophy, carrier, del ($n = 2$)
Duchenne muscular dystrophy, carrier, del/N ($n = 2$)
Emery–Dreifuss muscular dystrophy, X-linked, affected male
Emery–Dreifuss muscular dystrophy, X-linked, carrier[a]
Fragile–X syndrome, affected male, expansion
Fragile–X syndrome, female carrier
Ocukar albinism, X-linked, affected ($n = 2$)
Subtotal: 12
Chromosomal Disorders
69,XXX
47,XXY
46,XX, der(4) t(4:13)
46,XX, t(10:22) (q25;q13)
47,XX,+14
45,X
47,XXY*
47,XXX
47,XY+der21 t(2;21)
46,XX t(15;17)
47,XX +21
Subtotal: 11
Total: 57[a] hESC lines

[a]The same hESC line (with XXY and Emery–Dreifuss muscular dystrophy).
CFTR, cystic fibrosis transmembrane regulator.

one of which was derived from the same embryo that was the source of hESC line with Emery–Dreifuss (carrier) type muscular dystrophy (Table 2.1).

As can be seen from the recent review of the status in the establishment of hESC (28), the presented repository of 212 hESC lines seems to be the world's largest collection of hESC lines available for stem cell research. Among other reported lines were three hESC lines with cystic fibrosis, one with trisomy 13, and one with triploidy (29, 30). Thus, the presented collection represents the world's first and only repository of hESC lines with genetic and chromosomal disorders available for research in the primary mechanisms of the realization of genetic abnormalities.

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3

DERIVATION OF STEM CELLS FROM EPIBLASTS

MICHAL AMIT

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EMBRYONIC STEM CELLS FEATURES

Embryonic development initiates soon after fertilization when blastomer cleavage, proliferation and differentiation occur. The blastomers within the developing mammalian embryo remain totipotent until the morula compaction stage. In the compacted embryo, the blastomers initiate polarization which results in the creation of two distinct cell-populations: cells of the inner cell mass (ICM), which contributes to the embryo proper, and the cells of the outer thropoectoderm layer, which develops into the extra embryonic tissues. Soon after implantation is achieved, the ICM is divided into two distinct layers: a layer of primitive endoderm, which gives rise to the extra embryonic endoderm, and a layer of primitive ectoderm, which gives rise to the embryo proper and to some extra embryonic derivatives. After implantation and gastrulation take place, the cells become progressively restricted to a specific lineage, thus their pluripotency is lost and they are regarded to as multi-potent progenitor cells. Therefore, it should be noted that in the intact embryo pluripotent embryonic stem cells proliferate and replicate only for a limited period of time.

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Embryonic stem cells (ESCs) constitute a unique type of stem cells derived from the ICM of the mammalian blastocyst. These cells are characterized based on the criteria used for adult stem cells, the features of teratocarcinoma cells, and the well-characterized mouse ESC (mESC).

ESCs are characterized according to their source; they are isolated from the preimplantation embryo, from the ICM of the blastocytes. There were reports on ESCs isolated from an embryo at an earlier stage of development (morula stage embryo); however, these reports were restricted to mice. It is still to be clarified whether these cell lines are identical to cells isolated from the ICM.

When cultured in appropriate conditions, ESCs are capable of prolonged undifferentiated proliferation, creating uniform colonies with cells demonstrating high nucleus-to-cytoplasm ratio, the presence of two or more nucleoli, and typical spaces between cells.

ESCs maintain normal diploid karyotype even after prolonged culture (1). Cases of karyotypic instability are scarce (1–4), suggesting that they represent random changes which often occur during cell culture.

ESCs uniqueness lays in their pluripotency. These cells have been shown to be pluripotent, both *in vitro* and *in vivo* by embryoid body (EB) formation (5, 6) and by teratoma formation (7, 8), respectively. Undifferentiated mESCs have been shown to contribute to formation of chimeras, particularly to the germ cell line (9), and later some mESC lines were demonstrated to form whole viable fetuses (10).

ESCs express specific surface markers while maintained in the undifferentiated stage. Mouse ESCs express high levels of surface marker stage-specific embryonic antigen-1 (SSEA-1), and nonhuman primate ESCs as well as hESCs strongly express SSEA-4, tumor recognition antigns 60 and 81 (TRA-1-60 and TRA-1-81, respectively), weakly express SSEA-3, and unlike mESCs do not express SSEA1 (8, 11). ESCs were also found to express a number of specific genes, the most recognized being Oct-4, a transcription factor known to be involved in the process of ESCs self maintenance (12). In recent years, another transcription factor, Nanog, was recognized as an additional major key player in ESCs renewal (13, 14). Additional genes were found to be highly expressed in undifferentiated hESCs and mESCs and are offered as a set of markers that classify ESCs (15).

It has been reported that ESCs do not exhibit X inactivation. While maintained at the undifferentiated stage, both X chromosomes are active and, upon differentiation, one chromosome undergoes inactivation (16). Recent reports demonstrate, however, that some hESC lines vary in their X-inactivation status (17–20). This might point to a different, later source for some of the lines than the ICM, possibly the epiblast stage.

Mouse ESCs are reported to remain in the S phase of the cell cycle for the majority of their life span. The specific stage of the cell cycle in which hESCs spend most of their time is yet unknown.

DERIVATION OF hESCs

Human ESC lines were first derived using the culture methods developed in the 1970s for embryonal carcinoma cell lines and in the 1980s for mESC derivation. ESC isolation involves the selective removal of the trophoectoderm layer followed by proliferation of the ICM cells. To this end, two principle methods can be used: (1) immunosurgical or mechanical isolation of the ICM, and (2) culture of an intact embryo following the removal of the zona pellucida, and the isolation of the resultant ICM cell outgrowth during the second passage.

For the derivation of the existing hESC lines, embryos from in vitro fertilization programs were used, including surplus embryos (8, 11), or embryos of low quality that were excluded from clinical usage (21). Due to the progress in assisted reproductive medicine techniques, embryos are available for hESC lines establishment. Currently, there are more than 150 lines available for research worldwide. The utilization of embryos for research, however, raised ethical aspects that were addressed by the publication of specific guidelines for the use of embryos for hESC studies. The increasing number of available hESC lines indicates that the derivation of these lines is a reproducible procedure, where success rates vary from less than 10% (22) to over 40%, depending on the quality of the embryos and on the technique used for lines isolation (11, 23).

DERIVATION OF LINES FROM LATE-STAGE EMBRYOS

During early mammalian embryonic development, a short window of time arises when each cell of the developing embryo possesses the capacity to differentiate into every cell type of the adult body. Although hESCs have been isolated and characterized (8, 11), the pluripotency of human postimplantation embryonic cells that are present between the time of implantation and the gastrulation process has never been examined before. The ability to culture human embryos *in vitro* until day 9 had been previously reported, demonstrating healthy proliferating ICMs (24), yet these reports did not provide answers to a few critical questions, such as whether pluripotent stem cells still exist in the postimplantation embryo and whether they can be isolated and cultured continuously to allow their characterization. Additional work demonstrated that within a 9-day-old embryo, groups of relatively undifferentiated cells could be identified within the ICM, closely resembling the appearance of ESCs. The examined embryo attempted to form an amnion with a cavity, but it did not present a bilaminar, discoidal structure as is expected in week 2 of development, and hence was regarded as abnormal (25).

More recent publication reported the establishment of an hESC line from an 8-day-old embryo. The authors used a three-step culture conditions tech-

TABLE 3.1. Lines Isolated from Embryos Beyond 6-Days-Old Cultures

Source	Resultant Line	Reference
Human 8-days-old embryo	ESC-like cells	(26)
mESCs cultures with HepG2 cells conditioned medium	Primitive ectoderm-like (EPL) cells	(27) and (28)
Mouse embryo	primitive ectoderm-like (EPL) cells, cultures with HepG2 cells conditioned medium	(29)
Egg-cylinder-stage mouse embryos E5.5	Epiblast cells	(30) and (31)

nique, which successfully supports the development of 8-day human blastocysts. The resulting blastocyst ICMs consist of significantly more ICM cells in comparison with 6-day-old blastocysts (26). One cell line which was successfully isolated from an 8-day-old embryo exhibited similar features as hESCs, including the expression of specific markers, normal 46, XX karyotype and teratoma formation (26). However, more intensive study should be conducted in order to clarify whatever these line characteristics are indeed identical to those of hESCs. Thus, stem cell lines can be isolated from embryos beyond the 6-day-old preimplantation embryo.

One of the cell populations that appears later in the process of embryonic development is the population of primitive ectoderm cells. Rathjen and colleagues demonstrated a method for homogenous differentiation of mouse ESCs into early primitive ectoderm-like (EPL) cells using conditioned medium of HepG2 cells (27). These cells demonstrated epithelial-like structures, a higher tendency to differentiate into mesodermal tissues and a reduced ability to integrate into the embryonic germ layers after injection into mouse blastocysts (28). When primitive ectoderm cells were isolated from mouse embryos, they demonstrated similar characteristics to those of EPL cells (29). These primitive ectoderm cells' ability to be cultured for limited passages *in vitro* also depended on the presence of HepG2 conditioned medium (29). The major difference between these types of cells, however, is the reversibility of the characteristics of the EPL cell produced from mESCs; for example, they regained ESC properties when the HepG2 conditioned medium was removed (27, 28). The lines isolated from embryos beyond 6-day-old embryo are summarized in Table 3.1.

DERIVATION OF STEM CELL LINES FROM EPIBLAST

During implantation, the embryo consists of the epiblast, a derivative of the ICM, which will later differentiate both to the embryo tissues, and to the extraembryonic layers. Recently, two scientific groups reported the isolation

of mouse epiblast cell lines (30, 31). Mouse embryos, at the Egg-cylinder-stage (E5.5) were plated with inactivated mouse embryonic fibroblasts (MEFs). Under these conditions, the epiblast cells (EpiSC) grow as an epithelial colony and proliferate. EpiSC colony morphology resembles that of hESCs colonies, appearing as a large monolayer.

The resulting cells expressed transcription factors associated with pluripotency, including Oct3/4 and Nanog (30, 31). Similar to EPL cells, EpiSCs were found to express FGF5 and Nodal, genes specifically expressed in the late epiblast layer post implantation (31). Gene expression analysis demonstrated that EpiSC lines were similar to each other and distinct from mESCs. The difference in the expression of specific genes suggests that mESCs and EpiSCs are two distinct pluripotent states representing cells of the preimplantation and postimplantetion embryo, respectively (30, 31). Similar to mESCs, EpiSCs efficiently form EBs while cultured in suspension and are able to create teratomas when injected into athymic nude mice (30). When injected into mouse blastocytes, EpiSCs either failed to form chimeric mice altogether (30), or form chimeric mice with extremely low efficiency (2 out of 385 mice) (31).

Genes associated with the germ line, including Stella, Piwil2, Stra8, and Dazl that are expressed by mESCs are significantly decreased or undetected at all in EpiSCs (30). As with hESCs, blocking STAT3 phosphorylation at tyrosine 705 with a Janus Kinase (JAK) inhibitor allows culturing of EpiSC as undifferentiated cells (30). Similarly, the activin receptor inhibitor SB431542 induces rapid differentiation of EpiSCs, demonstrating that EpiSCs pluripotency relays strictly on activin/Nodal signaling and not on the leukemia inhibitory factor pathway as with mESCs (31). The features of the resulting epiblast cells are compared to those of mESCs in Table 3.2.

TABLE 3.2. Comparison between Mouse Epiblast Cells and Mouse ESCs Features

ESCs	Epiblast Cells
Small, compact, pile-like colonies	Large, monolayered colonies
Expression of transcription factors associated with pluripotency; Oct-4 and Nanog	Expression of transcription factors associated with pluripotency; Oct-4 and Nanog
Express low or not at all FGF5 and NODAL genes	Express FGF5 and NODAL genes
EBs formation	EBs formation
Teratomas formation	Teratomas formation
Chimeric mice formation	Do not form Chimeric mice
Expression of genes associated with the germ line	Do not express or express low levels of genes associated with the germ line
Require leukemia inhibitory factor (LIF) signaling to remain in the undifferentiated stage	Relays on activin/Nodal signaling and does not require LIF signaling to remain in the undifferentiated stage

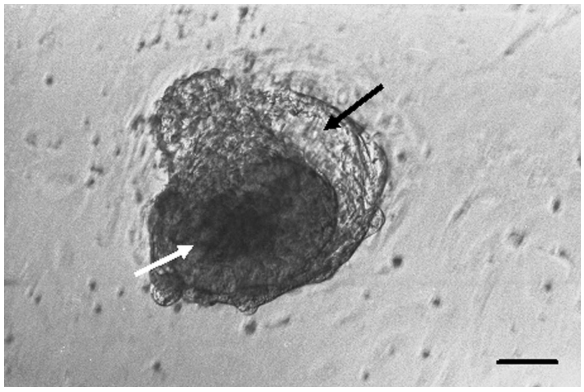

Figure 3.1. Extended embryo culture. Embryo cultured with inactivated MEFs, note the developed cyst (black arrow) and a disc-like structure containing the potent cells (white arrow). Bar 60 μM.

The results of these studies clearly demonstrated that epiblast cells can be maintained as stable cell lines and are distinct from mESCs in their epigenetic state and in the signals controlling their differentiation (30).

Furthermore, these cell lines could contribute significantly to the study of early development and shed a light on early developmental events.

Unfortunately, from obvious ethical reasons, EpiSCs could not be isolated from a human embryo, and to date, although hESCs has existed for over 10 years, EPL cells were not reported to be derived from hESCs. This may indicate that EPL derivation protocols will need to be modified from the protocol developed for mESCs. We therefore designed a method for extended blastocyst culture in order to derive stem cell lines ("extended blastocyst cell" lines, or in short "EBC lines") from embryos beyond the blastocyst stage (Amit et al, unpublished data). An example for a cultured blastocyst is illustrated in Fig. 3.1. Using this method embryos were cultured with MEFs for up to 13 days post fertilization. Compacted cells were mechanically removed from these embryos and continue to proliferate while cocultured with MEFs. Lines derived utilizing this method can be propagated in culture for prolonged time periods, sustain normal karyotypes, and form embryoid bodies while cultured in suspension. Although these EBC lines express some markers characteristic of hESCs, their expression levels are significantly lower, and some early differentiation markers such as Brachyury are also expressed; they are less likely to form teratomas and when they do, the complexity of the teratomas obtained diminishes in comparison with other lines derived at earlier stages of blastocyst maturation.

Gene expression profiles were generated using Illumina bead arrays that contain probes for 24,000 unique transcripts. The expression of subsets of individual genes was validated using parallel analysis both by focused micro-

array and by reverse transcriptase–polymerase chain reaction (RT-PCR) and identified a limited ability to differentiate into trophoectoderm and an expression of markers typical of maturing epiblasts. Overall the data suggests that EBC lines resemble the epiblast stage.

The major obstacle in determining the exact source of the EBCs relates to their derivation methods. Unlike hESCs, EpiSCs or EPL cells which were derived from an embryonic ICM at a certain stage, in the extended embryo culture technique, it is impossible to determine the embryonic stage of development and to be able to isolate cells from it simultaneously. In addition, any *in vitro* system for human embryonic cell culture cannot be regarded to as identical to *in vivo* human embryonic development.

Nevertheless, the newly designed method allows insight into the cell features of the postimplantation human embryo between implantation and gastrulation using *in vitro* models, and determines that multipotent human cell lines can be isolated at a period in embryogenesis later than the blastocyst stage. Further intensive study should be conducted to clarify the embryonic stage of these cells. The resultant cell lines comprise a distinct cell population, which may prove valuable for establishing differentiation systems in general and differentiation into mesoderm derivatives in particular. These lines therefore represent unique tools that will enable us to dissect early developmental events.

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4

DERIVATION OF EMBRYONIC STEM CELLS FROM PARTHENOGENETIC EGGS

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"Sex is a universal phenomenon" (1) … sometimes. Most eukaryotes do reproduce sexually; however, there are exceptions in which males, unfortunately for those of us in the gender, are dispensable. This phenomenon is called "parthenogenesis"—from the Greek for "virgin birth"—a process by which the female gamete can begin to develop in the absence of the male one.

If parthenogenesis results in a live offspring, then it is reproduction without the use of sperm. The term "parthenogenesis" should not be confused with "gynogenesis," in which a sperm fertilizes the egg and is later excluded from the final composition of the genome (1). While for lower vertebrates and other animals parthenogenesis is a proven fact, the chances that it could become another way to reproduce mammals are, based on the data available, slim to none. However, the concept of mammalian parthenogenesis is currently undergoing a revival, due to its possible use for the generation of embryonic stem cells that can, in turn, be used for cell therapy. Ethical considerations aside, there are real, practical benefits to deriving cells from an egg that was not fertilized—the main one being the potential for making cells that are 100% compatible with the donor of the egg.

Parthenogenesis can be classified as natural or artificial, depending on whether human intervention occurs in the process. "Natural parthenogenesis" refers to the cases where parthenogenesis is a normal form of reproduction.

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Offspring and/or embryos produced this way are called "parthenos." "Cyclic parthenogenesis" refers to animals that reproduce by parthenogenesis during particular stages of their lives, while, during other periods, they can reproduce sexually. In other cases, animals produce parthenos or biparental offspring depending on the needs of the species, which is called "facultative parthenogenesis." Certain types of insects, crustaceans, and lower vertebrates are known to reproduce by parthenogenesis. Perhaps the most common insect that can reproduce using both methods, that is, sexually and by parthenogenesis in a facultative fashion, is the honeybee. The queen can generate a large colony of female worker bees when the eggs are fertilized and a modest number of males or drones when reproduction is by parthenogenesis. Other examples of insects produced by parthenogenesis include grasshoppers and silkworms. Among crustaceans, the best-known examples are lobsters, shrimps, and water fleas. An example of an all-female vertebrate is the lizard *Cnemidophorus uniparens*, which evolved from the ancestral, sexually reproducing species *Cnemidophorus inornatus* (2).

Researchers began to pay attention to parthenogenesis as a way to understand the reproductive significance of fertilization. Jacques Loeb, in 1913, reported protocols for artificial parthenogenesis for the first time (1). His main interests were studying the continuity of the oocyte through generations and testing the hypothesis that the sperm was needed to maintain the immortality of the egg. Loeb reported, in a seminal paper in the *Journal of Experimental Biology*, that sea urchin eggs could develop in the absence of sperm by simply changing the osmolarity of the water. He reported later, in his book *Artificial Parthenogenesis and Fertilization*, that "in 1899, I succeeded in inducing unfertilized sea urchin eggs to develop into larvae by exposing them for two hours to hypertonic sea water" (1). Subsequently, Loeb was able to activate starfish eggs using acid solutions. His excitement was such that he went on to say that "the mysterious complex of the living spermatozoon might be replaced by well-known physic-chemical agents and that if it wasn't because parthenogenesis was laborious, parthenogenetically derived animals could exist today normally" (1). Given Loeb's scientific stature, we can allow him to overinterpret the importance of his work. It would take another 70 years to explain the reasons why most of the higher vertebrates cannot reproduce by parthenogenesis.

Not all of the fault for the lack of a proper explanation for why mammals cannot reproduce by parthenogenesis should be attributed to the exuberant statements of Loeb. In 1940, Gregory Pincus and Herbert Shapiro published a report describing the birth of one rabbit by parthenogenesis. The mother doe gave birth to a female rabbit that was "normal in appearance and behavior" (3). The protocols used by Pincus were based on physical changes to the oocyte environment, that is, the use of hypotonic solutions, hypertonic solutions, or both. The protocol that apparently yielded the partheno offspring involved the use of an *in vivo* cooling mechanism in which the fallopian tubes of a recently ovulated doe were laparoscopically accessed and cooled for 20 min. It is dis-

concerting that almost 70 years after these experiments were reported, no other group has replicated the finding. In retrospect, this was probably an honest mistake; Pincus also reported that, in some instances, they used vasectomized males to induce ovulations. These males were probably not as sterile as the researchers thought they were.

Several attempts were subsequently made by different groups using different species to try to generate mammals via parthenogenesis. In 1975, Modlinski reported the *in vivo* culture of haploid gynogenetic embryos. These embryos were allowed to develop for 5 d, but only one reached the blastocyst stage. The failure to develop was then attributed to "the lack of male embryos transferred," something that bears no biological justification indeed. Two years later, Hoppe and Illmensee reported the production of 100% gynogenetic and androgenetic embryos (4). The authors reported that, using micromanipulation, the female or male pronucleus of a fertilized embryo could be easily identified and microsurgically removed. If the embryos were left in cytochalasin B overnight, they would be diploidized; when later transferred into recipient females that were previously bred with vasectomized males, an offspring could be obtained. Two males and five females were born using this procedure, and some of them were reportedly capable of giving birth to normal offspring. Thirty years after the announcement of this work, nobody has been able to replicate it. In 1976, a report by Kaufman et al. showed that parthenogenetic embryos developed only to the forelimb bud stage, and, while heartbeat was observed, no pups were born alive (5). Later, the same group described their attempts at producing gynogenetic offspring, obtaining a similar outcome: No development was observed beyond day 9 of pregnancy (6). Failures were attributed at that time to the lack of paternal contribution and the possible homozygosity of some genes. A clearer picture of the roles of paternal and maternal genomes started to emerge from the experiments in which paternal and maternal pronuclei were exchanged. When 100% androgenetic embryos were produced and transferred into the uterus of a pseudopregnant female, only extraembryonic tissues were obtained. The opposite experiment yielded better results: Fetal development up to day 10 was obtained when embryos that were 100% female were allowed to develop in the uterus; however, these consistently lacked extraembryonic tissues. As a control, one-cell embryos reconstituted with both male and female pronuclei were transferred into the uterus, and a normal offspring was born (7,8). These experiments categorically demonstrated that both maternal and paternal contributions are necessary for normal development of an embryo. Surani et al. went on to say that "specific imprinting of the genome occurs during gametogenesis, so the presence of both a male and female pronucleus are essential," a statement that to this day remains unchallenged.

While it is true that male and female contributions are necessary for the development of fertilized embryos to term, the fact that 100% female-derived mouse embryos—by parthenogenesis or gynogenesis—can develop up to day 10 of pregnancy is remarkable. Upon further histological examination, these

fetuses seem to have all the organs and tissues of a normally fertilized one, although they are slightly retarded in growth. Observations reported by Stevens and Varnun in 1974 gave credence to the notion that the oocyte has the intrinsic potential to develop into multiple tissue derivatives in the absence of sperm (9). It was known that ovarian teratomas rarely occurred in the mouse (1 in 25 000 animals). However, the authors found that the inbred strain LT was developing teratomas at an unusually high rate. Of interest was the discovery that these teratomas had within them oocytes and embryos at the two-cell stage. Among many differentiated tissues, they described the presence of pigmented cells, hair, bone, adipose and glandular tissue, and cartilage. The most abundant tissue found was neuronal. They also described the presence of some structures in the uterus resembling five- to six-day-old embryos; some of these embryos were later characterized as diploid. That preimplantation embryos were found in the reproductive tract of these females—without having had contact with a male—and that teratomas were only present *after* females reached puberty strongly suggested that the tumors originated from matured fully grown oocytes and not from primordial germ cells. A connection between these findings and the embryonic stem cell (ESC) field was still far from being established though.

After the initial report of the derivation of mouse ESCs by Evans and Kaufman in 1981, Robertson announced the derivation of ESCs without paternal contribution. Oocytes from the 129/SvEv and C57BLxCBA strains were induced to divide artificially using a solution of 7% ethanol, a process we now call "activation." Later culture of these embryos demonstrated that they were capable of reaching the blastocyst stage, and, when the authors attempted to plate these embryos and derive cells from them, they succeeded. Some of the activated embryos had one pronucleus and extruded the second polar body (PB), giving rise to haploid blastocysts, whereas others that showed two pronuclei and no PB generated diploid embryos. It is worth mentioning that the authors reportedly used 10% fetal bovine serum and 10% newborn calf serum, a considerably higher proportion of protein and growth factors when compared to the culture medium currently used. Overall, they generated six haploid and two diploid cell lines: "the world's first parthenogenetic stem cells." The authors were surprised to find the cells morphologically "indistinguishable" from those derived from biparental embryos. Of interest was the finding that, while the autosomal component was stable, one of the X chromosomes was not (10). Protocols for the generation of diploid parthenogenetic embryos were later refined with the use of cytochalasin D (CCD). Kubiak and collaborators argue that CCD is capable of inhibiting the first or the second PB extrusion, depending on whether it is used at the first or the second meiotic division, respectively. Such a protocol calls for allowing the embryos to extrude the PB and therefore producing embryos that will be diploid. In addition, the authors argued that embryos genetically identical to the mother at every locus could be produced if the first PB extrusion is inhibited. Kubiak et al. did try to produce offspring using parthenogenetically activated embryos using this

protocol; however, fetuses did not develop beyond 9 d of gestation. Other mammalian species in which attempts were made to produce parthenogenetic individuals after transferring embryos into the uteruses include rabbits, sheep, and pigs. In pigs, when activated oocytes were transferred into surrogate mothers, 29-day-old fetuses with heartbeats were obtained (11); in sheep, fetuses developed up to the 21-somite stage, although, in this case, the embryos were gynogenetic. Nonetheless, 100% female-derived parthenogenetic embryos were also capable of undergoing organogenesis, including liver, heart, and mesonephros, among other structures (12). One of the most remarkable experiments on parthenogenesis with species other than mice was performed by Ozil and Huneau using rabbit oocytes. Using electricity as the activation stimulus, they found that the degree of development of the parthenogenetic fetuses would depend on the intensity and amplitude of the electricity used. Using their best activation protocol, they transferred these oocytes into the uterus of a surrogate mother, and they found that these developed up to day 11.5 of gestation with a morphology very similar to a fertilized embryo (13). Kono and collaborators have shown that they could extend the development of parthenogenetic embryos up to day 13.5 of gestation when they used the genome of a nongrowing oocyte into the cytosol of a fully grown one. When those embryos were analyzed for the expression of some imprinted genes, they found that H19 was upregulated severalfold when compared to fertilized embryos of the same age (14). Later, using a hemizygous mutant that had only monoallelic expression of H19, the same authors were able to obtain embryos that developed up to day 17.5, an unambiguous result that underscores the impact that proper expression of imprinted genes has on development. Many other groups have attempted to obtain fetal development without fertilization, only to conclude, in agreement with what Surani stated years before, that in order to obtain full embryonic development, an embryo must contain the male and female contribution to its genome.

Given this evidence, if imprinted genes play such a pivotal role in the fate of a fetus, why can mouse ESCs differentiate in what seems to be a normal process? The first parthenogenetic ESCs, derived by Robertson et al. in 1983, were apparently capable of expanding normally *in vitro*. Whether or not they were able to terminally differentiate normally was not known until Mann et al. injected some of these pluripotent cells into syngeneic hosts and observed that teratomas with a larger proportion of ectoderm derivatives were formed at the expense of mesoderm or extraembryonic tissue. Later experiments injecting parthenogenetic cells into a host-fertilized embryo showed that some parthenogenetic cells integrated into the host and that, of those cells that did develop to term, some even developed into gametes (15). Later, others showed that the contribution of parthenogenetic cells in the developing pups diminishes as the animal develops—that is, adult animals will have less parthenogenetically derived cells than fetuses (16).

Allen and collaborators decided to fully test the developmental potential of diploid parthenogenetic ESCs after the cells were cultured for several

passages *in vitro* (17). Cells were introduced into host-fertilized embryos, and then the degree of chimerism of the offspring was measured. Considering previously published data, the cells were not expected to contribute much to the overall composition of the offspring. Surprisingly, they found that parthenogenetic ESC derivatives were able to contribute to heart, liver, brain, spleen, blood, lungs, and gonads; most importantly, germline transmission was obtained. Based on what it is known about imprinting, these data appear to be contradictory. However, it is now known that certain imprinted genes can be reactivated—or silenced—during culture conditions. These findings, along with recent findings showing the isogenicity of these cells, introduced the possibility of making ESCs from primates that could potentially be used for cell therapy.

The first primate ESC line was derived in 1996 from a fertilized rhesus monkey embryo. These cells were capable of maintaining a normal karyotype for more than 1 year, and, when induced to differentiate into a teratoma, cell derivates from ectoderm, endoderm, and mesoderm were obtained (18). The same group later reported the derivation of ESCs from marmosets and humans (19, 20). Later, in 2001, four cell lines from *in vitro*-fertilized cynomolgus monkey were produced (21). All of these primate ESCs share similar morphological characteristics—that is, cells have a small nuclear-cytoplasmic ratio, have similar markers and stemness gene expression profiles, and have the capacity to form teratomas with multiple tissue derivatives. Our group reported for the first time the derivation of ESCs from parthenogenetically activated cynomolgus oocytes (22, 23). At the time, we were developing protocols to attempt somatic cell nuclear transfer, and we used a protocol for activation of oocytes very similar to the one developed for the activation of bovine oocytes. From 18 metaphase II (MII) oocytes, we generated four blastocysts; out of those, one cell line was produced (Cyno1). This cell line was extensively characterized and was found to have all the markers and phenotypical characteristics that biparental cell lines have. The only difference was that Cyno1 cells showed a distinct gene expression profile when it came to imprinted genes. During their pluripotent state, imprinted genes can significantly drift in their expression profile, suggesting a very fluid state (24). Only a few imprinted genes, such as the paternally expressed gene SNRP, were always absent, regardless of whether the cells were undifferentiated or not. H19, however, can be expressed either at high levels or almost to the level of biparental cells (unpublished observations). Whether our observations on the potential differentiation capacity of Cyno1 cells are similar to the observations made of mice by Allen et al. (17) remains to be determined. Since nonhuman primate chimeras between monkey ESCs and host-fertilized embryos have yet to be shown to work, the degree of terminal differentiation these cells can achieve *in vivo* remains to be determined. Experiments with teratomas have demonstrated that Cyno1 ESCs are capable of making tissues from all three germ layers, a stark contrast from the observations made by Jeffery Mann when partheno-

genetic cells were first reported in mice (25). Cyno1 cells showed the capacity to make neurons *in vitro*; specifically, they generated functional dopaminergic and serotonergic neurons (22).

Subsequent to our studies on monkey, we began to test protocols for activation of human oocytes. Others had previously tried to activate human oocytes with moderate success (26, 27). We began activating human MII oocytes from consenting donors in 2000. We applied exactly the same protocol used for nonhuman primates, and six blastocysts were obtained out of 22 MII oocytes (28). Their quality, though, as measured by morphology, was significantly lower than that of parthenogenetically derived cynomolgus macaque blastocysts (22). Subsequent attempts using frozen/thawed human MII oocytes yielded remarkably high rates of development to the eight-cell stage with very little fragmentation. From the eight-cell stage forward, however, parthenos did not develop as well. A blockage was observed at that stage, and only a few embryos were capable of making a blastocoel cavity (29).

While our group, as well as others, were pursuing the generation of human parthenogenetic ESCs, Kim and colleagues categorically demonstrated in the mouse that a parthenogenetically derived ESC can be recognized by the mother as self in the majority of the cell lines produced from such a female. Previous reports from Kubiak described the generation of diploid, genetically identical mouse embryos using CCD to prevent the extrusion of the first PB (30). They also claimed that parthenogenetic embryos were genetically identical to the mother. Kubiak argued that the extrusion of the second PB restored the heterozygosity of the embryos, creating a "true clone" (30). Kim's work marked a turning point in the potential use of parthenogenetic stem cells (31). This research used two different protocols for the derivation of parthenogenetic embryos: one that inhibited the extrusion of the first PB; and another one, the second PB. They obtained blastocyst and embryonic stem cells at high rates—65% of the MII-derived blastocysts and 37% of the MI-derived blastocysts produced cell lines. Kim looked at the major histocompatibility complex (MHC) molecules of these cell lines and of the donor of the oocyte and concluded that 21 of the 23 ESC lines derived from MI oocytes were heterozygous. Conversely, 24 of the 72 cell lines derived from MII oocytes were heterozygous (33%). Further, when parthenogenetic ESCs (from MI or MII oocytes) were transplanted back to MHC-matched recipients, teratomas were obtained in almost 100% of the cases, whereas those coming from fertilized ESCs were rejected. These data demonstrated that parthenogenetic ESCs can be generated that are genetically identical, at least at the MHC locus, with the donor of the oocyte. A higher proportion of MHC-matched cell lines can be obtained when the first PB is retained instead of the second. Still, even in those circumstances where implementing the protocols for MI PB retention is impossible, it is reasonable to expect some of the MII-derived cells to be heterozygous. This was reinforced with work published by Revazova et al. later the same year (32). Six different cell lines were produced using 42 MII oocytes. These

cell lines were quite similar in differentiation potential to those derived from human fertilized oocytes; surprisingly, they had identical MHC molecules (Class I and II) to those of the oocyte donor. Revazova's group achieved high rates of development of these parthenogenetically activated oocytes and of ESC derivation. Their success can be attributed in part to the use of low oxygen (5%) during embryo culture and to the use of umbilical cord serum during the first few days of culture. Another interesting finding of this group was that these oocytes came directly from females who approached the clinic with the purpose of becoming pregnant. While doing so, they generated their own parthenogenetic ESCs. Four out of the five female donors enrolled in the study who produced ESCs also became pregnant using the cohort oocytes. An independent group, the same year, generated two human parthenogenetic ESCs as well. Mai et al. used activation protocols similar to Revazova's, obtaining similar efficiency of cell line derivation—that is, 10% of cell lines obtained from activated oocytes (33). A subsequent publication, also from Revazova's group, showed that human leukocyte antigen (HLA) system homozygous cell lines can also be obtained (34). Unfortunately, it is not yet clear what the main difference was in the protocols designed to produce either heterozygous or homozygous cell lines. It appears that the protocol for oocyte activation in the homozygous lines was modified with the use of puromycin instead of 4-dimethylaminopyridine alone (Table 4.1). Whether or not this explains why HLA homozygous ESCs were produced remains to be determined. A second manuscript describing the derivation of parthenogenetic human ESCs (hESCs) that are HLA homozygous was published by Chinese researchers. Their protocol was quite simple, relying on the expertise of the embryologists to keep those oocytes that were fertilized; they developed only one pronucleus. Lin et al. showed that, from one embryo with an extremely poor morphology, parthenogenetic hESCs could still be derived (35).

While parthenogenetic human ESCs show promise for being derived from a woman of reproductive age with the same MHC molecules as the donor of the oocytes, the cells with more immediate applicability are those described by Dighe et al. in rhesus monkeys (36). These nonhuman primate parthenogenetic ESCs are the ultimate tool for studying the real safety and efficacy of the cells, since they have been shown to possess the same set of phenotypic characteristics as human and mouse cells.

Parthenogenetic experiments with invertebrates and lower vertebrates started almost a century ago. However, it was only recently that a real interest in the potential application of this technique has gained momentum. The development of human parthenogenetic cells that can match the MHC molecules of the donors has brought the possibility of using these cells clinically much closer. In any event, much basic research is still needed to fully understand the potential risks and benefits of this technology. The role of imprinted genes and the functional value of differentiated parthenogenetic cells must be tested using long-term transplantation experiments in nonhuman primate models.

TABLE 4.1. Protocols for Oocyte Activation

Species	Protocol	Reference
Mouse	2 mM of SrCl2 in cytochalasin B for 6 h	(37)
Rhesus monkey I	5 μM ionomycin for 2 min followed by 2 mM of DMAP for 4 h	(38)
Rhesus monkey II	3 electrical DC pulses of 2.7 kV/cm for 50 μsec every 30 min in solution containing 7.5 μg/mL of cytochalasin B and 7.5 μg/mL of cycloheximide	(38)
Cynomolgus monkey	5 μM ionomycin for 2 min followed by 2 mM of DMAP for 4 h	(38) (22)
Human I	5 μM ionomycin for 4 min followed by 2 mM of DMAP for 3 h	(28)
Human II	5 μM ionomycin for 5 min followed by 1 mM DMAP for 4 h	(32)
Human III	5 μM ionomycin for 5 min followed by 2 mM of DMAP for 5 h	(33)
Bovine I	5 μM ionomycin for 4 min followed by 1.9 mM of DMAP for 5 h	(39)
Bovine II	5 μM calcium ionophore for 5 min followed by 2.5 μg/mL of cytochalasin D and 10 μg/mL of cycloheximide	(40)

Notes: Protocols for parthenogenetic activation of MII oocytes in different species. We have selected only those protocols that yield the best blastocyst development. We apologize to some of our colleagues who have not been mentioned in this short summary due to the lack of space.

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5

REPROGRAMMING DEVELOPMENTAL POTENTIAL

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INTRODUCTION

The developmental potential of mammalian cells can be viewed in a hierarchical fashion (Fig. 5.1). At the top of this hierarchy lies the fertilized egg or zygote, which results from the union of a male and a female gamete (sperm and oocyte, respectively). The zygote is totipotent, which means that it has the potential to form a complete organism, including all of the greater than 200 cell types and the extraembryonic tissue (i.e., amniotic sac and placenta). As the zygote develops, it begins to take shape, forming a hollow ball called the blastocyst. At one pole of the blastocyst lies the inner cell mass (ICM), the area from which pluripotent embryonic stem (ES) cells are derived. Cells of the ICM and their *in vitro* counterparts—ES cells—can give rise to any differentiated cell type found in the three primary germ layers of the embryo (endoderm, mesoderm, and ectoderm) and germ cells (sperm or oocyte). As the cells of the ICM develop further, they begin to specialize, forming organized structures, tissues, organs, limbs, and eventually an entire organism. However, the process of cellular specialization comes at a developmental cost. As a cell differentiates along its destined path, its potential is gradually restricted, culminating in the terminal differentiation of a single cell type (Fig. 5.1).

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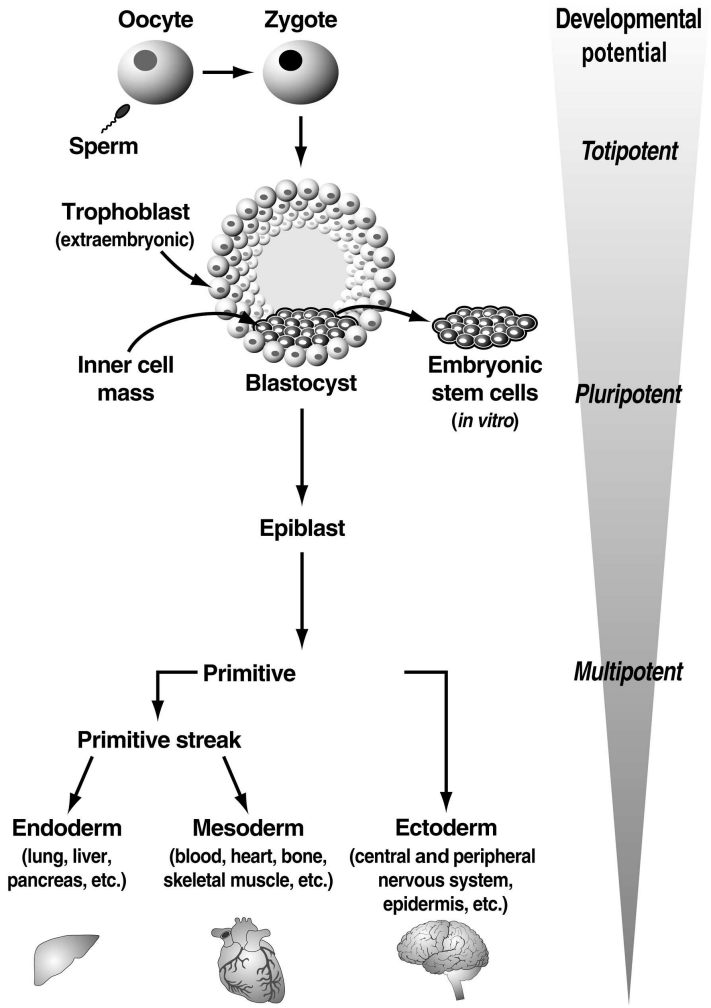

Figure 5.1. Mammalian developmental. A hierarchical depiction of development highlighting the gradual decrease in potential that accompanies differentiation from a totipotent zygote to the postmitotic somatic cells that make up an adult organism.

The specification of a given lineage during development results from tissue-specific gene activation and the silencing of "stemness" genes and those associated with alternative cell fates. A stem cell does this by imposing a distinct and heritable pattern of gene expression in a daughter cell without altering the primary DNA sequence. Instead, changes in the higher-order structure of DNA-protein complexes (called chromatin) result in differing accessibility of the primary DNA sequence to the transcriptional machinery. The molecular

details of this process involve the organization of chromatin architecture (i.e., nucleosomal remodeling, nuclear compartmentalization, and dynamics) and a range of alterations to the chromatin, including DNA methylation and a variety of posttranslational histone modifications (1, 2). Together, these processes determine gene expression and are central to tissue/lineage specification. For example, even though the vast majority of cells in a multicellular organism share an identical genotype, organismal development generates a diversity of cell types with disparate, yet stable, profiles of gene expression and distinct cellular functions. Analysis of these processes has given rise to the field of epigenetics, which seeks to provide a bridge between genotype and phenotype. Thus, lineage specification can be considered an epigenetic phenomenon that plays a deterministic role in establishing and maintaining cell identity during development and throughout the lifetime of an organism.

Since establishment of the epigenome does not alter the primary DNA sequence, every cell should, in theory, retain complete developmental potential. Evidence for this notion was first demonstrated in 1952 by Briggs and King, who showed that the nuclei from an early embryonic frog cell could be reprogrammed upon transplantation into an enucleated oocyte, a process later named somatic cell nuclear transfer (SCNT) (3). The subsequent cell was able to generate a normal, hatched tadpole, and, years later, application of this technique witnessed a cloned frog (4). Due to the technical constraints of applying SCNT in mammalian cells, it was not until 1997 that Ian Wilmut et al. reproduced this phenomenon in mammals with Dolly the sheep (5). The creation of a cloned mammal conclusively demonstrated that the lineage/tissue restrictions imposed during development, while stable, are reversible, and can be reprogrammed if provided with the appropriate cues.

The demonstration of reprogramming, via nuclear transplantation, in mammals has given rise to the notion that banks of patient-specific ES cell lines could be generated as a virtually inexhaustible source of cells/tissues/organs to replace those lost to old age or damaged by disease (6). However, therapeutic application of SCNT is hampered by inefficiencies associated with the cloning process and ethical objections surrounding the use of human oocytes. Furthermore, the cellular, molecular, and biochemical events that occur in the somatic genome upon transplant into an oocyte remain largely unknown. As such, other techniques have been developed in an effort to address reprogramming from a mechanistic standpoint. These include somatic cell–ES cell fusion (7, 8); the environment-induced reprogramming of germ cells (9, 10); and retroviral delivery of defined transcription factor cocktails (11). The identification of alternative, simplified reprogramming methods has rapidly accelerated advances in this field, both by circumventing the technical and ethical considerations of using human oocytes, and by providing convenient and widely applicable means to study reprogramming.

Below we discuss the different strategies that have been used to reprogram developmental potential in mammals, including the work on interlineage conversion that paved the way for defined factor reprogramming (Table 5.1).

TABLE 5.1. A Summary of the Various Techniques Used for Cellular Reprogramming

Technique	Advantages	Limitations
1. Nuclear transplantation (SCNT)	Therapeutic cloning (customized ES cells)	Ethical issues; supply of human oocytes; lack of efficiency
2. Cellular fusion	Relative ease	Reprogrammed cells are tetraploid
3. Culture mediated	Relative ease	May be limited to germ cells
4. Genetic (overexpression or knockdown)	Relative ease; abundance of cell source; access to cells on a patient-to-patient basis	Use of oncogenic factors, alteration of host genome
5. Small molecule	Temporal control; decreased chance of inducing transformation	Difficulty of replacing all required factors

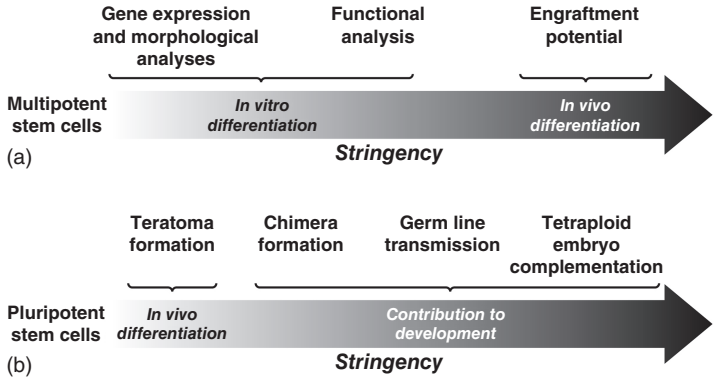

Figure 5.2. Methods used to validate developmental potential. (a) Strategies used to measure any degree of (e.g., multipotential) developmental potency. (b) Strategies used to measure pluripotency.

Importantly, we focus on the use of chemical methods to dissect pathways involved in reprogramming. We then summarize current progress on reprogramming to a state of pluripotency and compare and contrast the varying techniques (i.e., nuclear transfer, cellular fusion, culture-induced, and defined factor reprogramming) used to achieve this goal. Furthermore, we provide a comprehensive overview of the recent progress in reprogramming with defined transcription factor cocktails. Throughout, we critically discuss the criteria for assessing cellular potency at the functional and molecular levels (Fig. 5.2).

Finally, we close with a perspective highlighting the potential therapeutic applications of cellular reprogramming to generate patient-specific tissue for regenerative medicinal applications.

CELLULAR PLASTICITY

The prevailing dogma in mammalian biology dictates that during organismal development, cells progressively lose potential as they mature into more specialized tissues and organs. In other words, once specified, a cell irreversibly loses the capacity to generate other cell types in the body. This is in contrast to the regenerative potential enjoyed by other non-mammalian organisms. For instance, the cells on the outer edge of a severed salamander arm can dedifferentiate, form a multipotent blastema, and regenerate the lost limb in its entirety (including the bone, blood vessels, and nerves) (12). Clearly, a means to do this in mammals is highly desirable, and much of the early work in the field of regenerative medicine has focused on identifying the mechanisms that govern dedifferentiation in fish and amphibians (13). More specifically, the field has aimed to find similar mechanisms in mammals or a means to hijack the mechanisms used by regenerative organisms therapeutically.

Unfortunately for humans, mammals are found at the lower end of the regenerative spectrum. We can regrow large parts of the liver and pancreas and repair limited damage to skeletal muscle and the peripheral nervous system. This pales in comparison with the amazing capacity of amphibians and fish to regenerate tissue by dedifferentiating a somatic lineage back to a state of multipotency. While dedifferentiation of mammalian somatic cells may occur during oncogenic transformation, it is not a major force in physiological repair. Based on this notion and the observation that a clear corollary between regenerative organisms and mammals does not exist, pioneering work in understanding mammalian cellular plasticity has relied on alternative, exogenous means to expand developmental potential. In general, two methods have been applied—pharmacological intervention and/or genetic modification. The latter is done by expressing stem cell factors in somatic cells, whereas the former is done by extrinsically influencing somatic cells to behave like stem cells via pharmacological perturbation of signaling and/or the epigenetic architecture.

The discovery of mammalian cellular plasticity raises the possibility of reprogramming restricted cell fate, and may provide an alternative to many of the obstacles associated with using embryonic and adult stem cells in clinical applications. With a safe and efficient dedifferentiation process, healthy, abundant and easily accessible adult cells from a given individual could be used to generate different functional cell types to repair damaged tissues and organs. Clearly, there is considerable interest in understanding these complex processes, as well as ultimately controlling them for regenerative therapies. Below we discuss the genetic and chemical approaches that have been used to direct

lineage interconversion and highlight recent insights into the reprogramming of developmental potential, following a brief summary of the metric required to establish the expansion or conversion of cellular potential.

Methods Used to Establish Lineage Interconversion

The following chapter provides numerous examples of manipulating developmental plasticity ranging in scope from the conversion of progenitor cells to stem cells (14) to complete epigenomic overhaul (i.e., conversion of a tissue-restricted somatic genome to pluripotency) (11). However, such claims require rigorous qualification in order to demonstrate that the differentiation potential of a cell has truly been expanded. Toward this end, the characterization of a lineage and its developmental potential are assessed using a variety of functional and molecular standards (Fig. 5.2).

As our understanding of the epigenetic process of lineage specification and maintenance has matured, so has our scrutiny for confirming lineage swapping. We can now map the methylation patterns of DNA, characterize the modifications on histones, and determine gene expression profiles with much greater precision. In addition, advances in cell/tissue transplant, ES cell transgenics, and lineage tracing have given us more rigorous means to characterize a cell's developmental potential. Traditionally, differentiation potential was characterized based on cell surface marker expression and/or morphology following *in vitro* differentiation. While this strategy is a critical first step in establishing potential, it is the least stringent assay for a cell in culture and can give rise to *in vitro* artifacts (Table 5.2). For instance, assessment of cell-type-specific markers following *in vitro* differentiation can result in stress-induced aberrations in gene expression due to suboptimal culture conditions (e.g., when a fibroblast is cultured in serum-free media) (15, 16). Such artifacts can generally be ruled out by using more rigorous assays that rely on cellular function of the differentiated cells (e.g., action potential in neurons or insulin secretion in pancreatic beta cells). Even more stringent assays depend on engraftment potential and the ability to contribute to normal tissue/organ replacement, repair, or development.

The aforementioned methods can be used for cells with any degree of developmental potential, although they are more commonly used to demonstrate multipotency. When establishing pluripotency, more rigorous analyses are used. These include the ability to generate tumors that express cell types representative of all the primary germinal layers (germ cell tumors or teratomas); chimera formation; injection of cells into a developing blastocyst where the resulting organism consists of both host and donor cells; and the ability to contribute to the germ line of subsequent generations. The most stringent of all tests for developmental potential is the injection of cells into tetraploid blastocysts (4n DNA content), which results in an organism formed entirely from the donor cells (tetraploid embryo complementation). The rigorous application of these methods to a population of cells can determine its devel-

TABLE 5.2. Reports of Successful Cellular Reprogramming Efforts Using Various Approaches

Method of Reprogramming	Starting Cell Type (Means)	Reprogrammed Fate	Most Stringent Method of Differentiation	Reference
Nuclear transplantation (SCNT)	Mammary gland epithelial cell (oocyte)	Zygote	Cloned organism	(5)
	T lymphocyte (oocyte)			(69, 70)
	Olfactory neuron (oocyte)			(71)
Cellular fusion	Thymocyte (EC cell)	Pluripotent tetraploid cell	Teratoma	(86)
	Fibroblast (hES cell)			(8, 87)
	Fibroblast (mES cell)			(7)
Culture mediated	Primordial germ cells	Pluripotent EG cells	Cloned organism	(95–97)
	Spermatoginal stem cells			(9, 10, 101)
Genetic (overexpression or knockdown)	CLP (GATA-2)	Mast cells	*In vitro* differentiation	(21)
	CLP (GATA-1)	MEP		(22)
	CLP (C/EBPα)	GMP 9 (neutrophils/monocytes)		(23)
				(24)
	PreT (PU.1)	Dendritic cells		(24)
	ProT/PreT (C/EBPα)	Monocyte/macrophage		(26)
	ProT/PreT (GATA-3)	Mast cell		
	ProB/PreB (C/EBPα)	Monocyte/macrophage	*In vivo* differentiation	(25)
	B cells (Pax5 ablation)	T cells		(27)
	Fibroblasts (Oct-4, Sox2, c-myc, KLF4)	Pluripotent ES cells	Cloned organism	(11)
Small molecule	Pancreatic cells (dexamethasone)	Hepatocytes	*In vitro* differentiation	(28)
	Multinucleated myotubes (myoseverin)	Unipotent myoblasts		(36)
		Tripotent mesenchymal progenitor cells		(37, 40)
	Myoblasts (reversine)			
	Bipotent OPCs (HDAC inhibitors)	Tripotent neural stem-like cells	*In vivo* engraftment	(14, 49)

EC = embryonic carcinoma; hES = human embryonic stem; mES = mouse embryonic stem; EG = embryonic germ; CLP = common lymphoid progenitor; GMP = granulocyte/monocyte precursor; MEP = megakaryocyte/erythroid precursor; OPC = oligodendrocyte precursor cell; HDAC = histone deacetylase inhibitor.

opmental potential and are described in greater detail in the following examples.

Genetic Approaches

Reprogramming cellular potential using genetic methods involves either the ectopic/forced expression of a particular gene product or its targeted depletion. In general, this strategy aims to disrupt the existing epigenetic state of a cell by changing the gene expression profile. This technique is most successfully achieved by modulating the expression of transcription factors since they can control gene activity directly by binding to regulatory regions of DNA. A recent example of this approach by Takahashi and Yamanaka demonstrated that the simultaneous expression of four pluripotency-associated transcription factors in a somatic cell can convert the lineage restricted epigenome backward to pluripotency (11). In addition to this major reprogramming milestone, genetic reprogramming methods have also been used to gain insight into the mechanisms that control lineage specification and plasticity.

Lineage interconversion in hematopoietic lineages is by far the most well established (17, 18). Indeed, decades of work with hematopoietic stem cells (HSC) have identified many of the key players in the transcriptional networks that control lineage specification of HSCs and their progeny (19, 20). Based on this knowledge, careful selection and ectopic expression of transcription factors has been a valuable approach to expand cellular potency in hematopoietic lineages (Table 5.2). Early work using this strategy led to the observation that introduction of GATA-1 into common lymphoid progenitors (CLPs) redirected lineage commitment to megakaryocytic/erythroid progenitors (MEPs) whereas enforced GATA-2 expression reprogrammed CLPs into the mast cell lineage (18, 21, 22). Further analysis of this transition using granulocyte/monocyte progenitor (GMP)-derived eosinophil, basophil, or mast cell progenitors demonstrated that the timing of GATA-2 and CCAAT enhancer-binding protein α (C/EBPα) expression can differentially control lineage commitment. For instance, GATA-2 instructed C/EBPα expressing GMPs to commit exclusively into the eosinophil lineage, while it induced basophil and/or mast cell lineage commitment if C/EBPα was suppressed at the GMP stage (21). Whereas, C/EBPα overexpression can reprogram CLPs into GMPs, which could subsequently give rise to neutrophils and monocytes but not eosinophils or basophils (21, 23).

Later work in more restricted lymphoid cells demonstrated that C/EBPα overexpression in committed B and T cells resulted in their functional reprogramming to a macrophage fate (24, 25). Furthermore, preT cells could be reprogrammed to dendritic cells via PU.1 expression (24). Tracking these lymphoid to myeloid conversions identified that a transit intermediate state exists, in which markers of both myeloid and lymphoid lineages were expressed. Isolation of the intermediate population, however, led exclusively to cells with unipotential. Taghon et al. later demonstrated that preT cells, like macro-

phages, can also be reprogrammed to mast cells via ectopic expression of GATA-3 (26). In an elegant study using conditional Pax5 deletion in mice, Cobaleda et al. demonstrated that mature B cells from peripheral lymphoid organs can dedifferentiate *in vivo* back to early uncommitted progenitors in the bone marrow. These reprogrammed cells were able to rescue T lymphopoiesis in the thymus of T cell-deficient mice, and most importantly, the B cell-derived T lymphocytes carried immunoglobulin heavy- and light-chain gene rearrangements, providing a genetic marker to indicate true lineage reversal (27).

Chemical Approaches

Initial attempts at expanding differentiation potential focused on overexpression or knockdown of developmentally relevant genes using traditional genetic approaches. However, far fewer examples of reversible lineage control have been demonstrated with small molecules. Among these, the synthetic glucocorticoid, dexamethasone, was reported to facilitate interconversion between developmentally restricted fates (28). During development, the liver and pancreas both originate from neighboring regions of the foregut endoderm. Under various experimental contexts *in vivo*, hepatic foci have been described in the pancreas (29, 30), although the mechanism for this transformation was largely unknown. Shen and coworkers demonstrated that pancreatic cells treated with dexamethasone could be converted into hepatocytes *in vitro* (in the AR42J-B13 pancreatic cell line) and *ex vivo* (in organ cultures of pancreatic buds from mouse embryos). Furthermore, they also provided insight into the mechanism of this conversion. Dexamethasone treatment of pancreatic cells induced the expression of C/EBPβ that promoted hepatocytic conversion. In contrast, loss of C/EBPβ function (by overexpression of liver inhibitory protein—a dominant-negative form of C/EBPβ) blocked dexamethasone-induced hepatic conversion. In another example, Skillington et al. found that retinoic acid can synergize with BMP2 to stimulate cell proliferation, repress adipogenesis, and promote osteoblast differentiation in preadipocytes (31). Similarly, purmorphamine (an activator of Hedgehog signaling) (32) can function synergistically with bone morphogenetic protein (BMP)4 to convert preadipocytes into osteoblasts (33).

During the formation of skeletal muscle, proliferating myoblasts stop dividing and fuse into myotubes. *In vitro* studies with terminally differentiated murine myotubes suggest that they can be induced to undergo dedifferentiation into mesenchymal progenitor cells by the ectopic expression of Msx1 (34) or via the addition of extracts from regenerating newt limb (35). In order to identify compounds that reversed the cell fate of terminally differentiated myotubes, Rosania et al. screened a library of 2,6,9-trisubstituted purines in search of compounds that disassembled multinucleated myotubes into individual progenitor cells. A small molecule, myoseverin, was identified that induced the cleavage of multinucleated myotubes into myoblast-like cells

capable of proliferating and redifferentiating into myotubes (36). Affinity chromatography of cell extracts and examination of cytoskeletal proteins using an immobilized matrix of myoseverin revealed that it bound to and disassembled microtubules. Microtubule disruption thereby induced the reversion of terminally differentiated myotubes to mononucleated cells that were again responsive to both growth and differentiation conditions without any observable cytotoxicity. Genome-wide mRNA expression analysis further demonstrated the downregulation of myogenic differentiation markers and the upregulation of proliferation-associated genes. These results suggest that the effects of myoseverin most likely stem from cytoskeletal remodeling rather than dedifferentiation to multipotent progenitor cells. Although the myoblasts generated by myoseverin are still unipotent and the observed cleavage of myotubes may only represent a single step of lineage reversal, this experiment demonstrated that terminally differentiated states could be altered by a small molecule.

To identify small molecules that induce true dedifferentiation of murine myoblasts, a new screen was designed based on the notion that lineage-reversed myoblasts should regain multipotency, and that these dedifferentiated myoblasts should acquire the ability to differentiate into multiple mesenchymal cell lineages under typical adipogenic (fat), osteogenic (bone), or chondrogenic (cartilage) conditions. To assay multipotency of dedifferentiatied myoblasts in the primary screen, a two-stage screening protocol was developed. C2C12 murine myoblasts were treated with compounds for 5 d to induce dedifferentiation at which point the compounds were removed, and cells were assayed for their ability to undergo osteogenesis upon addition of known osteogenesis-inducing agents (which only affect mesenchymal progenitor cells; Fig. 5.3a). A 2,6-disubstituted purine, later termed reversine, was found to have the highest activity in the primary screen (37). Reversine inhibited myotube formation of C2C12 myoblasts, where treated myoblasts could later redifferentiate into osteoblasts or adipocytes upon exposure to appropriate differentiation conditions. Importantly, the dedifferentiation effect of reversine on C2C12 cells was shown at the clonal level, suggesting that it was inductive rather than selective. Moreover, at the most effective concentration of reversine, no significant cell death was observed.

In the absence of osteogenesis-inducing medium, continuous reversine treatment alone had no osteogenic activity; for example, transdifferentiation of C2C12 myoblasts to osteoblasts or adipocytes was also not observed under the conditions used to induce osteogenesis or adipogenesis. These observations suggest that reversine acts by directing dedifferentiation, rather than by simply enriching certain progenitor cells through the selective killing of myoblasts. This example provides a proof-of-principle demonstration that dedifferentiation of lineage-restricted cells to a more primitive, multipotent state by a synthetic chemical can be achieved via a rationally designed phenotypic screen of chemical libraries, and that such concepts and technologies are readily applicable to other models.

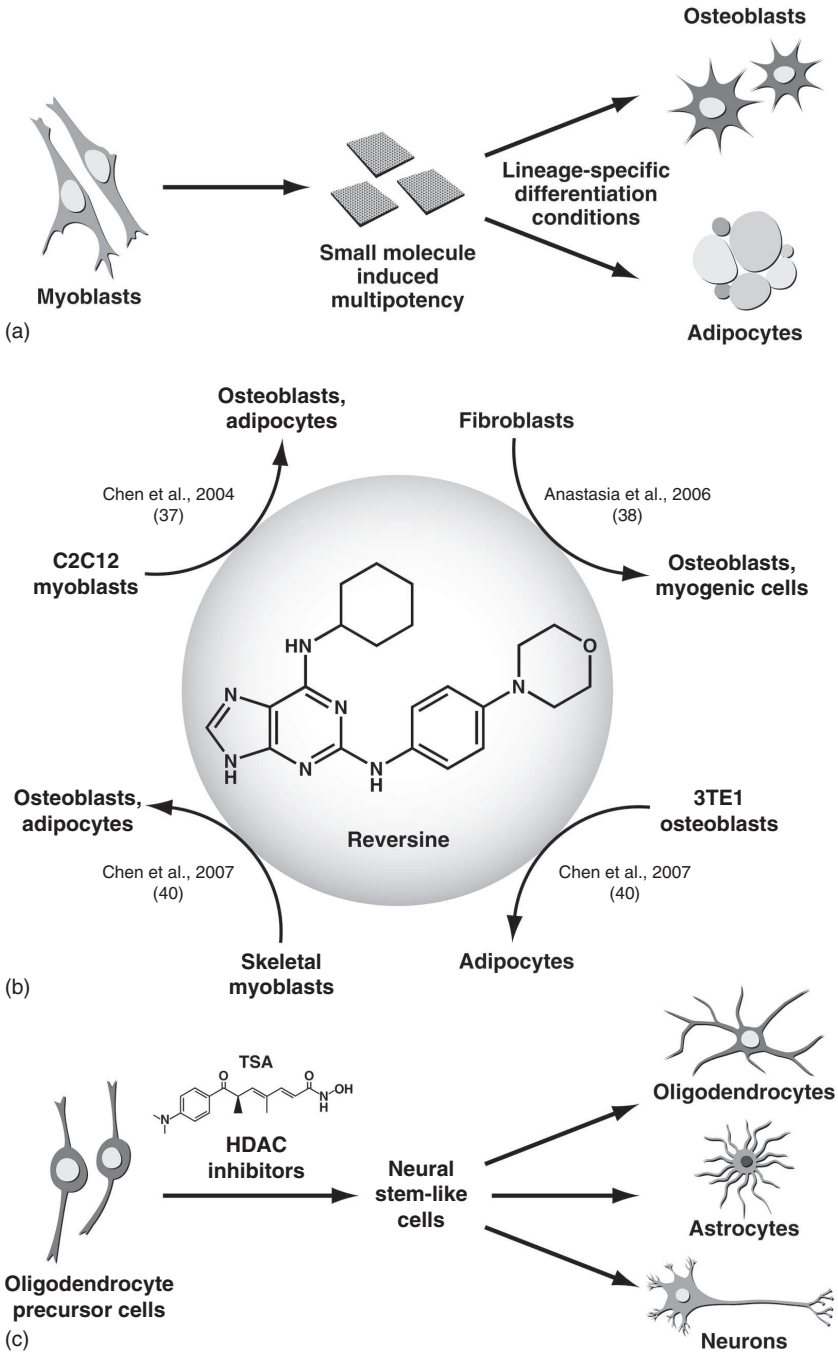

Figure 5.3. Chemical reprogramming. Summary of the strategies and successful reports of small molecule induced cellular reprogramming. (a) Two-stage screening approach used to identify 2-(4-morpholinoanilino)-6-cyclohexylaminopurine (reversine). Reversine treatment converted unipotent C2C12 myoblasts into multipotent mesenchymal precursors that could be differentiated into osteoblasts and adipocytes upon exposure to lineage-specific cues. (b) Reported applications of reversine in cellular reprogramming. (c) HDAC inhibitors, including trichostatin A (TSA), are capable of reverting oligodendrocyte precursor cells to a tripotent neural stem-like state with neuronal differentiation potential.

The novelty of reversine immediately sparked an interest within the field and is currently being used by numerous other investigators (Fig. 5.3b). For instance, Anastasia et al. demonstrated that reversine treatment converted primary murine and human dermal fibroblasts into myogenic-competent cells at high frequency both *in vitro* and *in vivo* (38). Additionally, the treated fibroblasts could also be differentiated into osteoblasts under appropriate conditions. In 2007, Kim et al. found that reversine treatment of the 3T3-L1 preadipocyte line under adipocyte differentiation conditions induced a synergistic enhancement of adipocyte differentiation (39). Interestingly, this process did not involve well-established adipocyte differentiation signaling paradigms (PI3K/Akt or mTOR/p70s6k), suggesting that novel pathways are operative in the adipocyte differentiation process. While these studies did demonstrate different uses for reversine, the mechanism of action still remained largely unknown.

Follow-up work by Chen et al. has extended the work of Anastasia et al., confirming that reversine is active in multiple cell types, including 3T3E1 osteoblasts (treated cells demonstrate adipogenic potential) and human primary skeletal myoblasts (treated cells demonstrate osteoblast and adipogenic potential) (40). Further, Chen et al. identified the cellular targets of reversine as mitogen-activated extracellular signal regulated kinase (MEK1) and nonmuscle myosin II (NMMII) heavy chain (40). Biochemical experiments showed that reversine treatment inhibited the enzymatic activity of purified MEK1 *in vitro*, MEK-dependent signaling (as determined by the inhibition of ERK1/2 phosphorylation), and the ATPase activity of Myosin II (heavy chain). To confirm that the reversine-induced plasticity was mediated by its interaction with MEK1 and NMMII, additional genetic studies were also performed. Ectopic expression of either MEK1 or NMMII could partially block the activity of reversine. Moreover, simultaneous treatment with a siRNA targeting NMMII and a MEK inhibitor (U0126) expanded the differentiation potential of C2C12 cells to include the adipogenic and osteoblast lineages. In contrast, cells transfected with nontargeting siRNAs and treated with dimethyl sulfoxide (DMSO) did not gain multipotency, and C2C12 myoblasts treated with either siNMMIIs or U0126 alone did not gain the ability to undergo osteogenesis or adipogenesis. These results indicate that simultaneous inhibition of both MEK1 and NMMII is required for reversine's activity.

Further investigation also found that reversine induced cell cycle arrest in G2/M, which was reversible upon compound withdrawal. A series of cell-cycle stage-specific inhibitors were used to provide evidence that the effects of reversine on the cell cycle contributed to the increase in cellular plasticity. In addition, removal of insulin from the growth media also blocked reversine's action. PI3K is a major kinase activated by insulin signaling and involved in diverse cellular processes, including proliferation, cell division, and apoptosis. Addition of a PI3K inhibitor (LY294002) blocked the effect of reversine in C2C12 myoblasts. These results suggest that the activation of the PI3K signaling pathway is essential for the reversine-induced increase in cellular plasticity,

adding another level of complexity. Furthermore, treatment of C2C12 cells with either reversine or U0126 resulted in decreased histone H3 lysine 9 acetylation, whereas simultaneous application of a histone deacetylase (HDAC) inhibitor (trichostatin A—TSA) and reversine blocked the effect of reversine. This experiment suggests that histone acetylation may also be involved in the reversine's activity via suppression of cell fate-determining genes. In summary, it appears that reversine acts as a dual inhibitor on NMMII and MEK1. By blocking NMMII, reversine induces G2/M phase accumulation of C2C12 myoblasts and, at the same time, modulates acetylation of histone H3 by inhibiting MEK-dependent signaling. PI3K signaling is also essential for reversine's activity, although a detailed mechanism of its involvement remains to be established. Moreover, the effect of other mechanisms related to the inhibition of NMMII and MEK1 remains to be determined. Nonetheless, this work is a pinnacle achievement in the field of regenerative medicine and provides a clear demonstration that small molecules will provide significant insights into the complex processes that mediate developmental plasticity.

As discussed, development and cell fate determination require genetic and epigenetic coordination. Among the epigenetic mechanisms that regulate lineage specification, posttranslational modification of nucleosomal histones represents the most well-studied class. Histones make up the majority of protein mass in chromatin and act as spools around which DNA winds. They are subject to a variety of posttranslational modifications, including acetylation, methylation, phosphorylation, sumoylation, and ubiquitination, which ultimately govern transcription and lineage control (41). For example, methylation of lysine 9 and 27 on histone subunit H3 (H3K9me and H3K27me) are associated with transcriptional repression, while methylation of lysine 4 on H3 (H3K4me) is associated with gene activation. More generally, lysine acetylation correlates with chromatin accessibility and transcriptional activity (42). Along these lines, we have demonstrated that global histone acetylation, induced by HDAC inhibition, can partially reverse the lineage restriction of oligodendrocyte precursor cells (OPCs), thereby expanding the differentiation potential to include the neuronal lineage (14).

In vivo, OPCs arise from neural stem cells (NSCs) in discrete locations before eventually differentiating into mature oligodendrocytes. *In vitro*, however, OPCs can give rise to oligodendrocytes and type 2 astrocytes (43, 44), and recent evidence has suggested that OPCs can even access the neuronal lineage, via a multipotent neural stem-like state, after exposure to BMPs (45, 46). In an effort to provide a better understanding of the mechanisms governing OPC developmental potential, we developed a small molecule screening strategy to identify chemical inducers to control this process (Fig. 5.3c) (14). Toward this end, primary rat OPCs were transfected with a reporter construct that used a 5.5-kb region of the *Sox2* promoter to drive enhanced green fluorescent protein (eGFP) expression (P/Sox2-eGFP). Sox2 is a transcription factor that is expressed early in the developing brain and is essential for the maintenance of the multipotent state in NSCs (47). Additionally, Sox2 is highly

expressed in NSCs but not in lineage restricted precursors, such as OPCs (46–48). Therefore, it was assumed that an increase in the multipotency of OPCs would require Sox2 expression, consistent with the previous observation that Sox2 reactivation is essential for the BMP2-induced conversion of OPCs to NSCs (46).

In-house combinatorial and known drug libraries were then screened to identify molecules that activate Sox2 expression in OPCs. Compounds that triggered Sox2 reactivation in OPCs (~200 of 40 000 compounds tested) were then screened in a secondary assay for their ability to promote neurogenesis of OPCs that had been expanded in aggregate cell culture. The four compounds that passed this test (butyrate, TSA, MS-275, and Apicidin) were all known HDAC inhibitors, indicating that inhibition of HDAC activity can expand the developmental potential of OPCs to include the neuronal lineage. Moreover, clonal analysis indicated that OPCs treated with butyrate (56 of 136 clones, 41%) or TSA (41 of 129 clones, 32%) exhibited much higher potential to form neurons than the DMSO-treated control (9 of 366 clones, ~2.5%). This experiment suggested that the HDAC inhibitor-mediated neuronal potential of OPCs did not result from the amplification of a residual multipotent side-population of cells.

Genome-wide transcription profiling was then used to characterize the mechanism of HDAC inhibitor-mediated OPC plasticity, and to compare this profile to that which results from BMP2. Indeed, OPCs rapidly acquired several molecular characteristics of NSCs when treated with TSA, including activation of 13 genes that identify or maintain the NSC state, while simultaneously silencing a large group of oligodendrocyte lineage-specific genes. Among the genes activated by TSA were a group of BMP-responsive genes, which provided evidence for cross talk between these two processes. Additionally, the reactivation of Sox2 was also central to both BMP- (46) and HDAC inhibitor-mediated OPC plasticity, where the knockdown of Sox2 dramatically reduced the potential for OPC neuronal differentiation. As such, it appears that BMP2 and HDAC inhibitors trigger similar changes in OPC gene expression that makes them responsive to neuron-inducing stimuli, a process that is mediated, at least in part, through the reactivation of Sox2.

Further insights into this interesting phenomenon were provided by Liu et al. who both confirmed our findings and demonstrated that HDAC inhibitor-treated OPCs formed functional neurons that were able to engraft *in vivo* (49). Interestingly, HDAC inhibitor-induced neurogenesis occurred at the expense of oligodendrogenesis. Together with our results, these data suggest that the oligodendrocyte identity of OPCs is critically dependent on HDAC enzymatic activity. When HDAC activity is high, the oligodendrocytic epigenetic memory of specified progenitors is established by repressing neuronal and astrocytic genes as the cells progress to mature oligodendrocytes. However, when HDAC activity is inhibited, progenitors are unable to establish an oligodendrocyte-specific program of gene expression, and they are reprogrammed into a multipotential state that is responsive to neurogenic or astrogenic signals. This

series of experiments, and previous work using HDAC inhibitors in neural stem and progenitor cells (50–54), suggest that the establishment of an oligodendrocyte memory through histone deacetylation allows oligodendrocyte differentiation to remain silent during the neurogenic and astrogliogenic period of development. These experiments not only provide insight into the mechanisms operative during brain development, they also suggest potential avenues that can be exploited in future endeavors to manipulate and reprogram cell fate.

Environment-Induced Cellular Plasticity

Stem cell homeostasis is controlled by both intrinsic regulators and the extracellular environment (niche). In recent years, it has been suggested that the niche actively restricts multipotent adult stem cells from nonclassical differentiation (e.g., differentiation across germ layer restricted barriers). Moreover, much of this work purports that these cells are inherently plastic, that the niche is responsible for imposing the lineage restrictions, and that access to otherwise developmentally restricted lineages simply requires removal from the niche. This phenomenon is referred to as transdifferentiation and has been reported for a variety of cell types ranging from hematopoietic to intestine, skin, and NSCs. Although these claims are very exciting, the experimental evidence supporting them is often inadequate and requires qualification. For instance, *in vitro* differentiation (the least stringent of the assays for developmental potential; Fig. 5.2) is often the only criterion used for transdifferentiation. While this technique is a critical first step in establishing potency, it is insufficient for decisively concluding that a cell has been converted to a different fate. A case in point is neuronal transdifferentiation of mesenchymal stem cells (MSCs). This process is generally characterized immunocytochemically for expression of nestin, neuron-specific βIII-tubulin and/or NeuroD1. However, expression of these markers may more accurately be interpreted as a stress response from culture in suboptimal conditions (e.g., serum-free neural growth media) (15, 16, 55). In fact, there is a reasonable alternative explanation for the observed phenotypic changes: "cellular mimicry," defined as the phenomenon when one cell type morphologically appears like another without possessing any of the functions that make it unique from all other cell types (56).

More convincing claims for transdifferentiation between blood, bone marrow, and brain lineages has relied on transplantation of *ex vivo* expanded cells from one organism into another. While such experiments meet higher degrees of developmental scrutiny than simply assaying for lineage-specific markers *in vitro*, these claims have remained controversial because of flaws in interpretation and experimental design (57, 58). Subsequently, most of this work has either proven irreproducible or has been largely attributed to cell fusion events (59–64). Thus, while unexpected transformation events may occur in somatic lineages by simply changing their environment, such events

are exceedingly rare, and transdifferentiation between somatic lineages remains an unproven concept.

REPROGRAMMING TO PLURIPOTENCY

Historically, lineage-specific gene expression was thought to occur via elimination of genes not expressed in a particular tissue type with concomitant retention of those that were expressed. Cloning experiments in amphibians and mammals have decisively demonstrated that this is not the case (13, 65). Rather, tissue- or lineage-specific gene expression occurs at the level of the epigenome where genes not expressed in a particular tissue are silenced (via DNA methylation, histone marks, etc.) rather than excised. A variety of other techniques to reprogram the epigenome of various mature tissues back to pluripotency has confirmed the findings established by cloning and provided further insight. Among these techniques are ES–somatic cell fusion, culture induced reprogramming, and, more recently, reprogramming with defined factors. Below, we briefly summarize these techniques, the advances in our understanding resulting from their application, and conclude with a comprehensive summary of the recent work in defined factor reprogramming.

Nuclear Transplantation

SCNT, also referred to as nuclear transplantation, denotes the introduction of a somatic genome into an enucleated oocyte. Upon transfer, cellular factors in the egg reprogram the somatic epigenome back to a state of totipotency. The resultant cell behaves as a fertilized zygote in that it is then capable of giving rise to a cloned organism. Dolly the sheep was the first mammal to be cloned (5), and provided proof of principle that the epigenetic state of somatic cells, while stable, is not irreversibly fixed, and, more importantly maintains a complete set of instructions to create a mature organism. Alternatively, SCNT can also be used to generate ES cell lines via *in vitro* culture of SCNT-blastocysts. Since the creation of Dolly, 16 other mammalian species have now been cloned (66), including a nonhuman primate (67) and a human blastocyst (although ES cell lines were unable to be subsequently established) (68). In addition to providing a platform to study the mechanisms active during early development, SCNT has also received a great deal of attention as a potential source for patient-specific pluripotent cell lines, which would serve as an inexhaustible supply of tissue for use in age-related and degenerative diseases.

The generation of a cloned animal from a somatic genome laid to rest the idea that lineage specification was permanent; however, it remained an open question whether the genome of a terminally differentiated cell could be reprogrammed. The generation of mice from mature lymphocytes carrying differentiation-associated immune-receptor rearrangements (69, 70) or post-

mitotic neurons (71) unambiguously demonstrated that all cells, including terminally differentiated cells, maintained the code for complete developmental potential. In other words, the epigenetic changes that direct exit from the cell cycle and terminal lineage specification are reversible if provided with the right complement of factors. Interestingly, cloning with terminally differentiated somatic nuclei is significantly less efficient than with less differentiated cells and often involves the establishment of an ES line prior to the generation of a complete organism. This suggested that the differentiation state of a somatic genome affects cloning efficiency, where a less differentiated genome is more easily reordered to a pluripotent state by an oocyte than its more differentiated counterpart. Consistently, NSCs generate cloned animals more efficiently than do neurons (72, 73), and skin stem cells do so more efficiently than keratinocytes (74). Interestingly, early experiments with amphibians demonstrated this same inverse relationship between donor cell differentiation state and cloning efficiency (75). However, because cloning is affected by so many other parameters (e.g., stage of the cell cycle, physical state of the donor nucleus), it is still unclear whether cloning efficiency decreases with increased cellular specialization of the somatic nucleus in all cases (76).

SCNT has also provided insight into the mechanisms that promote oncogenic transformation and drive uncontrolled growth. For instance, the generation of normal chimeric mice from brain tumor (77) and melanoma (78) cells indicated that the phenotypic characteristics of some cancer cells result from epigenetic alterations that are reversible upon nuclear transplantation and re-establishment of the correct epigenetic architecture during a subsequent round of development. Nevertheless, nuclear cloning remains an inherently inefficient process where most clones die soon after implantation, and the few that survive have severe abnormalities, such as obesity and premature death (79, 80).

SCNT can be used to create organism-specific ES cell lines, which serve as a powerful tool to study early development and genetic disease. Importantly, these lines behave like normal ES lines in culture, produce teratomas when injected into immunocompromised mice (81), are able to generate organisms using tetraploid embryo complementation (69, 71, 82), and have indistinguishable gene expression profiles (83), suggesting that they have the same scientific and therapeutic potential as those derived from fertilized embryos. Furthermore, the application of this technology in human cells has presented the potential for therapeutic cloning—the use of patient-specific ES cell lines to generate cells/tissues lost due to disease or aging (discussed below). However, initial attempts to produce SCNT blastocysts from human somatic cells were unsuccessful, owing to the difficulty in obtaining a suitable number of human oocytes and other technical constraints (84). More recently, human blastocysts were derived from healthy oocytes (i.e., they were from young donors and injected shortly after isolation), suggesting that new information and/or methods may assist in the generation of SCNT-human ES lines (68). Although limited successes exist, the mechanisms and critical experimental parameters required for

human SCNT necessitate further exploration. Thus, alternative strategies have been developed to study the mechanistic details of reprogramming and may eventually be applied in the generation of patient-matched pluripotent stem cell lines.

Cellular Fusion

Fusion between different cell types has historically been used as a means to study the plasticity of the differentiated state (85). An interesting finding from these studies has been that the phenotype (developmental state) of the "less-differentiated" partner is dominant over that of its "more-differentiated" partner. For example, fusion of embryonic carcinoma (EC) cells with thymocytes resulted in pluripotent hybrids as judged by the induction of teratomas upon injection into immunocomprimised mice (86). Consistently, recent work fusing a variety of differentiated lineages with mouse and human ES cells resulted in the formation of pluripotent hybrids that could generate cell types from the three primary germinal layers (7, 8, 87).

An important finding from cellular fusion is that factors expressed in the nuclear compartment of the ES cell are essential for reprogramming, and that this process requires DNA replication in order for somatic pluripotency-associated factors to be activated from the somatic genome (88). This is consistent with cloning experiments in amphibians (89) and mice (90), which indicate that reprogramming requires injection of the somatic nucleus into the germinal vesicle of a metaphase oocyte where nuclear factors are available in the cytoplasm. However, it remained an open question whether the somatic genome was actually being reprogrammed or whether it was simply retained as quiescent cargo. Reactivation of the silent X chromosome in fused female somatic cells (7), demethylation and reactivation of pluripotency-associated somatic loci (7, 8), and complete removal of both copies of chromosome 6 from the ES cell (91) (which harbors pluripotency expressed genes, including Nanog) has provided molecular evidence for reprogramming of the somatic genome in hybrid cells.

While a limited number of interesting insights have been garnered from cellular fusion, studies on the molecular mechanism governing this process have been severely impeded by the inefficiency of generating hybrids. Furthermore, tetraploidy of the reprogrammed cells presents a major shortcoming for use of this process in customized cell therapy. Incubation of somatic cells with ES and EC extracts has been suggested as an alternative reprogramming method (92), although convincing evidence of this front is still lacking.

Culture Mediated

SCNT and fusion experiments require that a somatic nucleus be exposed to nuclear/cytoplasmic factors from a pluripotent cell in order to induce repro-

gramming. However, it has been demonstrated that some rare cell populations can be reprogrammed simply by prolonged culture *in vitro*. In fact, it appears that all stages of development from which it is possible to derive teratomas can also give rise to pluripotent ES cells *in vitro* (93). For instance, oncogenic transformation of spermatozoa/oocyte progenitor cells (primordial germ cells [PGCs]) gives rise to EC cells, the driving population in teratocarcinomas (94). Furthermore, *ex vivo* culture of normal PGCs can give rise to lines of embryonic germ (EG) cells (95–97), which behave like ES cells in their pluripotent capacity, but have imprinting differences resulting from their germ cell origin. Interestingly, both mouse and human EG cell lines initially require fibroblast growth factor-2 for proliferation, but, once established, respond to the species-specific ES cues. Furthermore, germline contribution has been demonstrated for some EG (98, 99) and EC (100) cells, thus demonstrating their pluripotency.

An interesting aspect of germline cells is that they undergo dramatic epigenomic overhaul during differentiation, a characteristic that may make them more suitable for reprogramming. More recently, three groups have also reported the derivation of pluripotent ES-like cells from mouse testes upon *ex vivo* expansion in glial-derived neurotrophic factor (GDNF) containing media (9, 10, 101). These lines, derived from postnatal spermatogonial stem cells, were established in leukemia inhibitory factor (LIF)-containing media and later deemed fully pluripotent as judged by their ability to contribute to the germ line in chimeric mice. These cell lines represent the only clear example of pluripotent cells derived from an adult mammal that are not the result of oncogenic transformation. In fact, it has been suggested that all pluripotent cell lines, including ES cells, are the product of germ cells (102). While cell lines derived in this manner allow insights into what it means to be pluripotent, they do not provide any additional insight in the mechanisms behind reprogramming. Thus, a simplified and tractable means to study reprogramming was still needed.

Defined Factors

In 2006, Shinya Yamanaka and Kazutoshi Takahashi presented a groundbreaking alternative means to convert a differentiated genome into one that has complete developmental potential using retroviral delivery of a defined transcription factor cocktail (11). To achieve this, they used mouse embryonic fibroblasts (MEFs) engineered to express neomycin resistance from the *Fbx15* locus, a downstream target of the pluripotency-associated factor Oct-4. In MEFs, the *Fbx15* locus is silent and the cells are vulnerable to neomycin. Following transduction with a pool of 24 candidate reprogramming factors, Takahashi and Yamanaka noticed the appearance of ES-like colonies that were resistant to selection with antibiotic, indicating that they had reactivated the Fbx15 locus. To identify the active transgenes, the experiment was repeated

with 24 pools of 23 factors. Plates displaying a reduced number of colonies were assumed to be missing a key factor. Multiple iterations revealed Oct-4, Sox2, c-Myc, and Klf4 as the minimum set of factors necessary to produce Fbx15 selectable colonies.

In addition to Fbx15 expression and mouse ES cell colony morphology, the neomycin-resistant colonies produced from Oct-4, Sox2, c-Myc, and Klf4 transduction also expressed multiple pluripotency markers (e.g., Nanog, SSEA1, Dax1) and were duly named induced pluripotent stem (iPS) cells. However, the majority of the resulting colonies did not show reactivation of the endogenous Oct-4 and Sox2 loci and required constitutive expression of Oct-4 and Sox-2 transgenes to maintain the pluripotent state. Subcutaneous injection of Fbx15-iPS cells into nude mice generated teratomas, but the cells were unable to produce viable chimeras upon injection into a developing blastocyst. Together, these results indicate that while Fbx15 iPS cells were pluripotent, they had not been fully reprogrammed to a self-sustaining ES cell state.

To improve the odds of achieving fully reprogrammed iPS cells, three groups independently reproduced Takahashi and Yamanaka's original results using a different selection strategy (103–105). In these studies, antibiotic-resistant genes were expressed from the loci of Oct-4 or Nanog, genes known to be critical for maintenance of the pluripotent state. Indeed, colonies derived using this method were indistinguishable from ES cells upon rigorous developmental characterization (Table 5.3). For instance, Nanog selected iPS cells revealed global histone methylation patterns (H3K4 and H3K27) and gene expression profiles that were indistinguishable from those of mouse ES cells (103). Additionally, female Nanog-selected iPS cells showed full reactivation of the silenced X chromosome during reprogramming and random silencing upon differentiation (103). More rigorous analyses demonstrated that Nanog or Oct-4 selected iPS cells could contribute to the germ line of chimeric offspring following blastocyst injection (104, 105), and Oct-4 iPS cells were able to generate an entire organism using tetraploid blastocyst complementation, although these mice did not survive to term (105).

Clearly, the Nanog or Oct-4-driven selection strategies were a great improvement on the initial work with Fbx15-driven selection. However, the drawback of any selection scheme is that it requires modification of the starting genome, a potential barrier for applying similar techniques in therapeutically applicable cells. To address this, several groups have successfully derived iPS cells from somatic cells by utilizing morphological criteria in place of drug selection (106–108). Interestingly, morphological selection also increased the efficiency of reprogramming roughly 5–10-fold, as compared to the analogous antibiotic selection approaches (107). This observation supported the notion that reprogramming is a gradual process where drug selection may remove cells that have not yet achieved pluripotency.

Another drawback from the original Yamanaka gene quartet is that c-Myc is a potent oncogene. Retrovirally delivered c-Myc is silenced in bona fide iPS cells, but reactivates and drives tumor formation in a considerable fraction of

TABLE 5.3. Summary of the Factors, Methods, and Validations Used for Induced Pluripotent Stem (iPS) Cell Generation

	Mouse			Human		
Factors	Oct-4, Sox2, c-Myc, Klf4	Oct-4, Sox2, c-Myc, Klf4	Oct-4, Sox2, Klf4, (c-Myc)	Oct-4, Sox2, c-Myc, Klf4	Oct-4, Sox2, Nanog, (Lin28)	Oct-4, Sox2, c-Myc, Klf4, (LTA/hTERT)
Selection	*Fbx15*	Nanog/Oct-4	Nanog/Fbx15			
Cell type	MEF, TTF	MEF, TTF	Hepatocytes, gastric epithelium	Dermal fibroblasts, synoviocytes	IMR90, foreskin fibroblasts	MRC5, BJ1, MSCs, dermal fibroblasts
Chimera	Mid-gestation	Germline transmission	Germline transmission			
Tetrapoid complementation		Mid-gestation				
Teratoma		✓	✓	✓	✓	✓
Microarray	✓	✓		✓	✓	✓
Epigenetic analysis	Fbx15/Nanog promoter (de)methylation, Oct-4/Nanog promoter H3 acetylation/ H3K9 (de)methylation	Imprinting, Xi activation, whole geneome (de)methylation		Oct-4/Sox2/Nanog H3K4 & H3K27 (de)methylation, Oct-4, Nanog, Rex1 promoter (de)methylation	Oct-4 promoter (de)methylation	Oct-4/Nanog promoter (de)methylation
In vitro differentiation	Embryoid bodies			Cardiomyocyte, NSC, embryoid bodies	Embryoid bodies	Hematopoietic lineages, embryoid bodies
Reference	(11)	(103–105)	(114)	(113)	(116)	(117)

MEF = mouse embryonic fibroblast; TTF = tail tip fibroblast; NSC = neural stem cells.

iPS-derived progeny (~20% of chimeras) (104). Subsequently, it was demonstrated that c-Myc is dispensable for reprogramming (108, 109), a finding that should bring the therapeutic use of iPS cells one step closer to clinical utility. Consistently, Yamanaka observed a significant reduction in cancer incidence in chimeras generated from three-factor (minus c-Myc) reprogrammed iPS cells (108).

Less than a year after the first report of mouse iPS cells, the labs of Shinya Yamanaka and James Thomson reproduced this feat in human cells (110, 111). While Yamanaka eventually settled on the same combination of transcription factors used to create mouse iPS cells, his initial attempts with this combination were unsuccessful (112). This observation was later attributed to the degree of viral infectivity, which is considered a critical determinant in the generation of iPS cells. For instance, transgene copy number totals are greater than 10 in every clone analyzed thus far (e.g., most clones have three or more copies of each gene) (103, 105, 112, 113). Consequently, the initial attempts by the Yamanaka lab to reprogram human fibroblasts using amphotropic pseudotyped retrovirus gave poor transduction efficiency and yielded no colonies. To overcome this, they introduced the ecotrophic retroviral receptor into human fibroblasts via lentiviral transduction, which allowed for much greater infectivity in cells subsequently infected with four factor-ecotrophic pseudotyped retroviruses. Remarkably, this method allowed for the successful generation of iPS cells from both 36-year-old female dermal fibroblasts and 69-year-old male synoviocytes.

Human iPS cells cannot be characterized as accurately as their mouse counterparts since exact animal models of development do not exist. Thus, Yamanaka relied on teratoma formation and a battery of *in vitro* assays. For example, human iPS cells were capable of differentiating to both neural cells and beating cardiomyocytes. Embryoid body formation generated cells expressing key markers from all three germ layers, based on mRNA expression. Bisulfite sequencing of the Oct-4, Nanog, and Rex1 promoters revealed that they were demethylated and in an active state. Furthermore, chromatin immunoprecipitation (ChIP) demonstrated that the Oct-4, Sox2, and Nanog promoters exhibited H3K4 and H3K27 methylation patterns indicative of an open chromatin state, whereas bivalent chromatin patterns were observed at key developmentally associated promoters (i.e., Gata6, Msx2, Pax6, and Hand1). Microarray analysis of female iPS cells generated an expression profile that was highly similar to a female human ES cell line, although some differences did exist.

The Thomson lab took a slightly different approach in making iPS cells. They generated a small, 14-member lentiviral library based on factors with known or suspected roles in pluripotency. Application of this library on myeloid cells differentiated from a human ES cell line that expresses neomycin from the Oct-4 locus (114) revealed a novel quartet of pluripotency inducing factors—Oct-4, Sox2, Nanog, and Lin28 (where Lin28, much like c-Myc, was not required, but increased the efficiency of reprogramming as determined by colony formation) (110). This four-factor combination was then applied to primary IMR90 fetal lung fibroblasts and newborn foreskin fibroblasts. Colony

generation was observed in each. Bisulfite sequencing of the Oct-4 promoter in both IMR90 iPS cells and fetal foreskin iPS cells revealed that it was active. Further, teratomas generated from IMR90 and fetal foreskin iPS cells contained tissue types representative of the three germinal layers, although it was noted that teratomas generated from fetal foreskin iPS cells displayed varying degrees of heterogeneity. Lastly, genome-wide transcriptome analyses of IMR90 and fetal foreskin iPS cells exhibited expression profiles that were similar to those of the H1, H7, H9, H13, and H14 human ES cell lines.

Since the initial publication of human iPS cells from the Yamanaka and Thomson labs, two other groups have successfully applied the Yamanaka transcription factor quartet (115, 116). Of note, several interesting observations were made by George Daley's group. First, they had considerable difficulty deriving iPS cells from primary adult cells in the absence of the immortalizing genes, SV40 large T antigen and the catalytic subunit of human telomerase reverse transcriptase (hTERT). This six-factor cocktail successfully reprogrammed BJ1 fetal foreskin fibroblasts, MSCs, and adult human dermal fibroblasts. Surprisingly, large T antigen and hTERT were not found in the reprogrammed clones, and likely provided a supporting role by stimulating the secretion of paracrine factors in nonreprogrammed cells (115).

Mechanistic Insights

Reprogramming with defined transcription factors is initiated when a single cell is bombarded with transgenes that integrate stably and randomly throughout the genome. The transgenes express protein products from viral promoters at extremely high copy number, resulting in an excess of those particular proteins in the cell. During a series of cell turnovers, these proteins reorganize the differentiated epigenome to the pluripotent state. The process concludes when the endogenous loci of the ectopically expressed factors (e.g., Oct-4) are reactivated and stably maintain the pluripotent state (105). In addition, silencing of the viral elements is required for multilineage differentiation *in vitro* and *in vivo* (106, 110, 117). Based on this broad description, three uniting themes have emerged: (a) that expression of the reprogramming factors initiates a stochastic sequence of events; (b) that reprogramming is slow and strictly dependent on cell turnover, and (c) that a somatic cell passes through intermediary stages as it progresses to pluripotency (65, 107, 117, 118). For instance, clonal analyses (and thus identical transgene integration pattern) have demonstrated that the activation of pluripotency markers can occur at different times and that only a fraction of the cells within the clone go on to become completely reprogrammed (107). This process also requires at least 10 days (117, 118) and will not occur if the transduced cells are not provided with ample room to proliferate (i.e., contact inhibition prevents proliferation and inhibits the process [Lyssiotis et al., unpublished results]). Furthermore, rapidly dividing cells reprogram faster than their slower dividing counterparts (10–12 d with c-Myc and >30 d without c-Myc) (108, 109, 117, 118), although c-Myc likely plays additional roles (see discussion below). Lastly, intermediate

stages have been isolated that possess unique characteristics as compared to their differentiated or iPS cell counterparts (11, 117, 118).

Since reprogramming is not a natural process, ectopic expression of the Oct-4, Sox2, Klf4, and c-Myc quartet is not simply hijacking an endogenous cellular mechanism. Their function during reprogramming can be garnered from the endogenous roles of these transcription factors. For instance, Oct-4 and Sox2 have long been known as critical mediators of pluripotency in both mouse and human ES cells. More recently, it was demonstrated that Kruppel-like factors are required for mouse ES cell self-renewal where there is functional redundancy between the varying isoforms (i.e., Klf2, Klf4, and Klf5) (119). ChIP on chip experiments also suggest that Klf4 is an upstream regulator of Oct-4, Sox2, and Nanog, indicating that Klf4 may be a key player in the canonical ES cell autoregulatory network (120). The functional redundancy of the Kruppel-like factors suggests that it may be possible to replace any one of the four factors with a functionally redundant transcription factor. Yamanaka confirmed this hypothesis with a number of Oct, Sox, Kruppel, and Myc family members. Interestingly, Sox1, Sox3, Sox15, Sox18, Klf1, Klf2, Klf5, L-Myc, and N-Myc were able to fill in for their respective counterparts with varying degrees of efficiency (108).

While the functional role of endogenous Oct-4, Sox2, Klf4, and c-Myc in ES cells gives some insight into how they may be functioning during reprogramming, it does little to explain the dynamics of reprogramming during the 10–20 d process. Recent experiments using inducible versions of the four factors by two groups have concluded that transgene expression is required for 10–14 d in order to achieve complete reprogramming (117, 118). Further, it appears that there is sequential activation of various pluripotency associated markers, where alkaline phosphatase appears at day 3 of transgene expression, followed by expression of the surface marker SSEA1, and finally activation of the endogenous Oct-4 and Nanog loci.

It was also suggested by these studies that the cells *must* go through multiple rounds of replication, providing justification for the difference in timing observed in the presence/absence of the proliferative oncogene, c-Myc (10–12 d with c-Myc and >30 d without c-Myc) (108, 109, 117, 118). However, this may not be the whole story with c-Myc. Reprogramming efficiency was reduced by one or two orders of magnitude without it (108, 109, 113), indicating that it may be playing additional roles (consistent with the fact that it can bind to 25 000 DNA sequences and promotes DNA synthesis independent of transcriptional regulation) (53, 121–123). These may include opening chromatin (124), contributing to establishment of the ES state and enhancing misreplication (125). Additionally, it has been proposed that c-Myc works in part with Klf4—which inhibits c-Myc induced apoptosis—by conferring somatic cells with rapid proliferation kinetics and immortal growth potential (123). Nonetheless, the fact remains that c-Myc (or c-Myc and Klf4) boosts the speed of the process, in part, through shortening the time for a cell to complete a cycle (109).

Although the greater majority of this work has been done in fibroblasts (mouse embryonic and tail tip fibroblasts) (11, 110, 112), Yamanaka recently demonstrated that hepatocytes and gastric epithelial cells can also be reprogrammed using this method (113). Most importantly, this work has provided strong evidence against two alternative reprogramming hypotheses (126). The first proposes that the reprogramming efficiency is so low because a specific subtype of cell (i.e., a stem cell or one with a unique epigenetic structure) is being reprogrammed. Now that this work has been done in cell types vastly different from fibroblasts, this appears quite unlikely. The second is that the transgene integration pattern and/or the activation of a pseudo-fifth gene results in a cell that is capable of being reprogrammed. Aoi et al. also provided a very strong case against this hypothesis by systematically analyzing and comparing viral integration patterns across a large number of iPS clones (113). They found that the transgenes did not have a consistent pattern of integration across clones, nor did they preferentially integrate either in terms of gene ontology class or cellular localization of the gene product. While this work provides strong evidence against such alternate hypotheses, further work will be required to completely rule them out.

Another interesting finding that came from the work of Aoi et al. is that there are intrinsic differences between the reprogramming of epithelial lineages and fibroblasts. For instance, formation of iPS cells from hepatocytes and stomach epithelia require fewer copies of the viral transgenes, are less reliant on c-Myc, and can be generated by selection for Fbx15 (113) (as opposed to MEFs, which require selection for Nanog or Oct-4) (104, 105). The likely explanation here is that epithelial cells are more prone to reprogramming. Consistently, epithelial cells share more characteristics with ES cells than fibroblasts, including tight intracellular contact and surface expression of E-cadherin (126) and levels of β-catenin and E-cadherin that are much higher than those in fibroblasts and nearly identical to that in ES cells (113).

PERSPECTIVE

Stem cells have received a significant amount of recent attention for their potential application as a source of replacement tissues or organs to treat diseases that result from the destruction of cells with limited regenerative capacity (e.g., cardiovascular diseases, neurodegenerative diseases, and diabetes) (127, 128). In particular, pluripotent cells represent a theoretically inexhaustible source of cells that can be differentiated into virtually any cell type. However, immune rejection of transplanted allogenic cells/tissues presents a major drawback to the widespread application of cell replacement therapy. Injected nonself cells are recognized as foreign and elicit an immune response that leads to their destruction. Even cell transfer (e.g., bone marrow transplant) among closely related individuals often requires lifelong immunosuppressive therapy.

The use of patient-tailored (autologous) cell-based therapies presents an attractive means to avoid issues associated with histocompatibility (129). For instance, somatic stem cells could be derived from a patient, expanded and/or differentiated, and then reintroduced. In fact, this approach is routinely applied to salvage/boost the immune system of patients undergoing radiation therapy where the individual serves as both the bone marrow donor prior to treatment and the recipient following treatment. While appealing, the applicability of this approach is limited to a small number of cases from which progenitor or stem cell populations are readily accessible and amenable to *ex vivo* culture or manipulation. A more general approach involves the creation of individualized cell lines with unlimited expansion and differentiation potential from a readily accessible and abundant cell population. The generation of such lines could involve either nuclear transfer or ectopic expression of an appropriate transcription factor cocktail. These pluripotent cell lines could then be subjected to defined differentiation regiments to produce an autologous cell population for transplantation to rescue a degenerative disease state. Alternatively, the need for technically challenging reprogramming and differentiation processes could be circumvented via the direct reprogramming of an appropriate accessible somatic cell type to a desired therapeutic cell type. However, this concept remains largely a theoretical notion.

Experimental support for the feasibility of deriving "customized" pluripotent cells is derived from its direct application in mouse models of genetic disease. In general, the strategy involves reprogramming mutant donor cells (e.g., fibroblasts) into pluripotent ES cells, either by SCNT or the iPS cell method; correction of the genetic defect in the donor genome using homologous recombination; followed by differentiation of the "repaired" cells into the cell type that is driving the disease. Subsequent transplantation of repaired and differentiated cells restores tissue function back to the wild type state. Importantly, this strategy provides the critical cell population lost to disease while allowing for correction of the disease-driving genetic defects. In addition, the immortal growth potential of reprogrammed pluripotent cell lines facilitates their direct genetic manipulation, and provides the opportunity to subject genetically modified cells to rigorous characterization and quality control.

This approach was first reported in the $Rag2^{-/-}$ mouse model of immunodeficiency (129). In this example, the nuclei of fibroblasts from immunodeficient mice were used as donors to establish an ntES cell line. Homologous recombination was then used to repair one of the mutated alleles, thereby recapitulating the normal Rag2 gene function. Transplantation of hematopoietic precursor cells derived by *in vitro* differentiation or those from the bone marrow of immunoidentical mice (derived by tetraploid embryo complementation) into the original donor mice resulted in the production of a complete immune system (129). More recently, this strategy has also been demonstrated in a mouse model of Parkinson's disease (130). Again, an ntES cell line was derived from donor fibroblasts, differentiated into dopaminergic neurons, and transplanted into individually matched host mice. Importantly, mice that

received autologous grafts showed significant improvements in behavioral scores and did not demonstrate an immune response upon transplant, as compared to mice that received allogenic cells (130).

The feasibility of combining therapeutic gene replacement with reprogramming has also been demonstrated using the iPS cell method (131). Adult fibroblasts obtained from a mouse with humanized sickle cell anemia were used to generate an iPS cell line. Importantly, the reprogrammed line was not selected using the original iPS cell strategy, but rather was chosen based on morphological criteria (107). The genetic defect driving the disease was corrected by homologous recombination, and the repaired line was used to generate hematopoietic precursors *in vitro*. Transplantation of the genetically matched cells rescued the disease state and returned the mice to a near wild-type level. More recently, this approach was also applied in a mouse model of Parkinson's disease (132). Similar to the sickle cell example, genetically matched iPS cells were derived, differentiated into dopaminergic neurons *in vitro* and transplanted into genetically matched mice.

Despite successful application of ntES and iPS cells in animal disease models, significant impediments complicate the practical realization of therapeutic cloning in humans (133, 134). For instance, SCNT is limited both by ethical issues and the scarcity of human oocytes, whereas therapeutic applications of the iPS cell method will require a means to bypass the use of retroviral vector-mediated gene delivery. Furthermore, robust and reliable ES cell differentiation protocols are still lacking for the greater majority of cell types. Clearly, significant hurdles still exist in the therapeutic application of human pluripotent cell-based therapies.

CONCLUSION

This chapter has provided an overview of the different strategies that have been used to reprogram the developmental potential of mammalian cells. Clearly, the identification of simplified and more amenable reprogramming methods has rapidly accelerated advances in this field. We are still, however, far from a detailed understanding of the genomic, epigenetic, and biochemical mechanisms that regulate reprogramming. The elegant studies described herein have broken the ground, and future studies will undoubtedly continue to pave the way for the use of patient-tailored reprogrammed cells in the clinic.

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PART II

6

ADULT STEM CELLS AND THEIR ROLE IN ENDOGENOUS TISSUE REPAIR

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The use of stem cells to treat various injuries and degenerative disorders holds great potential but is also a source of controversy. The use of embryonic-derived stem cells is debated from an ethical standpoint, and, further, their value is thought to be limited due to issues of immune rejection and transformation. The use of adult-derived populations of stem cells is a promising alternative in the development of regenerative medicine strategies. The discovery of multipotent stem cells within various adult tissues has been at the forefront of stem cell biology, and their presence offers the hope of providing autologous sources of expandable, ethically sound populations of cells that can be used to treat disease or injury. One of the most exciting prospects is the use of tissue-specific stem cells through endogenous activation and recruitment *in vivo* in the absence of their isolation and expansion *in vitro* (thereby circumventing the possibility of cell transformation). In this chapter, we review various adult stem cell sources and examine the work to date using endogenous stem cell activation as a means to promote tissue regeneration.

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BONE MARROW-DERIVED CELLS

Hematopoietic Stem Cells (HSCs)

Bone marrow has been shown to contain pluripotent HSCs, a population of cells that can reconstitute and maintain all lymphoid and myeloid lineage cells over the long term (1–3). HSCs are the best studied of the tissue-specific stem cells. HSCs residing in the bone marrow microenvironment display the cardinal stem cell properties of self-renewal and multipotentiality, with the capacity to repopulate the hematopoietic system of irradiated transplant recipients for the lifetime of the individual (2–5). The phenotype of HSCs is better classified than other tissue-specific stem cells, making them amenable to purification techniques (2, 3, 6, 7) based on a combination of cell size, fluorescent dye uptake, radioresistance and resistance to cytotoxic chemicals, and cell surface markers. The HSC compartment has been shown to be almost exclusively contained within the population of cells that are $CD150^{+}CD48^{-}CD41^{-}$lineage $^{-}Sca\text{-}1^{+}ckit^{+}$ (6, 7). Following hematopoietic ablation, for example, due to irradiation or chemotherapy, single HSCs from this population can repopulate the entire hematopoietic system (3–5). HSCs are responsible for maintaining hematopoietic tissue homeostasis and their activation following injury leads to a change in their proliferation kinetics, including a change in their mode of division. The factors that stimulate HSCs include, but are not limited to, granulocyte colony-stimulating factor (GCSF) (8–10), stem cell factor (SCF) (11), and interleukin-3 (IL-3) (12, 13). Individually, these factors induce HSC proliferation and/or mobilization from the bone marrow into the peripheral blood (14), and it is clear that these molecules can act synergistically to significantly enhance these responses. Accordingly, upon activation, increased numbers of HSCs are observed in the circulation, as well as in tissues and organs in the body (15), making them available to aid in tissue repair processes. Some of the benefits of HSC mobilization and activation are discussed below in terms of the work supporting the idea that HSCs can themselves be employed to activate endogenous precursor populations found within other tissues in the body. Notably, HSC mobilization following GCSF treatment has been used in clinical trials to treat a variety of disease states (8, 10, 14, 15), including human blood disorders such as leukemia (16).

Mesenchymal Stem Cells (MSCs)

MSCs are also derived from the bone marrow, and more specifically, from stromal cells. They display the stem cell properties of self-renewal and multipotency, giving rise to bone, cartilage, fat, tendon, and muscle (1, 17, 18). MSCs are characterized by the surface marker expression of CD105, CD73, CD29, CD44, CD71, CD90, CD106, CD120a, and CD124 (17) but do not express CD45, c-kit, or Sca-1 (1) as do bone marrow-derived HSCs (3, 19). MSCs are activated by many of the same cytokines as HSCs, including colony stimulating

factors, ILs, and SCF (18). A number of studies have indicated that MSCs can respond to cues following injury and can be induced to proliferate and migrate to damaged tissues and organs, including the pancreas, skeletal muscle, heart, liver, and the central nervous system (CNS) (1, 18, 19).

CNS-DERIVED CELLS

Forebrain Neural Stem Cells

The adult brain was long thought to be unable to generate new neurons and, therefore, incapable of regeneration. This dogma was proven to be wrong in 1992 when Reynolds and Weiss (20) showed that the adult brain contained a rare population of multipotent, self-renewing stem cells. Further work revealed that the stem cells and their progeny (collectively termed precursor cells herein) reside in the remnant of the embryonic subventricular zone (SVZ), the subependyma lining the lateral ventricles in the adult forebrain (21, 22). Stem cells in the SVZ consist of a relatively mitotically quiescent population of cells (22) that undergo cell divisions once every 15 days (23). Neural stem cells divide asymmetrically to self-renew and give rise to rapidly dividing neuroblast progenitors that migrate to the olfactory bulb along a well-defined pathway termed the rostral migratory stream (RMS) (23–26). The neuroblasts integrate into the olfactory bulb circuitry where they differentiate into functional interneurons (27). The behavior and dynamics of the forebrain neural stem cell is one of the best characterized in the entire CNS, and this has led to a number of studies examining the use of these cells to repair the damaged brain. Importantly, neural stem cells have been shown to reside in the human brain as well as in the murine system where they are best understood (28). This suggests that knowledge gained from rodent models had important implications for human application.

Initial work demonstrated that precursor cells within the adult rodent forebrain could be activated using a variety of exogenous growth factors. It was found that the infusion of epidermal growth factor (EGF) (29) or fibroblast growth factor (FGF) (30) into the lateral ventricles of the adult brain resulted in a dramatic expansion of the precursor population and migration into the surrounding parenchyma (29). Moreover, the migrating cells that survived postinfusion differentiated into neurons, astrocytes, and oligodendrocytes within the forebrain parenchyma (29). In a similar manner, neural stem cells can be maintained and expanded in number in the presence of leukemia inhibitory factor (LIF) (31, 32). Hence, adult neural stem cell behavior can be modified with the use of growth factors and cytokines, and they can be activated by stimuli to proliferate and differentiate *in vivo*.

Recently, the activation of neural precursor cells in injury models has been explored. Increased numbers of stem cells were observed in the adult forebrain following stroke (33), and a number of groups have shown that stroke

injury induces the expansion and migration of neural precursors from the SVZ into the surrounding parenchyma (33–35). Neural precursor cell activation was accompanied by the differentiation of migrating cells into neurons and glial cells in the lesioned tissue; however, the degree of activation, migration, and differentiation was quite limited in terms of its contribution to the lesioned brain following stroke (35). In more recent work, the combination of injury and growth factor infusion was explored following stroke. Using a pial vessel disruption model overlying the motor and sensory cortices in rodents to induce focal ischemia, Kolb et al. (36) infused EGF directly into the lateral ventricle for 7 days, followed by erythropoietin (EPO) for an additional 7 days. The rationale was to expand the numbers and migration of precursor cells using EGF and to subsequently promote their differentiation into neurons with EPO (37). The results were striking and revealed new tissue formation at the site of the stroke lesion, the generation of neurons and glia, and functional recovery in a number of behavioral tasks. Hence, the utilization of endogenous precursor cell populations to promote self-repair has the potential to be applied to disease and injury states and appears a viable approach to promoting self-repair in the injured CNS.

Another promising venue for regeneration following stroke lies in the use of bone marrow-derived stem cells to generate an environment that is conducive to regeneration. Systemic (38) or direct injection (39) of bone marrow-derived stem cell populations results in an enhanced recovery without the use of exogenous growth factors. Using a similar methodology of intravenous cell transplantation, Matsuyama's group reported that cord blood-derived stem cells can contribute to functional recovery and suggested that the transplanted cells promoted a favorable environment at the lesion site by modulating angiogenesis (40). Importantly, these studies showed that the bone marrow-derived stem cells used to treat stroke were not turning into neural progeny within the regenerating tissue, but rather, the cells were able to activate the endogenous cells within the CNS (i.e., endothelial cells and neural precursor populations) to promote regeneration.

Multiple sclerosis (MS) is a neurodegenerative condition that leads to the demyelination of axons within the CNS. The immune-mediated loss of oligodendrocytes and myelin results in a loss of function due to poor signal transduction. Much work has been done to isolate and characterize a cell type that can remyelinate areas of demyelination in MS. It has been proposed that a quiescent population of oligodendrocyte precursors (O2A cells) exists (41–43) that can be activated following injury (43–45) and/or after the addition of growth factors such as platelet-derived growth factor (PDGF) and FGF (46). These cells undergo a switch from mitotically inactive to a more rapidly dividing cell that gives rise to functional oligodendrocytes that are capable of generating myelin at sites of injury (47, 48). However, the O2A progenitor cells are activated through a mechanism that relies heavily on symmetric divisions of the precursor cells prior to their differentiation, suggesting that the pool of precursors may be depleted over the course of a chronic disease such as MS

(41, 45, 47). In addition, it has been shown that the activation of O2A progenitors only occurs locally and that these cells are not recruited from other regions of the brain (47). These observations, taken together, suggest that O2A progenitors may be a good source of regenerative cells but may not be sufficient over the long term.

Accordingly, a number of groups have looked at the possibility of using SVZ-derived neural precursors as a source of cells to promote remyelination. Interestingly, while SVZ precursors can generate all three neural lineages (neurons, astrocytes, and oligodendrocytes), it was found that they have a propensity to produce astrocytes and oligodendrocytes in the white matter of the brain (49). Nait-Oumesmar et al. (48) reported that following a demyelinating lesion in the corpus callosum of adult mice, SVZ-derived precursors were stimulated to proliferate more rapidly and subsequently migrated to the site of demyelination. The group further demonstrated that these migrating precursors differentiated into oligodendrocytes with the potential to produce myelin. Using a different mouse model of MS, experimental autoimmune encephalomyelitis (EAE), Pluchino et al. (50) transplanted forebrain-derived neural precursors into the circulatory system to examine the potential of the transplanted cells to migrate to sites of injury and incorporate into the host tissue. They found that the transplanted forebrain-derived precursors were able to migrate into the brain from the bloodstream, through the blood brain barrier, and differentiate into myelin-producing cells in areas of inflammation and demyelination at higher proportions than in undamaged areas. Moreover, they report that the transplanted cells were able to differentiate into myelin-producing cells that remyelinated axons in the damaged areas of EAE mice (50) and led to partial recovery of the EAE-induced paralysis. Most striking and relevant to endogenous repair strategies was the observation that the vast majority of oligodendrocyte precursors that formed *de novo* were derived from endogenous neural precursor cells and not the transplanted cells. The suggestion is that the transplanted neural precursors have the capacity to activate endogenous oligodendrocyte precursors through the release of growth factors or permissive factors and thereby stimulate endogenous repair (50). Following the onset of EAE, SVZ-derived precursors underwent enhanced proliferation and were recruited from both the RMS, their normal migratory route, and the SVZ to areas of damage (49). Importantly, the cells that migrated to the injury site differentiated into glial cells (49), appropriate for the lesion site, and did not turn into neurons, normally the fate of precursors that migrate along the RMS.

Spinal Cord

Neural stem cells can be isolated from along the entire neuroaxis of the mature CNS including the spinal cord. Similar to adult forebrain stem cells, spinal cord stem cells are a rare subpopulation of slowly dividing cells found within the lining of the central canal (51–53). Different from the forebrain, spinal cord

precursor cells (SCPCs) have different growth factor responsiveness and require EGF and FGF for proliferation *in vitro* and *in vivo*. Moreover, there is no evidence that SCPCs generate new cells under baseline conditions in the adult spinal cord. Regardless, it has been suggested that the spinal cord is capable of endogenous repair via the activation of SCPCs despite the fact that they are normally quiescent in their endogenous environment (54). Studies have demonstrated that infusion of growth factors such as EGF and FGF, in the absence of a lesion, can induce the proliferation, migration, and differentiation of SCPCs into glial cells (52) in the surrounding parenchyma. The lack of neuronal differentiation was striking. Other reports have shown that following spinal cord injury and in the absence of exogenous growth factor infusion, endogenous SCPCs will migrate toward the lesion site and again, differentiate solely into glial cells (53, 55). In this scenario, astrocytes are generated that contribute to the glial scar and the formation of an inhospitable environment for tissue regeneration (53–55). Given that these same SCPCs, when transplanted in a neurogenic region of the brain such as the dentate gyrus, are capable of neuronal differentiation (56), it is likely that the microenvironment of the spinal cord is dictating the prominent astrocyte formation, thereby limiting the potential of endogenous precursor activation for tissue repair.

Transplantation of MSCs (57) or immune-derived dendritic cells (58) has been shown to stimulate endogenous SCPCs, leading to proliferation and subsequent differentiation into astrocytes and resulting in repair of the blood brain barrier as well as leading to the production of myelinating oligodendrocytes (59). Takahashi et al. (53) have shown that in the acute phase following spinal cord injury, there is an increase in markers for ependymal cells (lining the ventricular spaces), in addition to markers of SCPCs such as nestin, proliferating cell nuclear antigen (PCNA), and glial fibrillary acid protein (GFAP). Interestingly, the activation of endogenous SCPCs was not limited to the site of injury but was observed throughout the spinal cord (53). Hence, enhanced proliferation, migration, and differentiation are all mechanisms that can be supported by the environmental cues following injury. The specific molecules that support these particular aspects of activation remain to be elucidated.

Given the importance of the microenvironment in dictating the response of CNS precursor cells following injury, a great deal of work has gone into determining the factors present in the injured spinal cord that may be inhibitory to tissue regeneration in general, and neuronal differentiation specifically. A number of molecules have been shown to be particularly important in establishing and/or maintaining the hostile environment, including IL-6 (60) and chondroitin sulphate proteoglycan (61). The inhibition of either of these two pathways enables functional recovery in animal models of spinal cord injury, and this is thought to be mediated by the creation of a permissive environment for enhanced precursor proliferation and differentiation into all neural cell types. Likewise, the addition of permissive factors such as transforming growth factor β1 (TGFβ1) and neurotrophins has been shown to enhance the survival of endogenous or transplanted cells as well as axonal

outgrowth following spinal cord injury (62). These studies highlight the importance of considering combinatorial strategies that will (a) stimulate endogenous cells and (b) concomitantly permit or promote their differentiation into functional cell types.

Thus, the work described in the CNS reveals that endogenous neural precursor cells can be activated to promote tissue regeneration and functional recovery. Importantly, activation of endogenous cell populations can be achieved following administration of specific growth factors and/or cytokines or, alternatively, can be achieved following cell transplantation. While the mechanism of precursor cell activation from transplanted cell sources is not well established, it is hypothesized to be due to factors released from the transplanted cells or cell–cell contact-mediated events. What is clear is that the transplanted cells are not turning into brain-specific cell types but instead are stimulating the endogenous precursor cells. As will be illustrated below when considering other tissues, the activation of endogenous tissue-specific stem cells by transplanted cell sources represents a novel approach to facilitating endogenous repair strategies *in vivo*.

PANCREAS

The discovery of multipotent precursors in the adult pancreas raised the hope that the pancreas has the potential to regenerate throughout adulthood via stem-like precursors (63). Seaberg et al. (63) demonstrated for the first time that a rare population of cells derived from the islets and ducts of the pancreas could give rise to clonally derived colonies of cells *in vitro*. These cells were further characterized and shown to have stem cell properties, including the ability to self-renew and give rise to all cell types in the pancreas as well as neural cells, including both neurons and glia. These pancreatic precursors generated pancreas-specific β-cells that expressed insulin and C-peptide, thereby demonstrating that these precursors could give rise to functional insulin-producing cells (63), which is of particular relevance when considering endogenous repair strategies. These findings have important implications for the development of treatments for disease states that involve injury or chronic damage to the pancreas, most notably, diabetes.

Pancreatic insufficiency in β-cell function accounts for the emergence of both type I and type II diabetes. Diabetes is counted among one of the fastest-rising conditions in North America, and much work is being done to find a cure. Type I diabetes is characterized by an autoimmune disorder in which immune cells attack and kill endogenous β-cells, leaving the body unable to produce insulin. People with type I diabetes are typically insulin-dependent from childhood. Type II diabetes, also known as adult onset diabetes, results in the desensitization of cells within the body to respond to insulin, in addition to decreased insulin production by β-cells. In both instances, the production of functional, insulin secreting β-cells is the goal to reverse the phenotype of

these diseases. Importantly, human-derived pancreatic precursors have also been shown to produce functional, insulin-producing β-cells *in vitro* (64) and, therefore, are good candidates for use in pancreatic restoration.

Recent human clinical trials were performed using transplanted pancreatic precursors following a procedure that has now been termed the "Edmonton protocol" (65). This procedure involved transplanting pancreatic islet cells from cadaveric donors into patients with type I diabetes. While their results were promising, the transplanted cells were unable to maintain insulin production for periods greater than 1 year. In fact, more than 75% of patients returned to using insulin after about the second year following transplant (65). The inability to maintain insulin production from the transplanted cells was thought to be due to the exhaustion of the transplanted grafts over time. This observation would demand that patients receive more than one graft to sustain insulin independence (65, 66). The very limited availability of cadaveric tissues further highlights the importance of goal-directed research aimed at activating the endogenous pancreatic precursors *in vivo*.

Early work in pancreatic regeneration revealed that HSCs contributed to the endogenous regeneration of pancreatic tissue in nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice (67). In this model, chemically induced pancreatic damage was followed by the intravenous injection of ckit+ HSCs and resulted in the production of functional β-cells that were capable of reversing the hypoglycemia in the chemically lesioned animals. Importantly, the HSCs did not directly differentiate into β-cells (68) or produce insulin (69) but rather acted to induce the differentiation of endogenous pancreatic precursor cells into β-cells (67, 70). This activation of endogenous precursors was mediated by hematopoietic factors, such as hepatocyte growth factor (HGF) (71). The specific cell that was producing new β-cells was not immediately evident, and it was initially proposed that new cell generation was the result of increased proliferation of existing islets or the dedifferentiation of islets into a more primitive, less committed cell type (72). However, the characterization of endogenous pancreatic precursors by Seaberg et al. (63) suggested that these cells were in fact the source of new pancreatic cells, and they had been activated in response to factors released by the transplanted HSCs (67).

LIVER

Diseases of the liver often result in a chronic degeneration of hepatic tissues leading to a loss of function and the need for replacement. Until recently, it was thought that the only means of regaining liver function was through liver transplantation. The chronic lack of tissue donors and the undesired effects of long-term immunosuppression make organ donation and transplantation a complicated and less than adequate means for tissue repair. More recently, the prospect of utilizing endogenous precursor populations to repair the damaged liver has been examined in detail.

The liver is an organ that possesses the innate ability to repair itself. Endogenous, mature hepatocytes are capable of undergoing rapid divisions to repopulate damaged liver (73–76). This ability to reenter the cell cycle in response to local cues and to proliferate multiple times before differentiating into a mature hepatocyte led to the hypothesis that hepatocytes were liver stem cells (74, 77). More recently, the theory that hepatocytes are stem cells has been disregarded, primarily due to their restricted differentiation capacity and the inability to sustain self-renewal capacity. Indeed, further studies have shown that hepatocytes are fully committed, giving rise to only hepatocyte colonies *in vitro*, and cannot reverse their commitment (78), which is consistent with the notion that hepatocytes are not stem cells.

Notably, the liver has been shown to contain a rare population of precursor cells that are important in repairing the liver following moderate to severe damage, such as that induced by inflammation or partial hepatectomy (77, 79). While the mechanisms of activation are not fully understood, the hepatocyte precursor cells are thought to be composed of a rare subpopulation of the biliary duct cells. These cells are termed "oval cells" based on their morphology and can be activated in response to extensive damage or destruction of the hepatocyte population (77, 80, 81). The oval cells generate both hepatocytes and cholangiocytes *in vitro* (77, 78, 82) and undergo self-renewing divisions (78). Hatch et al. (83) have shown that oval cells express CXCR4, the receptor for stromal-derived factor-1 alpha (SDF-1α), which is released by hepatocytes in response to damage, which suggests that oval cells are activated directly by injured hepatocytes. Activation of oval cells leads to their proliferation, migration, and the subsequent regeneration of functional liver tissue (83).

Interestingly, CXCR4 and SDF-1α are known to play a key role in the migration of HSCs (84–86). This has lead to a number of studies that examined the potential role of HSCs in liver regeneration. Petersen et al. (87) took advantage of an elegant model using sex-mismatched whole, unfractioned bone marrow from male rats injected into female rats, thereby allowing for the distinction of donor versus host-derived cells. The group demonstrated that the injection of bone marrow into rats with chemically induced liver damage resulted in the long-term incorporation of donor-derived cells in the host liver and, moreover, the findings suggested that transplanted HSCs differentiated into hepatocytes. This was supported by studies showing that HSCs *in vitro* (88) and *in vivo* (89, 90) were capable of differentiation into hepatocytes, albeit at a very low frequency approaching 0.01% (77). These newly differentiated HSC-derived hepatocytes were fully functional, expressing the hepatocyte markers HNF-3β, CK19, CK18, HNF-4, HNF-1α, and secreting urea and albumin, which are hallmark indicators of hepatocyte function (88). Importantly, it was demonstrated that the lineage commitment of HSCs to hepatocytes was restricted to the HSCs that homed to the liver (91, 92) and was not a property of HSCs outside of the liver "niche." This entire "transdifferentiation" hypothesis remains hotly debated in the literature as it pertains to all tissues of the body, not just the liver.

Despite the ongoing debate concerning the potential transdifferentiation of HSCs into hepatocytes during liver regeneration, there is strong evidence to suggest that endogenous oval cells proliferate following injury and contribute to liver regeneration (77, 79). What remains undetermined is whether the oval cells are lineally related to HSCs. It has been hypothesized that HSCs found in the liver can generate oval cells, which subsequently give rise to hepatocytes and cholangiocytes (87), suggesting that the oval cell is a more committed progenitor, and not the true stem cell (77, 82). The question is still under investigation.

MUSCLE

As early as 1961, work by Alexander Mauro (93) demonstrated the existence of a population of cells that lie between muscle myofibers and their basal lamina, termed muscle satellite cells. Studies have shown that satellite cells have the capacity to self-renew and differentiate into all myogenic cell types, suggesting that they are the *bona fide* stem cells in skeletal muscle (94, 95). It was further determined that satellite cells contribute to muscle homeostasis under baseline conditions, as well as contribute to muscle regeneration following damage or degeneration (94, 95). Recent work has further elucidated the mechanisms that these cells exploit to respond to damage.

Satellite cells are a mitotically quiescent population of cells under baseline conditions (96, 97) that can be induced to proliferate in response to muscle injury (98–103). Upon activation, satellite cells divide asymmetrically to self-renew and give rise to muscle precursors (104–107). The progeny that differentiates into mature myofibers initially expresses the transcription factors Pax7 and MyoD (a key myogenic differentiation protein) and then downregulates Pax7 during differentiation (107–109). The self-renewing progeny of the division, the satellite cell, expresses Pax7 but not MyoD and returns to mitotic quiescence, thereby maintaining the stem cell pool (107, 110). Sherwood et al. (106) demonstrated with single-cell precision, that Pax7-expressing satellite cells generate colonies positive for myogenic markers at a very high frequency, supporting the role of satellite cells in muscle regeneration.

Activation of satellite cells relies on extrinsic factors that are not present in the satellite cell niche under baseline conditions. The critical factors for activation were elucidated based on the expression of specific receptors on quiescent satellite cells including c-met (for HGF) (111), syndecan-3 and/or syndecan-4 (for members of the FGF family) (111), and CXCR4 (110). Release of HGF from injured muscle has been shown to activate satellite cells, inducing their proliferation, expansion in numbers, and differentiation into myofibers for tissue repair (112). Kästner et al. (113) have shown that various members of the FGF family also aid in the regulation of satellite cells during myogenesis. Taken together, these studies suggest that the muscle "niche" can activate the endogenous satellite cells and promote self-repair. Evidence from Webster and

Blau (114) suggests that satellite cells in the muscles of patients with Duchenne muscular dystrophy are continuously being activated and recruited to chronically damaged tissue, but cannot outpace the disease, hence the degree of tissue regeneration is limited by the rate of myogenesis *in vivo*.

HEART

Myocardial infarction causes the damage and death of resident cardiomyocytes in the heart, which leads to the loss of contractile force, resulting in decreased cardiac output, poor circulation, and, ultimately, can result in heart failure. Emerging studies suggest that the heart has regenerative capability even into adulthood. One hypothesis is that the heart contains a population of endogenous cardiac stem cells (CSCs), which possess the ability to self-renew, remain undifferentiated under baseline conditions, and generate multipotent progeny that can give rise to all the cell types that comprise the heart, including myocytes and vascular cells (115–118). CSCs are characterized by the lack of expression of cardiac structural genes, Lin^- (115) and the presence of Sca-1^+, similar to HSCs (119, 120). Upon isolation and differentiation *in vitro*, these cardiac derived Sca-1^+ cells are capable of producing beating cardiomyocytes (119). Following ischemic injury such as myocardial infarction, the putative CSCs are induced to proliferate (116, 118, 121) and migrate to the damaged tissue where they contribute to myocardial regeneration and functional recovery (i.e., enhanced heart contractility) (115, 116, 120). Linke et al. (116) have shown that CSCs in the dog heart are responsive to HGF and insulin-like growth factor-1 *in vivo*. Interestingly, proliferating CSCs are more abundant in acute ischemia than in chronic degeneration (121), suggesting that they either sensitize to the disease state or lose their ability to proliferate over time. A true loss of the ability to proliferate (and therefore self-renew) brings into question whether the CSCs are definitive stem cells or whether they are a population of cardiac progenitors. Regardless, once at the site of injury, CSCs differentiate and produce functional myocardium (115, 116). It has been further speculated that CSCs have inherent plasticity based on the observation that they contribute to cardiomyocyte regeneration as well as the formation of blood vessels (115, 116). Taken together, the presence of endogenous cardiac stem or progenitor cells in adult tissue that can be activated to contribute to functional recovery stands to revolutionize our treatment strategies for cardiac disease.

MSCs have also been shown to migrate to the damaged myocardium where they differentiate and integrate into cardiac tissue (122, 123). As mentioned previously, MSCs are self-renewing, multipotent cells derived from bone marrow stroma that generate adipocytes, chondrocytes, osteoblasts, tendons, and muscle (1, 17–19). Shake et al. (122) have shown engraftment of transplanted MSCs, labeled with a cross-linkable membrane dye, into host cardiac tissue resulted in an 8% improvement in cardiac contractility compared to

uninjected controls. Similarly, Toma et al. (123) demonstrated that human MSCs are capable of differentiating into mature cardiomyocytes in the ventricles of healthy, immunocompromised SCID/*beige* mice. These studies, while still under investigation, provide further support for developing regenerative medicine strategies using easily accessible bone marrow-derived populations of stem and progenitor cells that are capable of directly integrating into damaged tissue or capable of activating the resident, tissue-specific precursor populations to promote repair and recovery.

To effectively harness the potential of tissue-specific stem cells to regenerate damaged or diseased tissues of the body, the mechanisms that drive these multipotent, undifferentiated precursors must be fully understood and evaluated. Current research indicates that somatic stem cells in various organs and systems, including the bone marrow, brain, spinal cord, pancreas, muscle, liver, and heart, are capable of being activated and recruited to sites of injury when provided with the appropriate instructive cues. The utilization of endogenous stem cells would allow for unique advances in regenerative medicine and limit the need for organ transplantation. While it appears that the resident stem cells within various tissues can be activated to proliferate, migrate, and differentiate into appropriate tissue-specific cell types, the degree to which they are able to regenerate tissue appears quite limited. This general feature across tissues suggests that regenerative medicine strategies will ultimately demand the development of combinatorial strategies to ensure that sufficient activation and tissue regeneration occurs in order to obtain functional recovery. What is clear is that autologous stimulation and recruitment of stem cells is reshaping how disease, treatment, and outcome are viewed, and leading to the reevaluation of previous tenets claiming the static nature of the body.

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7

GREATER DIFFERENTIATION POTENTIAL OF ADULT STEM CELLS

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The general definition of a stem cell is a cell that is able to multiply without senescence and differentiate toward several distinct cell types. Stem cells can be classified according to their differentiation potential. Totipotent cells can differentiate to all embryonic or extraembryonic cell types; a fertilized egg is totipotent. Embryonic stem cells (ESCs), isolated from the inner cell mass of the blastocyst, are termed pluripotent as they can generate all cells of mesoderm, ectoderm, endoderm, and the germ line, but not extraembryonic tissue. Multipotent stem cells are obtained from organs, and even though they can differentiate in general to several more specialized cell types, their differentiation capacity is more limited (1).

Functionally, a stem cell can give rise to at least one daughter cell that is identical to the initial cell, a concept known as "self-renewal," and also generate progeny with more differentiated characteristics. Somatic cells can undergo 40–50 cell doublings before reaching critical telomere shortening, also known as senescence (2, 3). By contrast, stem cells can undergo self-renewing cell divisions exceeding the normal somatic senescence (4). While ESCs have proven unlimited proliferation capacity, such extensive self-renewal is less obvious for stem cells obtained from adult tissues (5, 6).

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The existence of adult stem cells has been recognized since the 1960s (7) with the proof that bone marrow (BM) contains cells that can rescue humans and animals from BM failure (8, 9). Since then, adult stem cells have been identified in the majority of adult tissues such as the hematopoietic system (10), central nervous system (11–19), gastrointestinal stem cells (20), skeletal muscle stem cells (21), adipose tissue stem cells (22), pulmonary stem cells (23) or dental stem cells (24), and perhaps, cardiac muscle stem cells (25–27), hepatic (28), and pancreatic (29) stem cells. However, the existence of the latter three is not fully proven, and at least for hepatic stem cells, such cells could be termed facultative stem cells only present under extreme liver damage conditions.

Traditionally, adult stem cells have been categorized as cells that can differentiate only into specialized cells of the organ from which the stem cells were harvested. However, during the last decade, numerous reports have challenged the view that adult stem cells are so-called "tissue-restricted cells," as evidence has been submitted indicating that adult stem cells may have the capability to differentiate into cells of tissues other than the tissue of origin. These phenomena have been termed stem cell plasticity (21, 30–49).

CRITICISMS AND PITFALLS

Although the reports suggesting greater differentiation potential of adult stem cells have been received with great enthusiasm by the lay and scientific community, there has also been skepticism and criticism regarding the veracity of these findings. For adult stem cells to be considered pluripotent, like ESCs, several criteria need to be met, which were not shown in most publications. Criteria for pluripotency include: (a) extensive self-renewal ability without signs of senescence and (b) functional *in vitro* and *in vivo* pluripotent differentiation capability at the single cell level. Most reports on stem cell plasticity did not fully fulfill these criteria.

A number of potential technical difficulties determining the donor origin of the presumed lineage switched should also be taken into account when evaluating claims of plasticity. Retrospective identification of donor cells has been achieved by several groups in the setting of sex-mismatch transplantation. The presence of the Y chromosome in presumed lineage switched cells when male donor cells are grafted in female recipients has been used to identify donor origin of cells. Combined identification of the Y-chromosome plus a tissue-specific antigen in a single cell is an accepted method to identify donor-specific differentiation, only when one can with certainty ascribe the nucleus to a given cell. This requires confocal analysis and 3D reconstruction of the cells to exclude that the signal from one donor cell nucleus is not mistaken for another superimposed recipient nucleus. Another widely used technique to detect donor cells are genetic labeling of the grafted cells with green fluorescent protein (GFP) or β-Gal. Here too, there are potential misinterpretations. The GFP signal can be difficult to detect in tissue sections and a green

fluorescent signal can also be derived from autofluorescence from recipient cells. However, careful analysis of the emission spectrum, which is different for autofluorescence and GFP, can discriminate between the two. The gene marker could be silenced, then not permitting detection of possible donor-derived cells that are engrafted and/or are differentiated (50). Today, maker genes are commonly expressed through a tissue-specific promoter, which allows detection of donor origin of the cell combined with specific tissue differentiation as well.

Morphology, phenotype, and function are three parameters needed to validate cell differentiation. However, many studies have demonstrated the differentiation potential of a cell exclusively based on morphology and phenotype criteria without demonstration of functionality. Morphology and phenotype alone are, however, insufficient to demonstrate lineage differentiation as many intracellular and membrane antigens can be found on multiple cells. For example, the CD34 antigen is not only present in hematopoietic cells (51) but is also expressed by angioblast and liver precursors (52, 53). Antigen expression is sometimes unstable and may be lost when cells become activated or proliferate (54). Furthermore, cells can uptake proteins such as insulin or albumin, *in vitro* and *in vivo*, from their microenvironment (55). The only proof for function is that the grafted cells and their progeny can rescue the function of a failing organ, which in many instances of presumed lineage switch was not proven.

To prove plasticity, it is also important that it is demonstrated that a single cell gives rise to cell types of the expected tissue as well as unexpected cell types. Researchers have used several methods to demonstrate clonal origin differentiated progeny, including limiting dilutions, cloning rings, single-cell deposition by fluorescence-activated cell sorting (FACS) or retroviral marking and characterization of the viral insertion site in the host cell genome. It is estimated that the probability that two retroviral sequences would insert in the same site is less than 1/10 000–1/100 000 (56, 57). Retroviral insertions and, to a more limited degree, single cell deposition by FACS, can demonstrate clonality. By contrast, the use of cloning rings or limiting dilutions are not considered proof of clonality. *In vivo*, single cell transplantation or viral marking is vital to determine the derivation of differentiated progeny. Although this represents a formidable task, in 2001, Krause et al. reported that a single homed hematopoietic stem cell (HSC) differentiated and engrafted not only into the hematopoietic system, but also into the lung epithelia, liver, gastrointestinal track, and skin, even though evidence for acquisition of functional characteristics of the cells in epithelial tissues was minimal (41). In 2002, Jiang et al. injected a single adult stem cell labeled with a reporter gene (LacZ), into the blastocyst and found contribution to the formation of most of the tissues formed in the chimera (46). It should be noted, however, that other similar cell lines failed to contribute robustly to chimeric animals following injection in the blastocyst (unpublished data).

A final criterion for stem cells deduced from the field of hematopoiesis is that a stem cell can self-renew. When applying this criteria to the field of stem

cell plasticity, this would require that one demonstrates either that a single cell gave rise *in vitro* to two equivalent cells that each can generate *in vivo* the expected tissue and the unexpected tissue. Alternatively, grafted cells capable of generating the expected and unexpected tissue can be recovered and grafted in a secondary animal, where they again demonstrate to have the ability to generate the expected and unexpected tissue. Few studies to date have been published that this is possible.

MECHANISMS FOR ADULT STEM CELL PLASTICITY

Another area of extensive discussion in the field of stem cell plasticity has been what mechanism(s) are underlying the perceived unexpected differentiation ability of stem cells. The first mechanism that could explain the plasticity phenomenon is heterogeneity of the cells used during experimental studies. When a nonclonal cell population is grafted, it is possible that several distinct progenitors with varied differentiation potential are cotransplanted. It is well known that stem cells from a given tissue may exist in an unrelated tissue. For example, HSCs are found in muscle (58, 59), and cells with hepatic characteristics have been found in the BM (60). Hence, transplantation of mixed populations of muscle-derived cells or BM cells may give the false impression that muscle cells can acquire a hematopoietic fate and BM cells a hepatic fate, where the real observation is that two cells present in each given tissue differentiate only in the expected progeny.

Second, transdifferentiation is the ability of mature or specialized stem cells to be reprogrammed toward a totally different cell type. This transdifferentiation can be direct or require a dedifferentiation step followed by an alternative maturation or specialization (redifferentiation) step. *In vivo* examples for such a phenomenon are, for instance, Urodeles, which can regenerate amputated limbs through a process of dedifferentiation of the cells located at the border zone of the amputated area, forming a blastema, from which redifferentiation in all cell types of the amputated limb occurs. These cells activate a process reminiscent of developmental development (61). Many other examples exist. *In vitro*, pancreatic epithelia can alter their phenotype to hepatocytes (62, 63). Similarly, when animals are placed on a copper-deficient diet, pancreatic epithelial cells appear to transdifferentiate into hepatocytes. Oligodendrocyte progenitor cells can be reprogrammed to neuronal cells when they are maintained at low density without serum *in vitro* (64). When Msx1 is expressed in C2C12 cells, a cell line that can give rise to myotubes, myotubes dedifferentiate and can redifferentiate not only into myoblast, but also into osteoblasts, chondrocytes, and adipocytes (65). Activation of RE-1 silencing transcription factor/neutron-restrictive silencer factor (REST/NRSF) target genes with a single transgene, REST-VP16, is sufficient to override the muscle differentiation pathways, and to activate terminal neuronal differentiation genes and triggering neuronal differentiation in myoblasts (66). Fibroblast can be repro-

grammed to activate T cells-specific genes by adding T cell protein extracts (67). Without any doubt, the recent studies wherein transdifferentiation of murine fibroblasts was achieved by ectopic expression of four transcription factors, Oct-4, Sox2, c-Myc, and Klf4, to confer a pluripotent state to cells that have been termed induced pluripotent stem (iPS) cells, provides the most definitive proof that transdifferentiation can occur under certain circumstances wherein specific transcription factors are activated. iPS cells exhibit the morphology and growth properties of ESCs. When grafted under the skin, iPS cells give rise to teratomas, and when injected in the blastocyt, iPS contribute to all somatic and germline lineage of the developing embryo, giving rise to germline chimeras (68–71). However, the degree of similarity with embryonic stem (ES) varied depending on the selection approach used. When the iPS cells were selected for the expression of Fbx15, there were notable differences with ES at the level of their transcriptome expression, DNA methylation pattern, and the formation of adult chimeras. On the other hand, when iPS generated from mouse embryonic fibroblasts were selected based on the endogenous expression of the pluripotent transcription factors Nanog or Oct-4, gene expression, DNA methylation, chromatin state, and formation of viable adult chimeras were highly similar to ESCs.

Tada et al. were the first to demonstrate that hybrid cells resulting from ESC and T cell fusion undergo nuclear reprogramming (72). Fusion with ESC resulted in the partial reactivation of the inactivated X chromosome and unstable Xist transcription in T cells, and the hybrid cells contributed to mouse chimeras. Two other independent groups, fused ESC with BM cells and neurospheres producing ESC-like hybrid cells, wherein the hematopoietic program and neural program, respectively, were eliminated (73, 74) Although the frequency of fusion events was low ($1/10^4$–10^5), the resultant ES formed embryo bodies *in vitro* and were able to contribute to some tissues when injected in the blastocyst. It follows from these observations that fusion between cells can impact the genetic program of one cell in favor of the other cell to which it fused. Similar observations have now also been made between two specialized cells *in vivo*. The first evidence for fusion event *in vivo* comes from a study by Lagasse et al. who initially demonstrated that crude BM populations as well as highly purified HSC (Kit^+ $Thy1^+$ Lin^- $Sca1^-$ cells or KTLS cells) could repopulate not only the hematopoietic system, but also the liver of animals with hereditary tyrosinemia (HT1), caused by a deletion in the fumarylacetoacetate hydrolase gene, and could rescue the animals from an otherwise lethal liver failure syndrome (37). However, a number of subsequent studies by this group and others demonstrated that the liver repopulation was not HSC autonomous, but required fusion between monocytic progeny from HSC and hepatocytes (75–78). Since then, other groups have published elegant studies in which fusion events were responsible for the apparent plasticity of BM cells in other nonhematopoietic tissue like the cardiomyocytes, skeletal muscle cells, hepatocytes, and Purkinje cells in the brain (79).

Even though many studies have now indicated that plasticity may at least in part be due to fusion events, other studies have shown that low levels of apparent transdifferentiation may also occur without fusion, including in the heart, the liver, and the brain. However, in view of the now well-acknowledged possibility that transdifferentiatioin is not cell autonomous but may be due to fusion, it is crucial that all possible methods are exhausted to demonstrate direct transdifferentiation versus fusion-based transdifferentiation. Demonstration that the transdifferentiated cell is not due to fusion can be obtained in sex-mismatched transplantation, demonstrating the presence only of the donor genotype (for instance XX) but not the host genotype (for instance XY). Alternatively, cells can be harvested from the animal, cultured *in vitro* with demonstration of a 2N genome, not more than 2N. Yet another method that has been used successfully to prove or disprove fusion has used a Cre-lox recombination system to detect cell fusion events. It cannot be forgotten that fusion occurs in nature and that fusion between donor and host cells could be exploited to develop therapies. Fusion between the egg cell nucleus and the spermatozoid is the initial event during vertebrate development. In the adult, cell fusion occurs as a common event during the generation of multinucleated skeletal myofibers from myoblasts (80). It is therefore not out of the question that fusion, for instance, between monocytes and skeletal muscle fibers would be one method to introduce missing genes in muscle tissue, as an option for therapy of muscular dystrophy, provided that the fused cell does not pose a potential threat to the host, such as, for instance, cytogenetic instability.

The final explanation would be that apparent plasticity is due to remaining cells with pluripotent capacity as vestiges from the embryonic development. However, full proof evidence for this hypothesis is currently still lacking. However, as will be discussed below, a number of investigators have now isolated in cultures of somatic or testicular tissue, cells that have the ability to differentiate subsequently into cells of mesodermal, endodermal, and ectodermal cell types. Some of these cell lines also generate teratomas and some can contribute to chimeric animals. This could suggest that cells with greater differentiation potential were present in the tissue used to isolate the cells. An alternative explanation is that the culture conditions imposed on cells induced activation of an earlier genetic program endowing the cells with the greater differentiation potential. Until it is possible to prospectively isolate a cell with such potential, without the intervening culture step, the question as to the mechanism of the greater potency of such a cell *in vivo* remains unanswered.

GREATER POTENTIAL OF ADULT STEM CELLS

In 2002, we described the isolation in culture of multipotent adult progenitor cells (MAPCs) (46, 47). Since then, MAPCs have been isolated in BM cultures from mouse (47), rat (46), human (43, 44), and other large mammals

such as swine (81). Unlike most adult stem cells, MAPCs expand without obvious telomere shortening for >100 population doublings (PDs). Mouse multipotent adult progenitor cells or (m)MAPCs are class I and II major histocompatibility complex (MHC) negative, CD44, CD45, Thy1, and Sca1 negative, and SSEA1 dim, and PDGFR-A and cKit positive. Human multipotent adult progenitor cells or (h)MAPCs are class II MHC and CD45 negative and MHC-I and CD44 dim. Using retroviral marking, we demonstrated that single MAPC differentiate into all mesenchymal cell types (44), endothelium, and cells with neuroectodermal and endodermal characteristics (43, 49, 82). When injected in the blastocyst, some mMAPC lines contributed to many somatic tissues in up to 30% of mice (46). MAPC also contributed to hematopoietic cells and some epithelia when grafted in nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice (83). Since the initial description, changes have been made to the isolation method in that we now isolate MAPC at 5% O2. Culture at 5% O2 resulted in the isolation of more clones of MAPC with significantly higher levels of Oct-4 (83, 84). Some mouse and rat MAPC clones express Oct-4 mRNA and protein at levels between 10% and 100% of those in mouse ESC (mESC). MAPC also express EPAS1 and Sall4, upstream regulators of Oct-4 and a number of the ECAT genes identified to be relatively uniquely expressed in ESC. However, transcripts for Nanog and Sox2 are low or undetectable. High Oct-4 MAPC differentiate much more robustly into arterial, venous, and lymphatic endothelium (85, 86), hepatocytes (manuscript in preparation), and contribute robustly to the hematopoietic system when transplanted in sublethally irradiated NOD/SCID mice (83). Since the isolation of MAPC, a number of cells with similar extended potency have been isolated from BM, umbilical cord blood (UCB), amniotic fluid, liver, and heart. For most of these, the culture conditions are similar although not identical.

Human BM-derived multipotent stem cells (hBMSCs) are clonally isolated from human BM. This cell can be expanded for >140 PDs without telomere shortening, in media containing 17% fetal bovine serum (FBS) on fibronectin coated plates, and at cell densities between 4000–8000 cells/cm^2. Clonally expanded hBMSCs are less than 20 micron in diameter and exhibit a high nucleus-to-cytoplasm ratio. The cell surface markers phenotype of hBMSC is notably different from mesenchymal cells or HSC found in the BM. hBMSCs are negative for CD4, CD8, CD11b, CD13, CD14, CD29, CD30, CD31, CD34, CD44, CD45, CD49e, CD71, CD73, CD90, CD105, CD117, CD133, CD146, human leukocyte antigen-Class I (HLA-ABC), and human leukocyte antigen-Class II (HLA-DR). In addition, hBMSCs do not express the pluripotent transcription factor Oct-4. *In vitro*, these cells differentiate to cells with phenotype of endothelial, smooth muscle, neural, and hepatic lineages. Also *in vitro*, coculture of hBMSC with neonatal rat cardiomyocytes induces cardiomyogenic differerentiation. *In vivo*, hBMSCs regenerate the myocardium by engrafting into an ischemic heart and differentiating to cells with a phenotype of cardiomyocytes, endothelial cells, and smooth muscle cells. However, it is not clear whether

the conversion of hBMSC to cardiomyocytes *in vitro* or *in vivo* is cell autonomous and not the result of fusion between hBMSC and cardiac myocytes.

Marrow-isolated adult multilineage inducible (MIAMI) cells, isolated from human BM initially with 5% FBS and subsequently maintained with 2% FBS on fibronectin coated plastic at 3% O2, can be expanded for >50 PD when cultured with 15% FBS, and at cell densities ranging from 1300–1400 cells/cm^2. MIAMI cells express high levels of CD29, CD44, CD49e, CD63, CD81, CD90, CD103, CD122, CD164, CNTRF, cMET, Flt1, KDR, BMPR1B, and NTRK3, and are negative for CD34, CD36, ICAM1, N-CAM, CD45, CD109, cKit, HLA-ABC, and HLA-DR. In addition to these cell surface markers, MIAMI cells also express telomerase (hTERR) and the pluripotent transcription factors Oct-4 and Rex-1. MIAMI cells differentiate *in vitro* to bone-forming osteoblast, cartilage-forming chondrocytes, and fat-forming adipocytes as well as cells with phenotypic features of neural cells and spherical clusters of cells expressing pancreatic genes (87–89).

Unrestricted somatic stem cells (USSC) are isolated from human UCB. These cells display fibroblast morphology, and are negative for CD14, CD33, CD34, CD45, CD49b, CD49c, CD49d, CD49f, CD50, CD62E, CD62L, CD62P, CD106, cKit, Glycophorin A, and HLA-DR, and they are positive for CD10, CD13, CD29, CD44, CD49e, CD90, CD105, vimentin, CK8, CK18, human Endo, KDR, and HLA-ABC. USSC can be expanded for more than 40 PDs, maintaining their telomere length and a normal karyotype. *In vitro*, USSC differentiate into osteoblast, chondroblast, adipocytes, and hematopoietic and neural cell with phenotypes of astrocytes and neurons expressing neurofilaments, sodium channel proteins, and neurotransmitters. *In vivo*, USSC differentiate into ectoderm, endoderm, and mesoderm in uninjured models. *In vivo* human bone reconstitution and chondrogenesis was appreciable after transplantation of USSC-loaded calcium phosphate cylinders in nude rats, and after transplantation of USSC-loaded gelfoam sponges into nude mice, respectively. Following their transplantation into an intact rat brain, human tau positives cells were detected up to 3 months afterwards. When USSC were transplanted into a preimmune fetal sheep, contribution to 5% of the hematopoietic system was seen, as well as contribution to 20% of the albumin-positive cells in the liver parenchyma and the presence of some apparently human cardiomyocytes in both atria and ventricles of the sheep heart (90–93).

Multipotent renal progenitor cells (MRPCs) are isolated from rat kidneys. MRPCs possess a spindle-shaped morphology and can be clonally expanded using general MAPC protocols. MRPCs express specific antigens for vimentin, Oct-4, Pax2, CD90, and CD44, and they are negative for cytokeratin, SSEA1, NCAM, CD11b, CD45, CD31, CD106, HLA-ABC, and HLA-DR. Some of the MRPC clones express high levels of Oct-4 and can maintain their self-renewal state for more the 200 PDs without evidence for senescence or apparent chromosomal abnormalities. *In vitro*, MRPCs can be differentiated to endothelial, hepatocyte, and neural cells. *In vivo*, these cells engraft and functionally dif-

ferentiate to renal tubular epithelium in the presence or absence of kidney injury (94).

Multipotent adult spermatogonial-derived stem cells (MASCs) are isolated from human BM, heart, and liver, using initially Mesencult media followed by 2% FBS containing "MAPC" medium. The cells can be clonally expanded for more than >40 PDs with significant levels of telomerase activity and keeping a normal human diploid DNA content. MASC from BM, heart, or liver all express CD13, CD49b, CD90, CD73, CD44, HLA-ABC, CD29, CD105, KDR, CD49e, and are negative for CD14, CD45, CD38, HLA-DR, CD133, cKit, and CD3, and express Oct-4, Rex-1, and Nanog. *In vitro*, MASC can differentiate to cells with phenotypic and functional features of endothelium, neurons, and hepatocytes. Notably, irrespective of the source from which the MASCs were isolated (BM, heart, or liver), the authors observed a common gene signature expression for maintenance of undifferentiated state, cytokines or growth factor, extracellular matrix-related, cell adhesion molecules, and genes related to the Wnt, Hedgehog, and Notch pathways (95).

Amniotic fluid-derived stem (AFS) cells are prospectively isolated from human and rodent amniotic fluid by immunoselection of cKit positive cells, and can be extensively clonally expanded for more than 250 PDs, maintaining long telomeres and a normal karyotype. These cells express HLA-I, CD29, CD44, CD73, CD90, and CD105. AFS cells are negative for HLA-DR (weak positive in some cases), CD34, CD45, or CD133. In addition, AFS cells expressed some embryonic cell markers like SSEA4 and the transcription factor Oct-4, but they were negative for the expression of other embryonic cell markers like SSEA3, Tra-1-81, or Tra-1-60. Retroviral marked clones show multipotent differentiation capacity and can functionally differentiate *in vitro* to mesenchymal, endothelial, neuronal, and hepatic lineages. *In vivo*, predifferentiated AFS cell can form bone after subcutaneous implantation (96). More recently, it has been reported that AFS cell can differentiate to functional endothelial cells, smooth muscle cells, and even cardiomyocytes in coculture with rat neonatal cardiomocytes. However, the authors, encouraged from the promising *in vitro* differentiation data, transplanted AFS cell into myocardial infarctions of Sprague-Dawley rats and rUN (immunodeficient rats), and observed a strong rejection in the myocardium of normal, ischemic, immunosuppressed, or immunodeficient rat (97, 98).

Anjos-Alfonso and Bonnet also prospectively isolated a nonhematopoietic/nonendothelial SSEA-1pos (0.5%–1% of all MSC) subpopulation from the mesenchymal compartment of BM, denominated as Pre-MSC. Pre-MSCs are isolated based on negative depletion of CD45/CD11b cells from adherent cultures or Lin/CD45/CD31 depletion from whole BM. These SSEA-1pos cells express Oct-3/4, Nanog, and Rex-1. The SSEA-1pos cells could also be sorted from uncultured BM and also express Oct-4 and Nanog mRNA and protein. These cells functionally differentiate at the clonal level into cells of the three germ layers *in vitro* and to multiple mesodermal cell types, including

osteoblasts, chondroblasts, endothelial cells, and hematopoietic cells *in vivo*. Of note, only when MAPC culture conditions were used could the cell phenotype and differentiation capabilities be maintained *in vitro* (99).

Similarly, very small embryonic-like (VSEL) cells have been prospectively selected using multiparameter cell sorting to obtain enriched populations with CXCR4, cKit, and SSEA4 (human)/SSEA1 (mouse) from mouse BM or human UCB. Freshly isolated VSEL express the pluripotent transcription factors Oct-4, Nanog, and Rex1, and markers of tissue committed stem cells like MyoD, glial fibrillary acidic protein (GFAP), and alpha feto protein (AFP). Murine VSEL are present in the side population of BM cells, enriched in BM of young mice, and they can differentiate to cells expressing transcripts of cardiac, neural, and endoderm cells upon coculture (100, 101).

Two other cell populations definitely merit discussion, as they are the most akin to true pluripotent ESC. In 2006 Guan et al., described how spermatogonial stem cells (SSCs) can be isolated from adult mouse testis using genetic selection. These isolated SSCs respond to ESC-like culture conditions and acquire ESC properties. The cells obtained under these conditions are named multipotent adult germline stem cells (maGSCs). maGSCs are able to spontaneously differentiate into specialized cell types of the three embryonic germ layers *in vitro* and generate teratomas after their transplantation in immunodeficient mice. When injected into an early blastocyst, SSCs contribute to the development of various organs in mouse chimeras and show germline transmission (102). Most recently, Seandel et al. have been able to isolate highly proliferative adult spermatogonial progenitor cells (SPCs) by cultivation on mitotically inactivated testicular feeders. When maintained *in vitro* in a long-term culture with mouse embryonic fibroblasts (MEF), SPCs give rise to MASCs. The authors could prospectively isolate SPCs based on the expression of GPR125, a cell surface marker found in adult testis. GPR125+ MASCs generated from the SPCs differentiated into different tissue-specific cell types from the three germ layers and contributed to many tissues in mouse chimeras, although germline transmission was not shown. In addition, MASCs also differentiated functional blood vessels *in vivo* (103).

In general, the cell populations described above fulfill many of the criteria we posed for demonstrating stem cell plasticity: (a) a single cell differentiates into multiple cell lineages; (b) differentiated cells are functional *in vitro* (and *in vivo*); and (c) at least for some of the cell populations, engraftment is robust and persistent. In addition, most somatic tissue derived share a common phenotype, absence of some cell surface antigens known to be expressed by MSC and HSC, and expression of some markers related to the pluripotent ESC (e.g., Oct-4 or SSEA).

As discussed previously, it remains unknown whether the differentiation potential attributed to adult stem cells with greater potential is actually present in the uncultured cells and hence represents cells with greater potency persisting into postnatal life, or if the differentiation potential bestowed on the cells is created in culture as a result of dedifferentiation. It should be noted that

Anjos-Afonso and Bonnet also isolated SSEA1-negative cells, which when cultured under MAPC conditions did not acquire Oct-4 or SSEA1 expression, suggesting that if the greater potency is induced *in vitro*, this is not possible for all cells. Likewise, Ulloa-Montoya et al. cultured MSC under MAPC conditions and were unable to induce an MAPC phenotype in the established MSC (104). Pre-MSC, VSEL, and AFS cells can be prospectively isolated directly from fresh BM or amniotic fluid, and these cells express already the ESC transcription factors Oct-4 (and Nanog and Sox2), which could be used as another argument for cells with greater potency remaining in postembryonic stages of development. However, Oct-4 levels increased when SSEA1+ cells were cultured under MAPC conditions, which could indicate that increased potency is acquired following *in vitro* culture. The answer to whether cells with a greater potential do or do not exist *in vivo* has not only an academic relevance but also biological and potential clinical implications. Indeed, if such cells are in fact remaining in postnatal mammals, they could be induced to proliferate and differentiate *in vivo* to affect certain diseases, without the need for *ex vivo* expansion. Alternatively, if the potency is acquired *in vitro*, it will be important to fully understand the reprogramming events that occur to ensure that inappropriate molecular events that could lead to malignancies do not occur.

Despite the progress, significant barriers remain to be overcome in order to establish stem cell-based therapies in the clinical setting. From now on, efforts will be directed on solving the difficulties with routine and efficient isolation of these adult stem cell populations, and standardization of quality control studies.

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8

CANCER STEM CELLS

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The cancer stem cell (CSC) hypothesis has, over the past several years, enjoyed an intense resurgence of interest from investigators probing the underlying mechanisms of carcinogenesis. Although originally proposed to exist in the late 1960s (1), the concept of tumor stem cells as an underpinning force of tumor biology was forgotten, and the technology to identify and isolate these cells was nonexistent, until recently (2–4). The CSC theory posits that only stem cells persist long enough to accumulate the set of mutations required to tip normal differentiation in favor of abnormal growth, and hence spontaneously arising human tumors likely arise from deregulation of stem or progenitor cell populations. In this paradigm, tumors are best conceptualized not as a clone of unstable cells with aberrant growth control, but more nearly as normal development gone haywire. More to the point, many cancers appear to result from stem cells that anchor the base of a differentiation program responsible for tissue regeneration and homeostasis, but have lost essential constraints on growth, differentiation, and/or localization.

Stem cells have the unique ability to both self-renew and generate all cell types of their denoted lineage. The term "self-renewal" implies that cell division must result in at least one daughter cell with equally potent potential. In normal tissues, self-renewal is usually offset by asymmetric cell division, or a symmetric cell division event wherein both daughter cells are committed to

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differentiate, resulting in progeny no longer able to self-renew (Fig. 8.1a). A suggested major difference between normal and neoplastic tissue is that this equilibrium is tilted in favor of symmetric, self-renewing cell divisions in cancer (Fig. 8.1b) (5). Whereas nonself-renewing progenitor cells or terminally differentiated cells usually constitute the majority within tissues and tumors, CSC may exist at the progenitor cell level, as these cells can acquire transformation events that confer self-renewal properties to an already proliferative and possibly multipotent cell type (Fig. 8.1c) (6, 7). Such changes generally result in aggressive, sometimes amorphous tumors. Together with the belief that CSCs underlie perpetual tumor growth, the CSC paradigm also holds the view that these cells are inherently more resistant to chemotherapy and radiation. These principles are slowly amassing support and might easily explain the refractory nature of many cancers to therapy (8, 9).

Cell lines derived *in vitro* from patient tumors and/or generated via oncogene overexpression or tumor suppressor knockdown have long facilitated cancer research; however, it is becoming increasingly clear that these lines differ from primary tumors in patients and may provide little insight on the biology of primary tumors (10–12). Conversely, CSC from minimally passaged, patient-derived xenogeneic tumors can generate heterogeneous tumors that may more closely resemble those *in vivo*. Because CSCs are functionally defined by their ability to self-renew and the ability to spawn tumors phenotypically and morphologically identical to parental tumors, both of which can only truly be assessed in a xenograft setting (13), overcoming the challenges to obtaining and experimentally probing these cells is paramount to understanding cancer.

Because conclusive evidence as to the existence of CSCs can only be delineated retrospectively by their ability to fuel tumor growth *in vivo*, techniques and instrumentation that facilitate isolation of healthy cells are paramount (13). Hematologic malignancies are among the best understood of the neoplastic diseases precisely because hematopoietic cells are easy to obtain and the *in vivo* and *in vitro* assays to determine the fate and potential of these cells have been developed. Similarly, the field of solid tumor biology has entered an era where not only can the cells responsible for fueling tumor growth be identified, but they can be isolated and their characteristics tested both *in vivo* and *in vitro*. There exists a number of technical hurdles that must be overcome before CSC can be thoroughly examined (13). First and foremost, obtaining CSC from epithelial tumors is not trivial. Isolation of single cells is critically important when experimentally attributing potential of one cell versus another. Cell dispersion protocols and the ability to discriminate clumps of cells are vital for clean analyses. Unlike hematopoietic stem cells (HSC), which are loosely adherent to other cells and can be obtained by flushing the bone marrow or sampling mobilized peripheral blood, solid tumor cells must be enzymatically digested to free them of their tight association with other cells and matrices within the tumor. This is not unproblematic given that harsh digestion conditions can kill cells and/or cleave surface proteins that might be

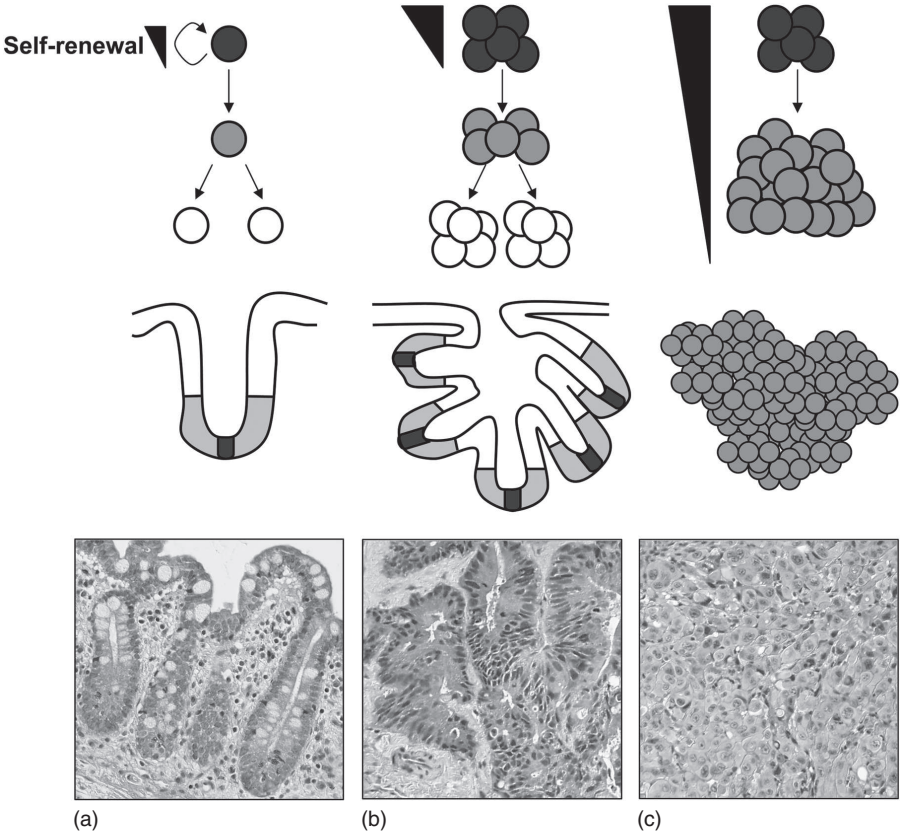


Figure 8.1. Colorectal-centric view of tumor progression. (a) Normal tissue-resident stem cell populations, such as the colon stem cell (red), which is localized at the base of the crypt, have both the unique ability to self-renew (relative ability indicated by a black triangle) and to generate the differentiated progeny that comprise this tissue. Progeny of the colon stem cell include highly proliferative transit amplifying cells (progenitor; gray) and terminally differentiated enteroendocrine or goblet cells (white). (b) In tumors where the CSC lies at the level of the tissue-resident stem cell, increased symmetric division fates result in expanded stem cell numbers and thus, a similar expansion of their resulting progeny. This sudden increase in cells vying for space within a tissue may manifest as an adenocarcinoma. (c) Aggressive, end-stage carcinomas might result from the gain of self-renewal capacity by an already proliferative progenitor cell pool with limited to no differentiation capacity, often resulting in amorphous, largely undifferentiated tumors. Hematoxylin and Eosin stains show histopathology of (a) normal colon crypts, (b) adenocarcinomas, and (c) aggressive, poorly differentiated carcinomas of the colon, each of which might reflect the cellular identity and self-renewal capacity of the stem cell population. (See color insert.)

subsequently used for discriminating and isolating CSC subpopulations. Furthermore, many solid tumor cells are adhesion dependent and undergo anoikis when ripped from their niche (14). Second, because CSCs constitute a small fraction of the overall tumor, reagents to precisely identify tumorigenic versus nontumorigenic cells are required. Third, better instrumentation to gently and accurately interrogate and/or isolate individual cells is needed. Finally, *in vitro* culture conditions that allow assessment of tumorigenic potential would greatly facilitate assays that quantify CSC and/or assess therapeutic efficacy without relying exclusively on murine xenograft transplants. These obstacles and the current or emerging technologies addressing them will be herein discussed.

DEFINING CSC IDENTITY

The initial isolation and characterization of a CSC was made in a hematopoietic malignancy; specifically acute myeloid leukemia (3). The essential ingredient enabling this advance was the knowledge base built upon the characterization of the normal HSC and its downstream progeny (15). Several factors contributed to the timely characterization of HSC versus stem cells from other tissues. First, appreciation of the unique characteristics inherent to HSC, such as their ability to contribute to long-term hematopoiesis, was coupled with the means of assessing this in a tractable, if not rapid, manner through transplantation into surrogate hosts made terminally leukopenic by irradiation. These transplants offered a rigorous path towards defining the true identity of these cells by asking them to fulfill their destiny, while providing the environment in which they could do so. Second, a body of knowledge surrounding normal hematopoiesis had been built up by identifying differentiated hematopoietic cell subsets, including B and T cells, and the various myeloid lineages. This work created a rich resource of well-characterized cell surface markers useful for discriminating these cell types, and also fueled the tremendous technological advancements in flow cytometry (16). The robust capacity of flow cytometry to define and isolate minor populations of viable leukocytes greatly aided the advancement of immunology and enabled efforts to define those cells capable of generating all the lineages that constitute blood.

Thus, in 1994, the conditions were ripe for the appreciation of CSCs in leukemia, leukemic stem cells (15). The fundamental assay of achieving long-term growth in a surrogate leukopenic host was, in straightforward fashion, adapted as an assay for determining the identity and potential of leukemic cell subpopulations, enabling the discovery of the leukemic stem cell. Knowledge of normal HSC surface markers, and those of its progeny, were then leveraged to query the features of CSC in other hematopoietic disorders (3, 17). For example, similar work later revealed that those cells driving chronic myelogenous leukemia (CML) had the phenotypic properties of normal HSC, but harbored the Philadelphia chromosome (t9:22; p210-BCR/ABL) (18). More

recent studies have shown that progression to acute blast crisis in CML is accompanied by a shift in the identity of the leukemic stem cell from the HSC to the common myeloid progenitor (7). Because the phenotypic identity of these cells was known, it was possible to isolate them at high purity and determine the molecular abnormality associated with disease progression (i.e., nuclear β-catenin). Studies such as these contributed to the profound shift in our perception of cancer and heralded in a new era of cancer research.

Nevertheless, the very foundations that enabled the initial appreciation of CSC in leukemia actually posed both technical and conceptual hurdles for the broader appreciation of CSC in other tumor types. Despite serious deliberation as to the presence of tissue-specific stem cells (19, 20), their existence in epithelial tissues such as the lung, breast, prostate, and skin were not demonstrated until recently (21–24). Given that these tissues contribute heavily to the overall morbidity of cancer, lack of knowledge as to the extent and role of normal stem cells in tissue homeostasis was a profound block to the broader appreciation of the fundamental role of CSC in cancer. The robust assay that drove characterization of HSC (i.e., long-term repopulation capacity in a surrogate host) is far less readily realized for other adult stem and progenitor cell populations; thus, the lack of progress in defining markers that enable tissue-specific stem cell isolation. This absence of phenotypic knowledge has, in turn, hampered detailed molecular and functional characterization of these cells.

It was recently recognized that the assay format used to identify leukemic stem cells (i.e., long-term growth in a surrogate host) could also be exploited with CSC isolated from solid tumors (25, 26). This is likely facilitated by an underlying relaxation of the stem cell niche requirements by CSC, which normally constrain the location and behavior of adult stem cells. Details regarding both the niche and the stem cells that reside in them are poorly advanced (27). However, it has been found that CSCs, like metastatic disease *in vivo*, are generally able to flourish in nonorthotopic locations. This relaxation of niche requirements has enabled the characterization of CSCs to surpass that of their normal stem and progenitor cell counterparts, in effect turning the order of discovery on its head relative to the way progress was made in the hematopoietic field. This has had a multitude of consequences. Notably, progress in the characterization of solid tumor CSC has not benefited from knowledge of cell surface markers defining analogous normal stem cells or their more differentiated progeny. Instead, research has had to confront the "chicken and egg" quandary of being unable to isolate the CSC until markers are known, while not knowing the proper markers in the absence of having first isolated the CSC. The first demonstration of CSC existence in a solid tissue cancer solved this dilemma using an empirical search strategy (25). Essentially, candidate markers were individually tested to characterize the tumorigenic capacity of those cells that exhibited heterogeneous expression, thus fractionating cancer cells into those either possessing or lacking the ability to drive long-term tumor growth. Following this early work, several markers were identified that help enrich the CSC from bulk tumor populations (Table 8.1). Among

TABLE 8.1. CSC Markers

Origin of Tumor	Enzymatic Treatment	Markers	Reference(s)
Breast	Collagenase III, DNAse	$ESA^+CD44^+CD24^-$	(25)
Colon	Collagenase III, DNAse	$ESA^+CD44^+CD166^+$ $CD133^+$	(28, 30, 31)
Prostate	Collagenase, trypsin	$CD133^+$ $CD44^+\alpha2\beta1^{hi}CD133^+$	(21, 34)
Pancreas	Collagenase IV	$ESA^+CD44^+CD24^+$	(32)
Head and neck	Collagenase III	$CD44^+$	(33)
Brain	Collagenase, hyaluronidase, trypsin	$CD133^+$	(29)

these markers, CD44 and CD133 have had prominent success (21, 25, 28–34). CD44, in particular, has proven to have considerable utility. First recognized as useful for fractionating tumorigenic cells in breast cancer, CD44 has subsequently been proven useful for similar purposes in neoplasias of the colon, head and neck, prostate and pancreas (25, 28, 32–34).

While the initial discovery of markers that enable enrichment of CSC from various solid tumors has established a foothold in our efforts to characterize these cells, much remains to be accomplished before we understand CSC well enough to develop effective therapeutic intervention strategies. The empirical search for CSC markers without real progress in the characterization of normal stem and progenitor cell counterparts is a problematic exercise. First of all, the lack of normal stem cell characterization hampers our ability to define the biochemical signals that control its behavior, define its niche, and regulate its decisions to self-renew or differentiate and/or migrate. These processes, core aspects of stem cell behavior, might well offer compelling points for curative intervention strategies when known. Our relative inability to dissect these properties in normal stem cells, without the confounding mutational heterogeneity of patient tumors that facilitate studies with cancer cells, is unfortunate. Similarly, the lack of information about downstream progenitor cells and differentiated progeny also hampers our efforts. For instance, it is currently unknown whether any, some, or substantially all spontaneously arising tumors first arise from a stem cell or a progenitor cell. While a compelling argument can be made that as the only cell with a long life span, the stem cell is uniquely positioned to accumulate the number of genetic lesions required to initiate a tumor, the final hit could, in principle, occur in any cell within the differentiation hierarchy. As both self-renewal and proliferation are lost in terminally differentiated cells, either a stem or progenitor cell seems a likely point at which to accumulate this final strike. This is further supported by the heterogeneous nature of many tumors, suggesting that the final instigating hit occurs in a cell with differentiation potential.

Whether the "cell of origin" in cancer is a stem cell or a progenitor cell is of more than an academic concern. For example, fundamental questions as to whether the CSC has any niche requirements influence our contemplation of therapeutic strategy. Similarly, one should not be surprised if the commitment from a stem cell to a progenitor cell is accompanied by large changes in pathway activation and/or responsiveness to the local microenvironment, which is again, of basic concern when contemplating effective intervention strategies. What, then, should be our path forward?

COUPLING INVESTIGATION OF CSC WITH THAT OF NORMAL STEM CELLS

There are a number of opportunities to achieve synergy between the examination of CSC and their normal counterparts. These parallel efforts would take advantage of strengths in current knowledge of each cell type. Consider, for instance, colon cancer and normal colon stem cells. Progress has been made in defining colon CSC markers that enrich for tumorigenic cells (28). Perhaps more importantly, we have effective means of assessing colon CSC in xenograft studies. In contrast, normal colon stem cell function is not easily assayed, nor do we have the markers to isolate these cells. However, with normal colon stem cells, we do have important location information (19). Normal colon stem cells are known to inhabit the base of the colon crypt, while more proliferative transit amplifying cells (i.e., progenitors) encompass the base pushing upward as they progress through differentiation toward absorptive colonocyte, enteroendocrine, or goblet cell fates (35). Strategies that seek to expand our knowledge by leveraging current understanding of each of these cell types will stand to fare better than those that pursue each in isolation. We will highlight some of these strategies below.

DISCOVERY OF NEW AND IMPROVED CSC MARKERS

A powerful method for identifying new CSC markers is a bootstrap approach, leveraging microarray analysis of enriched populations to identify novel candidate markers. These markers can then be tested empirically to determine whether they are sufficiently expressed to allow feasible utility, and/or whether they enable further fractionation and enrichment of the CSC. Because any success in this approach enables one to obtain a new set of microarray data that can then be interrogated for additional candidate markers that enable further enrichment of the CSC, this strategy provides a virtuous cycle wherein new markers enable better enrichment, thereby enabling discovery of even better markers that may not have been identified from less enriched populations. While this seems straightforward and feasible, it is not without significant shortcomings. Investigating candidate markers requires careful limiting dilu-

tion type experiments, where a titration of small numbers of cells is introduced into a large cohort of mice using parallel dilutions of cells expressing relatively high versus low levels of a candidate antigen. Many candidate markers are not necessarily expressed at high levels, thereby testing the limits of detection by flow cytometry, in addition to sorting fidelity. These factors, therefore, increase the burden of repeating large limiting dilution studies to confirm results. Further, such studies are inherently slow as each requires sufficient time for all tumors that might arise to give clear and robust growth. This latter point is of importance because only when a tumor has grown to significant size can one have confidence in the legitimacy of scoring the presence of a cell with self-renewal ability, rather than a cell that still possesses substantial proliferative capacity. For example, a committed progenitor cancer cell may proliferate for a period of time to result in a palpable tumor *in vivo*, but this proliferation should eventually exhaust itself to result in a residual mass composed of nontumorigenic and/or murine stromal cells (Fig. 8.2) (29). The consequence of this possibility is that the hunt for new markers requires an expensive and time-consuming campaign. Even then, there is no guarantee that the effort will avail new markers that enrich the CSC further. In fact, any prospective new markers buried in microarray data that would enable significant enrichment of the CSC, by definition, may be diluted by the majority of cells expressing less of this message. Of course, this assumes fidelity between protein and messenger ribonucleic acid expression, which could differ. Thus, the candidate markers that might be of greatest value may not show markedly different expression between the tumorigenic and nontumorigenic microarray data, or are significantly lower on truly tumorigenic cells and increase dramatically upon losing self-renewal capacity. Unfortunately, as anyone who has examined microarray data can attest, the consideration of gene sets exhibiting less dramatic changes between samples tends to expand the list considerably, and the confidence in the validity of the apparent minor expression differences diminish. How then might we improve the identification of new markers?

Coupling analysis of gene expression in enriched populations of CSC with positional analysis of gene expression in normal tissue can provide rapid insight into candidate markers. This is particularly true in those tissues where there is presumptive knowledge of normal stem cell location, as is the case for the colon crypt and skin (23, 36, 37). As progress is made in the stem cell field, such location information is being developed for many other tissues such as the lung, breast, and brain (24, 38, 39). The combined analysis utilizing our knowledge of candidate genes from enriched CSC populations, made possible by microarray data and the functional tumorigenesis assay, together with knowledge of presumptive stem cell localization in normal tissues, allows one to sort through long lists of candidate genes by immunohistochemistry (IHC) and *in situ* hybridization to spot those with intriguing expression patterns. In this fashion it should be possible to differentiate between those genes that exhibit gradients of expression from those that exhibit distinct expression patterns, and then consider the observed patterns in the context of the tissue's differentiation hierarchy. In this manner, genes with expression restricted to

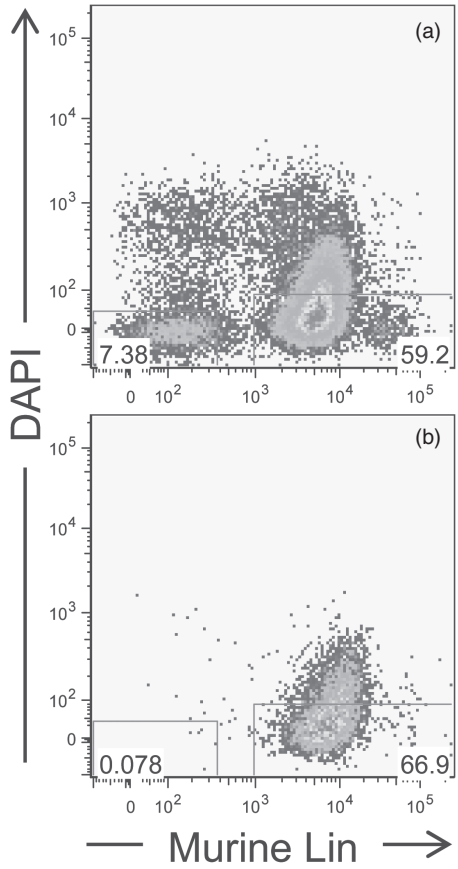


Figure 8.2. Palpable tumor generation should not alone be equated with CSC. Prospective colorectal tumor cells were isolated and transplanted into mice as described previously (29). Three months later, tumors were removed and analyzed by flow cytometry for live human and murine lineage (Lin) cells. Whereas tissue mass (a) was likely generated by a colorectal CSC because it contained live human cells resembling the parental tumor, tissue mass (b) consisted of only murine stromal elements that likely proliferated for a finite time period alongside human non-CSCs. (See color insert.)

potentially unappreciated progenitor stages within the normal tissue might be revealed. Furthermore, genes identified from CSC microarray data can also be ranked in terms of similarity to expression in normal stem or progenitor cells.

MONITORING BIOCHEMICAL PATHWAYS

Understanding the critical signaling pathways required for CSC maintenance is essential to progress in developing effective therapeutic strategies. As noted

previously, the CSC has a number of biological properties not generally present in most cells (2, 4). These prominently include the abilities to self-renew and to generate a multitude of differentiated progeny, but may also consist of unique niche requirements, migratory properties, and other traits not yet appreciated. Success in developing markers that allow the detection and visualization of CSC in the context of the tumor will provide an excellent platform for studying the underlying biochemistry that enables neoplasia. Again, a strategy that combines and leverages our understanding of both CSC and normal stem cells offers advantages to considering CSC in isolation.

There are effective strategies for altering gene expression and monitoring pathway activation with both CSC and normal stem cells. With CSC, one benefits from the ability to isolate and enrich these cells, in addition to the ability to reintroduce the cells in xenograft models to monitor their potential. The critical variable, then, is obtaining the means to modify gene expression and pathway activation. There are several promising approaches to address this challenge, including the addition or reduction of a target gene's expression. Overexpression can be achieved by introducing a particular gene's complementary DNA (cDNA), whereas reduction can be accomplished using RNA interference (RNAi) technology. The question then is how to introduce these molecular tools.

With progress in culturing CSC *in vitro*, it is possible to introduce a variety of genetic elements using lentiviral vectors (40). Transduced cells can then be followed for consequences of genetic alteration. With normal solid tissue stem cells, the situation is complicated by current difficulties in isolating and subsequently maintaining these cells *in vitro*, although recent advances appear promising (41). Further, it is generally not possible to reintroduce these cells and monitor their subsequent behavior in the context of normal tissue. Tracking consequences of genetic modification in normal stem cell populations in mice is therefore more tractable when the alteration is introduced into an entire organism (42). One challenge this approach faces is targeting the alteration precisely to a single tissue of interest. More difficult yet is restricting the expression of the genetic alteration to only the stem cell within the tissue without first knowing the genetic and molecular pathways one is trying to discern. There are approaches that offer help (43, 44). The use of tissue-specific and developmentally regulated Cre-recombinase, for instance, offers effective channeling of the alteration to the tissue of choice and can help circumvent confounding developmental consequences of altered gene expression in an entire organism from the time of implantation. Manipulating normal tissue stem cells in their natural context also opens an additional portal into stem cell biology: lineage tracking (45). With inducible systems, such as Tet-On/Off constructs (44), it is possible to provoke an alteration at a defined time point and then track the consequences of this change over time. This ability coupled with knowledge of the location of a presumptive stem cell in a tissue can help to uncover the time course, location, movement, and characteristics of the entire ontogeny of a tissue.

The flip side to having the ability to alter gene expression is having reasonable means to assess the impact of such a change. Many techniques can find ready utility in the study of both normal stem cells and CSC, including IHC, quantitative reverse transcriptase polymerase chain reaction (RT-PCR), fluorescent-activated cell sorter (FACS), and proteomics. A technique used in stem cell research, and likely to grow in popularity, is the use of pathway-specific reporter constructs (46). Not only would such reporters provide important location information in the context of living tissue, but with the advent of live animal imaging technologies and the increasing number of fluorescent adducts whose light can penetrate tissue (47, 48), these tools might have more powerful applications in the future. Often consisting of a well-chosen promoter driving an assayable gene product such as green fluorescent protein (GFP), luciferase or β-galactosidase, cells tagged with such a promoter-driven transgene would allow the study of consequences associated with a specific state of gene activation in either a living tissue or *in vitro*. Powerful examples of successfully utilized promoters in stem cell biology include Oct-4, Nestin, and Bmi1 (49–53). Another elegant example involves the use of the Axin2 reporter to highlight cells in which canonical Wnt signaling is active (54). Each of these is highly expressed in multipotent cells of specific tissues, but none appear universal in utility. The reason that the subject of pathway monitoring in normal stem cells is of considerable relevance to the study of CSC, given that it is possible to both modify and assess gene expression effectively in CSC, is that the study of normal stem cells provides a framework for appreciating whether changes induced in CSC are relevant.

Without being able to identify the normal stem cell, the various transgenic strategies discussed above are hindered in their ability to be applied in a focused and relevant fashion. Cancer research has moved beyond the microarray data derived from bulk tumor samples that constituted the state of affairs a few years ago to that derived from increasingly enriched populations of CSC (55). However, this microarray data, in isolation, simply provide evidence that large numbers of genes are detectibly expressed in tumorigenic cells at levels higher than nontumorigenic cells. Among these gene lists are certain to be known components of countless developmentally important signaling pathways and biological processes. Thus, this hard-won microarray data in and of itself offer myriad potential avenues for further research. One might reasonably expect that engaging in an effort to knock down expression of various genes expressed in CSC would have cellular consequences, and that by monitoring the consequences of these changes with pathway reporters and/or other approaches, insight on pathways critical to CSC might be identified. Certainly, valuable insights into CSC biology are obtainable, but which genes should be targeted? More critically, it is not clear whether the observed impact is of normal physiological relevance. Here, the power of combining efforts to impact and monitor pathways in both CSC and normal stem cells is likely to prove valuable. It is not unreasonable to suppose that pathways and specific genes found to be important to the function of the normal stem and progenitor cells

will also prove relevant in CSC. Conversely, pathways that are not of critical importance to the normal cohort of pathways utilized during development are less likely to be pathways of interest when considering CSC. Thus, lessons learned in the study of normal stem cells can be of great value in guiding CSC research. In turn, these lessons, which offer both a path to isolation of the self-renewing CSC population and means to assay it in xenograft models, may well offer useful insights into normal stem cell function and regenerative medicine applications.

DISPERSION OF SOLID TISSUE TO OBTAIN SINGLE CELLS

Although solid tumors can be propagated in mice by implanting tumor fragments, identification and isolation of CSC requires enzymatic treatment to obtain single cell suspensions prior to staining with antibodies. Prospective CSCs have been identified from multiple tumor types; the majority of which are epithelial (25, 28, 34). Because epithelial cells have tight interactions with each other and their microenvironment, dissociation of these tissues generally requires digestion using a combination of enzymes. As we summarize these tumor dissociation reagents, we emphasize that precaution must be taken when interpreting cell surface antigen expression profiles, as results can differ based upon which, and how, reagents are used.

Collagenase is a commonly used dissociation enzyme that degrades collagen fibrils in various connective tissues, including the skin, tendon, and blood vessels. Commercially available crude collagenase preparations often contain not only collagenase, but also several other proteases that are effective at breaking down intercellular matrices, the crucial goal of tissue dissociation. Although the presence of additional proteases is beneficial to achieve single cell suspensions for tissue culture, if cells are to be analyzed or isolated based on cell surface marker expression, precaution should be taken since these additional proteases might also target surface antigens. Currently, there are four types of crude collagenases, and each one has a distinct enzymatic profile. Therefore, each type of collagenase is typically used for certain types of tissues. Most tumor dissociation protocols use collagenase III or IV, which contain the least number of proteases. Purified collagenase can also be used to minimize secondary tryptic activities, but these reagents can be costly.

A number of other enzymes commonly included with collagenase in digestion protocols include hyaluronidase, trypsin, elastase, dispase, and/or DNAse. Hyaluronidase randomly hydrolyzes hyaluronic acid and chondroitin sulfates, major components of the extracellular matrix. Hyaluronic acid contributes to cell proliferation and migration, and may also be involved in the progression of some malignant tumors (56–58). Interestingly, hyaluronic acid is also a receptor for CD44, which has been demonstrated to be a reliable CSC marker in epithelial tumors analyzed to date (25, 28, 32, 33). Generally inef-

fective at dissociating extracellular matrix and tissue on its own, trypsin is a relatively strong and specific protease for peptide bonds. Care should be taken, however, as there is much variability in trypsin formulations and some suppliers will use this name for a crude mixture of pancreatic enzymes. Elastase has diverse proteolytic activity and is unique in its ability to hydrolyze elastin, which is a main component of dense fiber networks in elastic tissues. Because of this activity, elastase is commonly used in conjunction with collagenase and other enzymes when dissociating, for example, pulmonary tissue. Dispase is a neutral metalloprotease generated by *Bacillus polymyxa* and has been used as a secondary enzyme in cell isolation and tissue dissociation applications due to its mild activity and relative specificity for dissociating fibroblasts. DNAse is another reagent that is often added in dissociation protocols. As the name suggests, the target of this enzyme is DNA that is released from damaged or dead cells. When dissociating primary tissue, especially those with necrotic areas, the addition of DNAse reduces viscosity and prevents cell aggregation due to general "stickiness."

In our own experience with breast and colon tumors, primarily, we have observed that hyaluronidase and dispase can reduce signal intensity of certain surface markers from certain tissues. Whether collagenase has a deleterious effect on surface protein display is unknown, as this reagent is currently indispensable for tumor dissociation. When cells of interest are isolated based on positive marker expression, such as CD44, these artifacts are less of a concern. However, if cells are selected based on little to no surface expression of a certain antigen, such as CD24 on breast CSC (25), each enzyme needs to be tested for its effect on epitope maintenance, and heterogeneous expression among the population should be validated by an independent method such as immunofluorescence or IHC.

ISOLATION OF DEFINED CELL POPULATIONS

The past two decades has witnessed dramatic improvements, not only in the instrumentation that allows rapid, accurate analysis, and isolation of single cells, but also the degree to which these technologies are user-friendly. Although magnetic separation techniques are generally successful at enriching populations of cells, samples are always contaminated by dead and unwanted cells, or clumps of cells, that make clonal analysis problematic. The technology that, in essence, revolutionized not only targeted cell isolation, but also high-throughput cellular analysis, is the flow cytometer and FACS (16).

Flow cytometry allows rapid detailed analysis of single cells by detecting the light scatter and fluorescence emission characteristics of single cells as they pass by laser excitation sources in a stream of fluid. Light scattered due to the size and complexity of the cell is interpreted and displayed in two dimensions, easily allowing large, small, and/or granular cells to be discriminated from each other. In addition to these parameters interpreted from light scatter, emitted

fluorescence can also be assessed via an array of reflective mirrors and band-pass filters, such that multiple emission wavelengths can be analyzed from each excitation laser. The current record for analysis parameters on a commercial instrument is 18 fluorescence detectors. By assessing the light emission from fluorescent proteins, intracellular chemical reactions, dye retention, or fluorochrome-conjugated antibodies, thousands of cells per second can be interrogated on multiple parameters. On a flow cytometer with sorting capability, single cells with the distinct morphological and/or fluorescent characteristics defined by the user can then be isolated.

Developed primarily for use with hematopoietic cells, flow cytometers can also interrogate cells from solid tissues; however, their isolation raises complications. In addition to the issues surrounding the generation of a single cell suspension from solid tissues, these cells are often much larger and more fragile than hematopoietic cells. These properties make epithelial cells, for example, more susceptible to damage or death during the process of analysis and isolation. Optimized for small, uniform cell populations, cell sorters typically utilize high pressure (17–20 psi) and small nozzle sizes (70 μm) to facilitate high-throughput cell analysis and isolation by magnetic deflection of cell-containing droplets. Isolation of larger, more fragile cells from complex suspensions has proven more problematic. Although some companies have addressed this issue with ultra-low pressure capabilities and extremely large nozzle sizes, sacrifices are made when using such instruments, including reduced purity and throughput capacity (i.e., cells per second analyzed), and decreased sensitivity due to a wider beam path through the sheath fluid, resulting in less discrimination power on a cell-by-cell basis. Next-generation machines are already under development and some are attempting to circumvent the physical limitations afforded by current sorters altogether. One such technology focuses cells within a stream of fluid independent of sheath pressure, allowing precise analysis of cells in a closed system that can be sorted downstream using gated capillaries, and even reversed to reanalyze cells of particular interest. Not only could such a closed system sorter isolate cells independent of decompression and electrical charging stresses, but sterility could be easily maintained. Such advances would also address another limitation of current flow cytometers not discussed above: detection of antigens with low expression. Typically, definitive antigen detection by flow cytometry requires significant expression because any one cell's time in the excitation laser's path is extremely short. Although brighter antibody dye conjugates and improved lasers can address this issue to a point, the time-of-flight (i.e., length of time a cell is being interrogated) limitation remains. A cell sorter capable of analyzing cells without temporal laser excitation/emission limitations may require neither ultra-bright emission conjugates nor powerful lasers. Because data could thus be obtained for a longer period of time, more detail as to an antigen's relative expression might be obtained. Small expression differences may have large consequences where cell fate decisions are concerned, as lessons from Oct-4 in embryonic stem cells, for example, have taught (59).

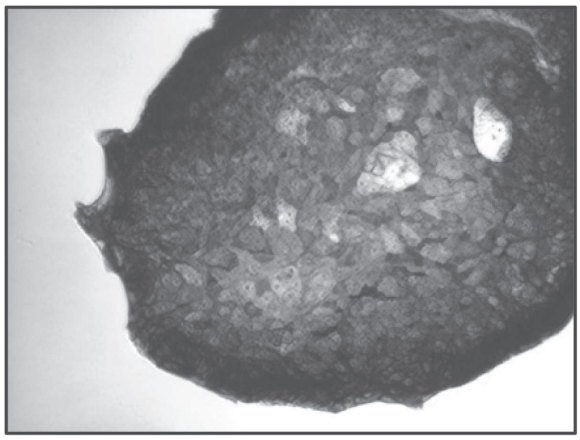
ASSAYS FOR CSC

Finally, after the cells of interest have been freed of their neighbors and isolated, human xenogeneic tumor-derived cells must be experimentally tested for their ability to generate secondary tumors or in functional *in vitro* assays that might predict behavior *in vivo*. The gold standard assay to enumerate CSC is xenogeneic transplantation (13). Only by demonstrating that a cell can completely reconstitute an actively growing, heterogeneous tumor can one be certain of CSC identity. Although the basic subcutaneous or orthotopic transplantation of the proper cells will generate tumors, the determination of whether the input cells were CSC, or more differentiated cells with high proliferation potential, relies on the use of limiting cell doses, serial transplantation, phenotypic analysis, and assays that combine these analyses to determine tumorigenic cell frequency. These types of investigations are important, as highly proliferative, but nonself-renewing cancer cells, for example, might multiply enough to produce a palpable tumor, but growth should eventually cease (Fig. 8.2). Driving CSC to such an end might even be the goal of therapeutic strategies: inhibit the self-renewal of CSC and send cells tumbling down the course of differentiation. Like limiting dose dilution assays for hematopoietic reconstituting cells (60), similar experiments can be performed with dilutions of either unfractionated solid tumor cell suspensions or cells isolated based on their phenotypic profile (30, 61, 62). The use of both options greatly facilitates an understanding of enrichment during CSC discovery efforts, although the complicating factor surrounding cell viability following extended isolation protocols should be considered. When combined with the introduction of RNAi or exogenous cDNA expression as discussed above, for example, insights can be realized on the basic signaling pathways a CSC relies on for survival, growth, and cell fate decisions.

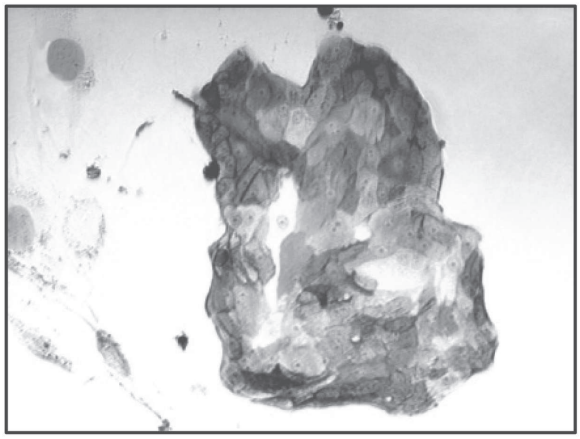
With regards to *in vitro* assays for stem/progenitor cell potential, the CSC field holds an advantage over developmental biology in that cells maintained *in vitro* appear able to generate morphologically and histologically diverse tumors after culture for as long as 3 weeks (31, 61). This is not yet possible with HSC despite decades of focused interest. Without diving into a discussion of cell culture medium, one should first debate cell culture in general. *Ex vivo* cell culture is certainly not physiological; however, steps can be taken to reduce the typical *in vitro* culture stresses cells experience in an attempt to more closely replicate the *in vivo* environment from which the cells were obtained. The first consideration is whether cells should be cultured in serum (63–65). Popular cell culture utilizes fetal calf serum as a supplement to support cell proliferation. Serum is full of growth factors and other proteins most cells normally do not encounter in their microenvironmental niches *in vivo* (66). In fact, most cells *in vivo* are probably exposed to only the cells and extracellular matrix immediately adjacent to them. To eliminate all the unknown factors associated with serum, various sources of media supplements or chemically defined media are now available that likely contain the minimal “goodies” a

cell needs to survive (41, 67). Further supplementation with growth factors is influenced by specific requirements of the tissue one is trying to culture (65). Next, the surface requirements, if any, for cell culture should be considered. If cell aggregation is the desired goal, nonattachment plates generally work best. However, for most cell populations that desire, and require, attachment to survive, simple hydrophilic tissue culture plastic is generally sub par. There are several general strategies to circumvent this limitation. Newer surfaces with alternating positive and negative charges, resulting from the inclusion of nitrogen-containing gas plasma during the coating process, better facilitate cell attachment and spreading (41, 68). Alternatively, plate surfaces can be coated with extracellular matrix components normally encountered *in vivo*, such as collagen, laminin, and/or fibronectin. Yet another option is the plating of cells atop a feeder cell layer that provides at least some of the complex interdependent cellular signals that might be found *in vivo*. However, the choice of feeder layer is not trivial, as there is considerable evidence that the stromal elements in tumors differ from those in normal tissue (69). Thus, the source and characteristics of the stroma support system chosen are likely to be of importance and influence the behavior of the culture system. Finally, the length of culture should be considered. Because conditions that completely replicate the physiological environment *in vivo* are presently impossible to copy, one must consider how long *in vitro* culture should continue. Even under the best of conditions, genetic alterations may occur and can persist to overtake a culture of cells. These changes can be very difficult or impossible to ascertain by visual inspection. One is therefore best served by efforts to characterize any genetic abnormalities upon initial passage and again at later passages, while minimizing duration of *in vitro* culture even if the purpose is a long-term assay readout or clonality study.

In vitro assays for CSC are relatively few and are an area where much work remains to be done. Although serial replating assays for neurospheres and mammospheres are commonly used indicators of *in vitro* self-renewal (70, 71), these assays may or may not be indicative of true self-renewal in the context of *in vivo* biology as they may simply be a readout of proliferative capacity, especially in cultures initiated with hundreds to tens of thousands of cells. This is particularly true given that CSC *in vitro* are virtually indistinguishable from their nontumorigenic progeny upon visual inspection, and cell surface markers are lacking that truly distinguish a tumorigenic from an nontumorigenic cell. To rigorously demonstrate self-renewal, *in vitro* assays should be performed at the single cell level, or at least in limiting dilution. Traditional colony forming cell (CFC) assays in the context of solid tissue biology may be extremely informative, especially if these assays can be linked to a differentiation capacity readout (70, 72). That is, if *in vitro* differentiation processes can be driven and the progeny identified, similar to the differentiation of myeloid and erythroid lineages in methylcellulose hematopoietic colony forming unit assays, then by combining self-renewal capacity with a differentiated progeny readout, one might be positioned to estimate not only the number of CSC in a particular culture, but also to assess the therapeutic efficacy both *in vivo* and



(a)



(b)

Figure 8.3. An example of colorectal colony morphology differences in vitro. Highly enriched colorectal CSCs were plated *in vitro* in either (a) serum-free medium or (b) medium containing fetal calf serum. After 16 d of culture, colonies were fixed and stained for human CD44 (magnification = 100×) and counterstained with Hematoxylin. (See color insert.)

in vitro. Although not as easy as visually distinguishing blast-forming unit-erythroid from granulocyte/macrophage colonies, technologies that quantify tumorigenic versus nontumorigenic cells based on cell/colony morphology, antibody staining, and/or reporter expression might be powerful. This may be especially true when high-throughput imaging technologies are incorporated into the screening process. For example, addition of serum to cultures of tumorigenic colon tumor cell cultures appears to reduce the overall expression of CD44, a marker known to demarcate colorectal CSC (28) (Fig. 8.3). Similarly, through the quantitative analysis of numerous colony characteristics, such as

overall colony numbers, cell number per colony, colony density, and/or cellular morphology using high-content screening instruments, one might be able to determine whether tumorigenic cells are affected by therapeutic agents *in vivo* or *in vitro*. Addition of more complex differentiation markers will only add to the power of such assays. At the end of the day, however, serial transplantation in limiting dilution experiments will likely remain unsurpassed for defining effects on CSC numbers.

STEPS FORWARD FOR CANCER STEM CELL BIOLOGY

Stem cell biology has, in recent years, enjoyed tremendous interest from both the scientific community and the general public. This interest has been underpinned by growing recognition of this research's potential to dramatically alter our ability to improve health-care options for patients suffering from a wide variety of ailments. In many cases, such as with Parkinson's disease, cystic fibrosis, and muscular dystrophy, we have not been able to advance effective therapeutics despite our success in developing a substantial understanding of disease pathology. Thus, the promise of stem cells in regenerative medicine as a strategy to fundamentally replace defective cells is tantalizing. Cancer stem cells, by contrast, present the opposite dilemma. Rather than being the potential cure, the CSC is itself the fundamental problem. Scientific endeavors to understand normal stem cell biology will present tremendous opportunities for synergy with our study of cancer. In time, it may come to be recognized that one of the most substantial victories for public health to emerge from our study of stem cells will be in the war on cancer.

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9

LARGE-SCALE PRODUCTION OF ADULT STEM CELLS FOR CLINICAL USE

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CELL THERAPY

Cell therapy involves the transplantation of cells, either alone or in combination with carrier materials, to repair or regenerate damaged cells or tissues. Although some therapies involve the isolation and utilization of more differentiated or end-stage cells, such as the use of chondrocytes for cartilage repair (1), most therapies are initiated with more primitive multipotent stem or progenitor cells. There are numerous sources of stem and progenitor cells, including bone marrow, adipose tissue, peripheral blood, and other specific tissues, including muscle, liver, and brain, that are currently being studied for their potential utility in regenerating or repairing tissues (2). Cell therapy is being considered for, or has already shown to aid in, the repair of multiple tissue types in such wide-ranging therapeutic areas as cardiovascular disease (3, 4), peripheral arterial disease (5, 6), liver disease, diabetes, neurodegenerative disorders, bone repair, and spinal cord injuries. In addition to tissue regeneration, a variety of cell-based approaches are also being investigated for the therapeutic treatment of cancer and infectious diseases, such as dendritic cell and T-cell vaccines (7).

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In cases where the number of stem and progenitor cells from the source tissue is low, *ex vivo* culturing has been employed to increase the number of these therapeutic cells. Depending on the conditions, culturing may also lead to purification, activation, differentiation, or functional enhancement of stem and progenitor cells. Culture conditions must be optimized and extensively tested to ensure the regenerative potential of the cells is maintained during the culture period.

MANUFACTURE OF CELL PRODUCTS FOR THERAPY

One of the greatest challenges of *ex vivo* cell therapy, particularly in a patient-specific approach where the patient is treated with cells derived from his/her own cells (autologous) or from matched allogeneic donor cells, is the ability to produce or manufacture these cell-based products in a clinically and commercially viable way. In many of these cases, the process for generating each therapeutic dose of cells is relatively complex with multiple processing steps, and will require advanced cell manufacturing capability in order to be commercialized successfully.

Some approaches to cell therapy require only minimal manipulation of cells (e.g., simple collection or washing steps) with no regulation of the cells as a product, and can affordably occur as part of the clinical procedures right within the point-of-care facility. However, when the cell manufacturing process is considered by the Federal Drug Administration (FDA) to be "more than minimal manipulation" of cells (21 CFR1271, Human Cells, Tissues, and Cellular and Tissue-based Products), the resulting cell product is regulated by the FDA as a biologic. Biologics require a Biologics License Approval (BLA) that includes compliance with Good Manufacturing Practices (GMPs) applicable for both drugs and biologics.

For allogeneic "off-the-shelf" approaches, one large batch of cells from a single donor is manufactured and used to treat many patients. Large-scale production of this type of cell product would typically involve increasing the surface area and/or volume of the culture vessel available to generate high numbers of therapeutic cells. Once qualified, these cells would be stored and available for use at any time. Although allogeneic cell products have definite benefits, issues remain as to the potential effect of immune responses on long-term engraftment and regeneration. In addition, the high number of cell doublings during extended multipassaged cultures that are frequently required to generate a large batch of cells from one donor add to the risks of genetic changes leading to cell transformation (8, 9).

A large number of cell therapies in development are patient-specific, which requires that a separate batch or a lot of cells be manufactured for each patient. Thus, for a commercialized therapy targeting thousands of patients or more each year, a "large-scale" cell manufacturing facility would need to

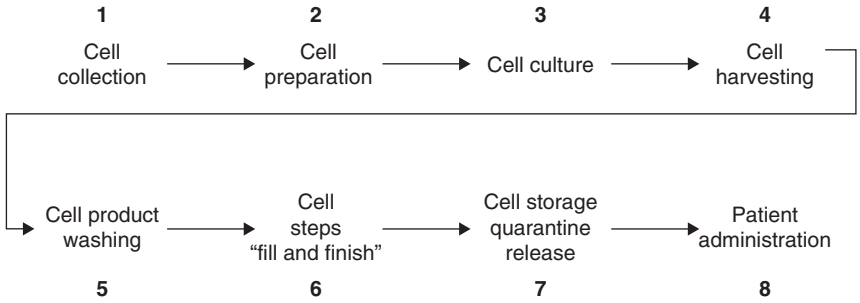

Figure 9.1. Cell production steps for *ex vivo* cell therapy.

support an equivalent quantity of individual batches of cells with zero risk of cross-contamination between cell doses and a very high certainty that each batch is successfully manufactured and released. Although this results in a unique manufacturing challenge, there are benefits to using patient-specific cells for some indications.

A manufacturing platform for cell therapies must be developed to successfully progress from development, through clinical trials, and into approved commercial use capable of treating large numbers of patients. The following process flow diagram summarizes the steps for a typical cell manufacturing process from cell collection to patient administration (Fig. 9.1).

Cell or specimen collection is performed in a clinical setting from a donor or the patient (for autologous therapy). The collected sample is typically shipped to a centralized manufacturing site for processing to prepare for cell culture. These steps may include a separation step, either using gradient density separation such as Ficoll® (GE Healthcare) to remove unwanted cell populations, or column purification to select for specific cell phenotypes. Cells are then inoculated into the culture device and incubated per the developed protocol. After culture, cells are harvested from the culture device and put through multiple processing steps to obtain a cell product ready for patient administration. These postculture steps include washing the cell product to remove impurities such as culture medium, and final steps such as concentrating the cell product by removing excess volume, or mixing cells with a cell delivery vehicle. The final cell product is stored until test results are obtained to allow release of the product for shipping and patient administration.

Very few cell products have been commercialized to date. Carticel® (Genzyme Biosurgery, a division of Genzyme Corporation, Cambridge, MA) is an example of an autologous cell product that consists of *ex vivo* cultured chondrocytes. It was the first FDA-approved cell therapy for repair of cartilaginous defects, with approval in 1995 (10). Over a span of 7 years from 1995 to 2002, 7500 patients were treated with this cell product.

REGULATORY REQUIREMENTS FOR CELL PRODUCTS

As mentioned above, *ex vivo* cell products are regulated by the FDA as a biologic, requiring a BLA. The FDA has provided requirements and guidelines for cell products in the Code of Federal Regulations (21 CFR 600). These include requirements for the generation and characterization of cell products prior to their administration to patients.

The cell manufacturing process must meet both FDA regulations and the requirements of a successful business model. Fundamentally, the manufacturing process must integrate the underlying biological process that drives production of functional cells in sufficient number along with the culture device capable of supporting the biological process. It also must integrate postmanufacturing processing of the cell product in preparation for patient administration. The total end-to-end process must be compliant with GMPs. Other key drivers that, while not mandatory, will greatly facilitate success include the use of closed-system technology, minimizing cell manipulation wherever possible, and the careful use of automation to achieve reliability, consistency, and cost-effective manufacture of the product.

In addition to a manufacturing platform, a number of other regulatory requirements around cell product characterization must be in place to have a commercially viable cell product. These are outlined in 21 CFR 610. Overall, the product must be safe, pure, and effective. To provide this level of assurance, full product characterization is necessary, typically using both *in vitro* and *in vivo* assays. The applicant must provide evidence of product safety, sterility, purity, identity, and potency (the quantitative measure of biological activity). These measurements are necessary for the approval of all biological products, and they must be completed for final product release of each manufacturing lot prior to administration to the patient. This is a particular challenge in patient-specific cell therapy, where a separate release is required for each cell dose. There is a restricted amount of final product, which limits the capability to test the final product. There can also be time constraints due to the potential short shelf-life of the final product.

CELL CULTURE PROCESSES

Initially, scientists are compelled to be highly focused on the biological process needed to generate putative therapeutic cell populations. These culture processes typically consist of manual methods of generating cells in flasks, culture bags, or other culture devices. These methods can be used early in clinical trials, and typically require many resources, manual steps, technician time, and open steps which introduce potential sources for errors, variability, or contamination. Moving into later-stage clinical trials, the focus must move to developing a device and manufacturing platform capable of incorporating the biologic culture process and producing sufficient numbers of cells as well as being

capable of meeting GMPs. The following section describes an example of the development of a culture process and integration of this process into a manufacturing platform.

IMPORTANCE OF MEDIUM EXCHANGE FOR MAINTENANCE OF HEMATOPOIETIC STEM CELLS

Previous work has shown the importance of medium exchange, not only on the productivity and longevity of bone marrow (BM) cultures (11), but also on the metabolic activity and growth factor production rates of marrow stromal cells (12). Figure 9.2 shows data from long-term BM cultures (adapted from Schwartz et al. 1991), which illustrate the effect of medium exchange rate on culture productivity. Data is presented as the cumulative production of colony forming unit-granulocyte/macrophage (CFU-GM) over time in culture. A slope of zero indicates no continued production of CFU-GM, suggesting a depletion of the hematopoietic stem cell compartment that gives rise to CFU-GM. Cultures fed every day, or only once a week, stopped producing CFU-GM very early during culture. A feeding schedule of every other day (3.5 times per week) resulted in continual CFU-GM production over the culture period, suggesting that hematopoietic stem cells are maintained under these conditions. Seemingly, the feeding rate for stem cell maintenance is capable of

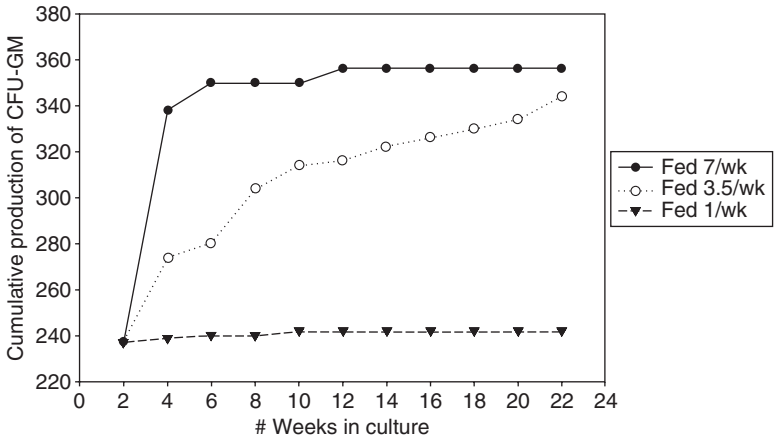

Figure 9.2. Medium exchange rates affect maintenance of hematopoietic stem cells. Bone marrow mononuclear cells were grown in long-term bone marrow cultures for 22 weeks as described (11). Cultures were fed either once per week (1/weekk), every other day (3.5/week), or every day (7/week). Every 2 weeks, CFU-GM colonies were measured from each culture and the cumulative CFU-GM production over the 22-week culture period is graphed.

maintaining the critical stem cell niche or microenvironment while providing fresh nutrients and depleting waste.

Controlled medium exchange rates have also resulted in the enhancement of cell production and/or biological function of other primary human cells including T cells and monocyte-derived dendritic cells. Monocyte-derived dendritic cells have demonstrated enhanced antigen presentation and maturation with increased medium exchange (13). Similarly, continuous medium exchange in human T-lymphocyte cultures yielded very high-density cultures and cells with superior replicative potential and enhanced biological function, as measured by release of granulocyte-macrophage colony stimulating factor (GM-CSF) and tumor necrosis factor (TNF)-α in response to anti-CD3 (14).

BIOREACTOR DESIGN CAPABLE OF SUPPORTING THE BIOLOGIC PROCESS

The next challenge was to develop a bioreactor system that could support very low rates of medium exchange while maintaining culture performance. Typically, medium flow is coupled with oxygenation, thus requiring high flow rates to provide the necessary oxygen transfer rates. This process causes significant shear stress on the cells and an inability to maintain the microenvironment. To address these issues, the oxygen supply is decoupled from the medium exchange process and a constant culture depth is maintained, thus enabling uniform oxygen supply to the cells throughout the culture while allowing slow medium exchange rates needed for optimal culture conditions (Fig. 9.3).

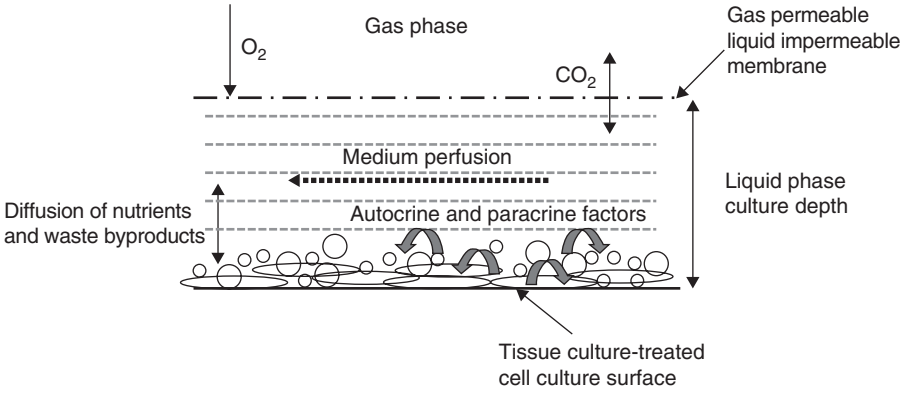

Figure 9.3. Schematic of single-pass perfusion technology. This figure illustrates the flow of medium, the cell growth area, and the gas exchange. Liquid depth is maintained by the gas permeable, liquid impermeable membrane. Medium perfusion is single pass and rates are such that there is diffusion of nutrients and waste byproducts, while maintaining the stem cell microenvironment containing important autocrine and paracrine factors.

This medium exchange method has been termed single-pass perfusion (SPP) in reference to a controlled single pass of medium flow across the surface of the culture without recirculation of the medium. This procedure can be simulated in manual systems by exchanging part of the medium without disturbing the cellular microenvironment area. This methodology allows for very slow medium exchange rates that maintain a tissue-like microenvironment, including the accessory cells, paracrine and autocrine factors, while providing optimal rate of nutrients and removal of metabolic by-products.

Because of the low medium exchange rates, SPP supports the growth of either suspension cells, anchorage-dependent cells, or a mixture of both, and results in cultures with negligible shear stress, increased cell numbers, and superior biologic activity. This is especially important in long-term BM cultures where a critical component is the establishment of an adherent stromal layer to create the niche supporting hematopoietic stem and progenitor cells.

A schematic of the resulting bioreactor design is shown in Figure 9.4 and has been reported previously (15, 16). The bioreactor is circular in shape and has 850 cm^2 of culture surface area. It has two primary compartments: (a) a bilayer liquid-filled cell growth compartment where the top layer contains the cell culture plastic surface and the bottom layer encloses a fluid path that consolidates effluent medium for delivery to an attached waste container, and (b) a gas compartment which is maintained with a controlled mixture of O_2, CO_2, and N_2 gases for oxygenation and pH stability of the culture. The gas compartment is situated above the cell growth compartment. These compartments are separated by a gas permeable, liquid impermeable membrane that facilitates both sterile closed-system design and creates a fluid-locked cell growth compartment that controls its depth at all times (e.g., during transport,

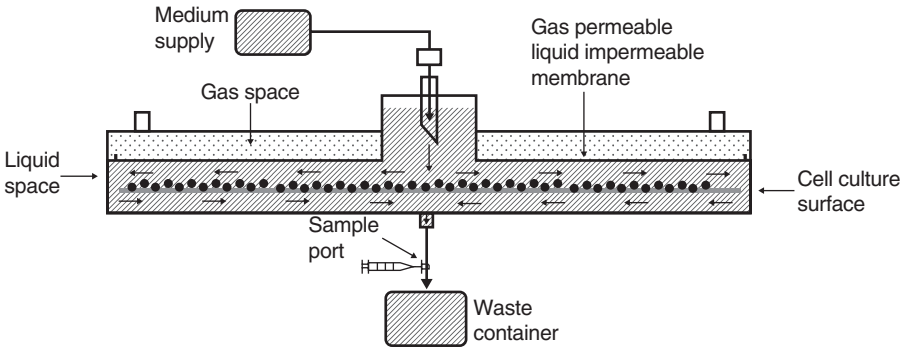

Figure 9.4. Cross-section of a bioreactor supporting single-pass perfusion. The bioreactor is a circular design with 850 cm^2 of cell growth area. Medium enters through a port at the center of the bioreactor, flows radially outward, and is collected in a waste container. The liquid space is separated from the gas supply by a gas permeable, liquid impermeable membrane. The culture maintains the same oxygen tension irrespective of medium perfusion rates.

pre- and post-culture manipulations). The incorporation of the separate gas compartment above the cell growth compartment enables continuous medium replacement while simultaneously providing uniform oxygen diffusion as necessary for cell expansion over a wide range of medium exchange rates. The surface of the cell bed is tissue culture-treated to facilitate adherence of anchorage-dependent cells. The bioreactor is accompanied by a fluid pathway, which includes the components for medium entry to the bioreactor and removal of spent medium at controlled rates. The entire system is closed and sterile.

The circular bioreactor is designed to feature radial SPP between parallel plates. The radial perfusion design overcomes the nonuniform distribution and nonhomogeneous environment experienced near the wall in axial flow rectangular parallel plate growth chambers. The medium enters at the center of the bioreactor, and flows radially outward toward the periphery. The cell bed is equipped with drain holes on the periphery, which facilitate drainage of the spent medium to the waste side, which in turn is connected to a waste container.

The linear velocity of the fluid in the bioreactor during continuous radial perfusion ranges from 1.8×10^{-3} cm/s near the center to 5.5×10^{-5} cm/s at the periphery for a rate of 50% volume exchange per day. At higher rates of medium exchange, up to 10 volumes of medium exchanged per day, the linear velocities would be 20 times higher or 3.6×10^{-2} cm/s at the center to 1.1×10^{-3} cm/s at the periphery. These velocities are slow enough that removal of nonadherent cells from the cell bed is not a concern. Shear stresses generated during perfusion are estimated to be on the order of 10^{-5} dynes/cm^2 at entrance and 10^{-8}–10^{-7} dynes/cm^2 at the periphery based on the average velocities for a 50% volume exchange per day. The minimum shear stress affecting the morphology of human umbilical vein endothelial cells *ex vivo* has been reported to be between 8 and 12 dynes/cm^2 (17). As another comparison, physiologic levels of venous and arterial shear stress have been reported between 1–5 dynes/cm^2 and 6–40 dynes/cm^2, respectively (18). The shear stress generated in the bioreactor, even at very high perfusion rates to support high cell density cultures up to 40 million cells per milliliter, is therefore not expected to compromise the cells.

Cells are inoculated through a port into the center of the cell culture compartment. After inoculation, the bioreactor is placed on a platform, which automatically oscillates, causing a bubble to distribute the cells uniformly over the cell bed surface. After distribution, the bubble is purged and replaced with medium and cells settle by gravity onto the surface. During perfusion, the medium enters through the center, flows radially toward the periphery, and exits into the waste side. Resistance eliminates loss of cells during distribution of inoculum on the cell bed surface.

From the waste side, the spent medium enters a fluid path that drains into a waste container. This fluid path incorporates a sampling port, which enables the user to draw effluent medium for microbiological testing or metabolite analysis. At the time of harvest, cells are drained into a separate harvest bag

through a harvest port at the periphery of the cell culture chamber. The culture chamber is also equipped with a tubing segment for delivery of trypsin and other wash solutions required for the cell harvest. At the conclusion of the harvest, the cell product is contained in a sterile bag.

CELL MANUFACTURING SYSTEM

Cell production in the bioreactor has been automated in a cell manufacturing system (15, 16). Briefly, the system embodies a modular, sterile closed-system process comprised of a presterilized, single-use disposable cell cassette operated by automated instruments incorporating microprocessor controlled hardware and custom software to run the processes. The fluid pathway in the cell cassette includes the bioreactor, a medium supply container, and a waste container (Fig. 9.5a). The instrumentation components of the manufacturing platform include a set of incubator units (Fig. 9.5b) and a cell processor (platform) unit (Fig. 9.5c), along with a computer-based system manager. Each incubator can control the flow of medium to the bioreactor, the temperature of the growth medium supply reservoir, the temperature of the bioreactor, and the flow and concentration of gases (O_2, CO_2, and N_2) to the bioreactor. The cell processor is a platform used to prime the bioreactor with medium, inoculate and distribute the cells throughout the bioreactor, and to harvest the cells after culture. The system manager provides a complete documentation of the events and aids in scheduling of tasks. An application key containing an electronic

(a) (b) (c)

Figure 9.5. Cell manufacturing system. (a) The cell cassette contains all parts of the fluid pathway, which include the circular bioreactor, the medium supply container, containers for waste medium and harvested cells, and tubing connecting each component. (b) Four incubators are contained within each unit. The cell cassette is placed in the incubator during the culture process. The medium supply container is inserted into a separate chamber within the incubator that maintains a temperature of 4 °C. The bioreactor portion of the cell cassette is placed into another chamber, which can be programmed for the desired culture temperature (i.e., 37 °C). (c) The cell processor contains a platform that holds the cell cassette and oscillates to assist in priming the bioreactor with medium, distributing the cells evenly throughout the bioreactor, and harvesting the cells.

memory device is affixed to each cell cassette and provides identification of the cell product, instructs the instruments through the cell production processes, maintains cassette status, and collects process data. As a result of the approach, mix-ups and operator error are prevented.

This flexible manufacturing platform has provided automated, reliable, and reproducible expansion of a variety of cell types (19). Protocols have been developed for the production of hematopoietic stem and progenitor cells from a small volume BM aspirate (13, 20) and from umbilical cord blood (21–23) for use in stem cell transplants. This platform also supports the expansion of mesenchymal components of BM and is currently being used in clinical trials for bone and vascular regeneration (24–26). Protocols for the production of dendritic cells and T cells have been developed for use in cancer vaccines (27). In total, Aastrom's manufacturing system has supported more than 625 *ex vivo* cell production lots and treated over 260 patients in Phase I/II clinical trials.

POSTCULTURE PROCESSING OF CELL PRODUCTS

Another critical step in the cell production process is the preparation of the cell product for patient administration after the culture process is complete. Typically, cells harvested from any culture process are not suitable for direct patient administration. To prepare the cultured cells to be ready for use when received at the treatment site, a set of postculture manufacturing steps are required, including removal of medium residuals (such as serum proteins and harvest enzyme), achieving an appropriate product volume (culture harvest volumes are typically much higher than is required for patient administration), and formulation of cells for delivery to patient which, depending on the application, may include mixing cells with delivery vehicles such as space-filling scaffolds or injectable matrices, prior to implantation.

Many of these postculture processing steps have not previously been automated or are not easily integrated into the manufacturing process. As a result, the postculture phase has been a relatively time-consuming process, subject to increased risks of variability, operator error, contamination, and excessive holding times. Additionally, the manipulations and transfers associated with these processing steps can result in cell loss and decreased cell viability. Although these issues have been managed for limited patient capacity, development of an automated unit process that integrates most, if not all, of these steps and leads to high recovery and viability of the cell product is critical for manufacturing scale-up.

DEPLETION OF CULTURE RESIDUALS FROM THE CELL PRODUCT

When harvested from the culture vessel, the cells reside in a solution that consists of various dissolved components that were required to support the

culture of the cells as well as dissolved components that were produced by the cells during the culture. Additionally, for adherent cells, the solution may also contain components used to detach cells from the culture vessel (e.g., trypsin). However, many of these components are unsafe or otherwise unsuitable for patient administration. Moreover, all final cell products must minimize "extraneous" proteins in the final product. For instance, in the FDA regulation 21 CFR 610.15b of 21, regarding General Biological Products Standards, the risk of extraneous proteins known to be capable of producing allergic effects in human subjects is addressed and provides an upper limit for serum concentration in the final product.

To create cells ready for therapeutic use in humans, it is therefore required to separate the dissolved components from the cells by replacing the culture solution with a new solution that has a desired composition, such as a pharmaceutical-grade, safe, injectable, electrolyte solution suitable for storage and human administration of the cells in a cell therapy application.

Current separation or washing strategies are based on the use of either centrifugation or filtration. An example of centrifugal separation is the COBE 2991 Cell Processor (COBE BCT) and an example of a filtration separation is the CYTOMATE® Cell Washer (Baxter Corp). Both are commercially available automated separation devices that can be used to separate dissolved culture components from harvested cells. However, as shown in Table 9.1, these devices result in a significant drop in cell viability and a reduction in cell numbers. The cell product used in these studies was a mixture of hematopoietic, endothelial, and mesenchymal cells derived after culturing BM mononuclear cells *ex vivo* for 12 d in long-term BM culture medium (Iscove's Modified Dulbecco's Medium containing 10% fetal bovine serum, 10% horse serum, and 5 μM hydrocortisone).

It is certainly desirable for a separation process to minimize damage to the cells and result in a cell product that is depleted of unwanted dissolved components while retaining high viability and biological function with minimal loss of cells. To address this, we have developed a novel method that performs the necessary separation steps within the bioreactor before the cells are harvested, as opposed to conventional methods where cells are washed after harvesting from the culture vessel. Because of the unique fluid dynamic char-

TABLE 9.1. Average Viability and Cell Recovery Using Currently Available Cell Washing Methods

Operating Principal	COBE 2991 Cell Processor ($n = 3$) % Centrifugation	Cytomate® Cell Washer ($n = 8$) % Filtration
Prewash % viability	93	93
Postwash % viability	83	71
Viable cell recovery	73	61

acteristics of the bioreactor, this approach results in very low cell damage and cell loss while simplifying the process by eliminating a separate piece of equipment for the washing process.

In preliminary studies, key variables affecting performance were tested to optimize cell viability and minimize cell loss while sufficiently depleting culture and harvest components (e.g., bovine serum and trypsin). To date, we have achieved greater than 90% viability and less than 5% loss of cells to the effluent wash solutions after washing and harvesting is completed. Several key variables have shown an effect on the outcome. Ensuring that the bioreactor remains static with the cells settled on the bottom surface is required. By completing all wash steps before any agitation, cell loss was reduced to a very low level. Additionally, as expected, it was important to perform two different wash steps, one before enzyme exposure to deplete culture residuals (e.g., the serum proteins in the culture medium) and one after enzyme exposure to deplete the enzyme.

The culture device can be viewed as a parallel plate flow chamber from a fluid dynamics standpoint and has a parabolic flow velocity profile across its height that results in a significant, but not total depletion of culture residuals for each volume exchange of the culture device with wash solution. In the preliminary studies, proportional reduction in residuals with increasing exchange volume was confirmed and a total wash volume of approximately 5 L was established that resulted in culture residual levels that were similar or lower than current processes. Gravity flow from reagent supply bags placed approximately 30 in. above the culture bioreactor was sufficiently slow so that no substantial cell loss occurred into the wash stream at any of the wash volumes tested, provided the bioreactor remained static. Figure 9.6 shows preliminary data comparing the new wash process to the Cytomate process, both post wash. First, cell recovery and viability were examined. As shown in Figure 9.6a, the yield of viable cells from the new process was almost double the yield of the Cytomate process. A dramatically lower viability of the Cytomate washed cells was observed compared to the new process (Figure 9.6b).

Since cell recoveries and viabilities were greater with the new wash process, it was of interest to examine the efficiency of the washing process. For this, levels of bovine serum albumin (BSA), which is a main component of fetal bovine serum used in the culture medium, were measured in the final product. Results in Figure 9.6c show that the new wash-harvest process actually had significantly lower residual levels of culture materials compared to the Cytomate process.

This novel wash-harvest method currently results in a harvested cell volume of approximately 250 mL. This volume may be suitable for intravenous infusion, but is typically much higher than is desired for patient administration in many tissue regeneration applications, where volumes as low as 5 mL may be indicated. The conventional approach of centrifugation and removal of excess supernatant is not ideal because of the manual steps involved, variability in

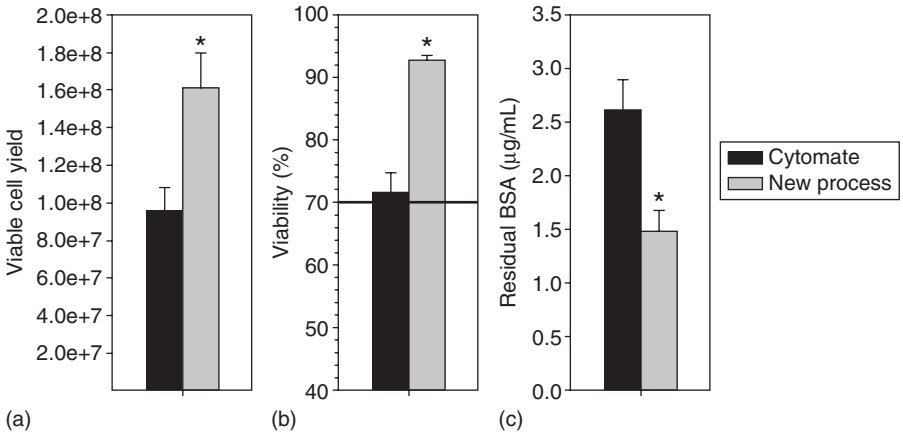

Figure 9.6. Comparison of New wash-harvest process to the Cytomate® Wash Process. Bone marrow mononuclear cells were grown in duplicate bioreactors for 12 d in long-term bone marrow culture medium (IMDM containing 10% fetal bovine serum, 10% horse serum, and 5 µM hydrocortisone). Cells from one bioreactor were harvested using either the New Wash-Harvest process while the other bioreactor was harvested by trypsinization then washed using the Cytomate®. Total cells were enumerated and viability was assessed. In addition, the amount of residual BSA present in the supernatant from the final cell product was measured. (a) The yield of viable cells from each process. (b) The percent viability of the cell product after each process. (c) The amount of residual BSA detected in supernatant from the final cell product after each process. Results are the average and standard deviation from nine individual experiments using nine different bone marrow donors. *Denotes significant difference with $p < 0.01$.

final product volume, and multiple steps required to achieve the 30- to 40-fold reduction. Future work will include the development of a volume reduction process that is sterile, closed, and able to be integrated and automated with the cell wash and harvest process.

SUMMARY

This chapter has reviewed the practical aspects of transferring an *ex vivo* cultured patient-specific adult stem cell product from preclinical bench top studies to patient administration in a clinically and commercially viable way. The first step in *ex vivo* cell therapy is defining a culture expansion process that results in the production of therapeutically active cell products. Once defined, this expansion process needs to be incorporated into a GMP-compliant manufacturing platform. Scale-up of the manufacturing process has multiple meanings depending on the application and therapeutic approach. In one approach, scale-up is increasing the number of cells produced in each particular lot or

batch. In other approaches, increasing the number of cell products that can be manufactured in a given amount of time is necessary. This latter definition has more recently been referred to as "scale-out" rather than scale-up. In autologous therapies, each patient receives his/her own lot of cells; therefore the ability to increase the number of lots that can be manufactured is essential.

Many steps are required after the cell product is harvested prior to administration to the patient. These include removal of residual medium components, such as animal-derived proteins, and formulating the cell product for delivery to the patient, such as volume reduction or combining cells with carrier or matrix material. Commercially available automated systems to assist in washing and concentrating cell products are not optimal. There is a definite need for the development of alternative strategies for postharvest processing of cell products.

Cell therapy is a promising approach to the treatment of a vast array of diseases and tissue injuries. As cell therapies move further along into later stage clinical trials, the ability to manufacture cell products under GMP, and the ability to scale-up or scale-out to support demand, will become critical components to their commercial success.

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PART III

10

GENETIC AND EPIGENETIC FEATURES OF STEM CELLS

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INTRODUCTION

Recent advances in technology have allowed science to begin grappling with the complexity of the human genome. This has raised anticipation for an era of "personalized medicine," when treatments will be chosen based upon each patient's genome and its influence on drug responsiveness, pathogen resistance, or oncogenesis. Stem cell technologies also offer a personalized medicine; it is difficult to imagine a form of medicine more personal than one's own cells, grown and directed *in vitro* for transplantation back into the site of disease or injury. Recent advances may make this possible for every patient, by allowing differentiated cells to be reprogrammed to the pluripotent state.

Clinical use of stem cell technology would vastly increase the number of human embryonic stem cell (hESC) lines in existence. Studies have revealed variations among existing lines in their undifferentiated phenotypes and differentiation potential, which may be inherent rather than a result of different derivation methods. Other studies have documented changes in potential that occur after derivation, during adaptation to culture conditions. These variations create risks for clinical practice, in that each hESC line may respond uniquely to directed differentiation efforts or carry a hidden danger of overproliferation in the recipient. New technologies for high-resolution mapping of the human genome and epigenome create the possibility of monitoring

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these risks. However, the science of interpreting genomic and epigenomic data is still in its infancy.

How might analysis of the genome and epigenome improve stem cell therapies? First, genomic and epigenomic technologies are necessary to demonstrate the stability of cultured stem cells and their derivatives. This will be essential in evaluating the cancer risk posed by cell replacement therapies. One hazard of hESC transplant is teratoma formation; steps must be taken to ensure that only differentiated derivatives are transplanted (1). However, the long-term cancer risk due to mutations acquired in culture must also be considered. hESCs frequently gain a copy or partial copy of chromosomes 12 and 17 in late passages, which is also a feature of teratocarcinomas (2, 3). Many smaller karyotypic abnormalities have been observed in hESC in culture (Table 10.1), although the phenotypic consequences are mostly unknown. In a few recent articles, mature cell types derived from hESC have been trans-

TABLE 10.1. Abnormalities Reported in hESC after Initial Derivation, or Following Extended Passaging

References	Chromosome Abnormalities	Epigenetic Changes	Other
(29)		CpG island methylation	
(37)		CpG site methylation, incl. imprinted genes	
(130)	Trisomy 12, +2q inv9		
(2)	+12p +17q		
(34)	+1, der(6)t(6;17)(q27;q1)	Loss of X-inactivation	
(57)	+13q21.3–31.3 Trisomy 17		
(131)	Trisomy 13		
(33)		Variable X-inactivation	
(3)	+i(12) del(7)(q11.2)		
(132)	+idic(X)(q21)		
(133)	Trisomy 12		
(26)	+17q, +1q, +20, c-myc amplification	Promoter CpG methylation	Mitochondrial sequence changes
(31)	+17 +12, +17 XXY, +12, +17 XXY, +12, +14, +17		
(134)	Trisomy 20		
(135)		Rare change in H19 imprinting	
(136)	XXY, +12, +17		

planted into animal disease models and successfully reversed degenerative conditions, only to have the "cured" animals die from hyperproliferation of the human cells. Overproliferation has been frequently reported following transplantation of undifferentiated ESC (4–7), but in several cases the evidence points to outgrowth of more committed precursor cells (8). The problem is not limited to embryonic stem cells. Mesenchymal stem cells from bone marrow can acquire a transformed phenotype during extended passaging *in vitro* (9, 10). Clinical hematopoietic stem cell transplants do not typically require any expansion in culture; nevertheless, donor-derived malignancies do occur at low frequency (11). Clearly, any cells intended for clinical transplantation must first be evaluated for intact chromosomes and undamaged growth control pathways.

Second, genomics and epigenomics may give information about the pluripotent or differentiated state of cells in culture. Changes in the karyotype of mouse ESCs, such as trisomy 8, correlate with loss of pluripotency and the capacity to colonize the germ line (12, 13). Genetic variations also play a role, as differences in germline contribution are observed among ES lines from different mouse strains (14). Pluripotency also depends upon the epigenetic state, as will be detailed below. Differentiation of hESC is accompanied by genome-wide chromatin reorganization (15). Subsequent fate choices are maintained via epigenetic inheritance, but methods now exist to reprogram adult cells to the pluripotent state (16–22). Because reprogramming is inefficient, it may be necessary to evaluate the epigenome of such lines to determine whether the cells are pluripotent or retain some commitment to their tissue of origin. An understanding of epigenetics on this level could also advance the therapeutic use of adult stem cells, by decoding the epigenetic changes required for stem cell plasticity.

The third area in which genomics and epigenomics intersect with hESC is in the study of human congenital or complex disorders. The creation of hESC with defined abnormalities has made the developmental biology of several human diseases accessible for the first time. These include myotonic dystrophy, Huntington's Disease, and cystic fibrosis, for which hESC models have been created from affected embryos identified by preimplantation genetic diagnosis (PGD) (23, 24). This approach could also lead to the use of embryonic stem cells to study diseases caused by the loss of parental imprinting, such as Angelman syndrome and Beckwith–Wiedemann syndrome, or failure of X-inactivation. Complex diseases which have genetic or epigenetic risk factors might also one day be studied *in vitro* by the creation of ESC lines with highly defined genomic and epigenomic states. The discovery of methods to reprogram adult cells means that stem cell models could potentially be created for any inherited disease with sufferers willing to donate a skin sample.

This chapter will discuss the use of new technologies for whole-genome analysis of genetic integrity and epigenetic regulation in the embryonic stem cell field. Molecular biology has improved upon the speed and resolution of karyotyping, which will allow more accurate safety assessments in stem

cells destined for the clinic. We will discuss a variety of tools for molecular cytogenetics, and some limitations of their use with embryonic stem cells. The chapter will also describe new methods in epigenomics that are being used to examine the ineffable state of pluripotency. New models of cancer that examine the role of epigenetic changes in growth control and genome stability suggest that these tools may be extremely valuable for appraising the clinical potential of hESC.

ALTERATIONS IN CULTURED STEM CELLS

Embryonic stem cells are not just cells of the blastocyst relocated to a dish. Cells of the inner cell mass do not grow well in isolation, so derivation of hESC lines creates selective pressure for an adaptation to *in vitro* culture conditions (25). Adaptation implies changes in the embryonic genome or epigenome, although the changes that must occur have not been defined and may be subtle. Prolonged expansion in culture often leads to profound genomic and epigenomic alterations, as detailed in Table 10.1. Late passage hESC lines often show chromosomal gains (2), amplifications of subchromosomal regions (26), loss of heterozygosity (LOH) (27, 28), alterations of mitochondrial DNA (26), and changes in promoter methylation (26, 29). The most common karyotype changes are duplications of the short arm of chromosome 12 and the long arm of chromosome 17, which confer a selective advantage in culture (2, 3). Such chromosomal changes are commonly associated with enzymatic passaging of hESC (30, 31).

Similarly, epigenetic changes that deregulate growth can be favored in extended culture of hESC. Pluripotent stem cells exist only transiently during development *in vivo*, during a period of dynamic epigenetic activity (32). Human embryonic stem cells are derived from six- to seven-day-old cultured blastocysts, comparable to preimplantation embryos. The epigenome is subjected to general erasure in the preimplantation period, followed by genome-wide *de novo* methylation occurring about the time of implantation. The exceptions are the imprinted genes, whose parent-specific epigenetic marks are spared from this remodeling process. In female embryos, the preimplantation period also sees epigenetic inactivation of one of the pair of X chromosomes, simultaneous with the earliest cellular differentiation events (33).

Epigenetic remodeling can go awry *in vitro*. Epigenetic dosage compensation of the X chromosome may not be occurring normally in hESC, as some female hESC lines exhibit X-inactivation prior to differentiation and some do not (33). In some female lines, X-inactivation fails during prolonged culture (34). This can affect other chromosomes; in some female mouse ES lines, failed dosage compensation leads to global hypomethylation of the genome (35). Culture conditions can also perturb imprinted genes; assisted reproductive technologies raise the risk of imprinting disorders (36). hESC lines are typically

derived from embryos left over in assisted reproduction, which means that epigenomic integrity should be considered prior to cell therapy applications.

Epigenetic modification of gene promoters is also essential for differentiation of cells both *in vivo* and *in vitro*; thus undifferentiated hESC are epigenetically distinct from their progeny (37). Various hESC lines show different patterns of promoter methylation, and these epigenetic marks are unstable during culture (29). Both gains and losses of methylation occur *in vitro* and changes are most frequent shortly after derivation of the hESC line. The changes are inherited through extended passaging and in differentiated progeny. Comparison of differentiated cells derived from hESC with equivalent primary cells also shows that aberrant methylation occurs in culture (38).

One possible consequence of the abnormalities that arise in hESC derivation and culture is that their differentiated progeny will fail to integrate and function properly after transplantation. The more frightening prospect is that the same changes that promote growth of hESC in culture will also promote overproliferation or cancer in patients. Tumors show a wide variety of genomic abnormalities, including mutations, copy number changes, and allelic losses; some of the same changes occur in high passage hESC; for instance, amplification of chromosome arms 12p and 17q are hallmarks of teratocarcinoma (39). The roles of epigenetic changes in cancer initiation and progression have only recently begun to be appreciated (40). Established tumors exhibit genome-wide alterations in DNA methylation, typically featuring both reduction of CpG methylation overall and hypermethylation of some gene promoters. Global changes in histone modification are also common in cancer (41). Major growth control genes may be epigenetically silenced rather than mutated, while general epigenetic changes can promote genomic instability. Later in the chapter we will examine epigenetic pathways which maintain pluripotency; components of these are frequently perturbed in cancer, which suggests that the unique epigenetic state of stem cells contributes to their susceptibility to oncogenic transformation (42).

The cell therapy field cannot expect to succeed without taking every precaution for patient safety. This poses several challenges for genomic and epigenomic assessment of stem cells. First, assays must be developed to detect abnormalities at high resolution; these must not be overly costly in terms of money, time, or cell material. Subsequent sections of this chapter will describe new whole-genome technologies for genomics and epigenomics, along with their capacities and limitations. Second, we must attempt to define the stem cell epigenetic state that supports true pluripotency, not just self-renewal and undifferentiated biomarker expression but also the capacity for unhindered differentiation and maturation. Global analysis of the epigenome is relatively new, and this chapter will attempt to summarize what has been learned so far about the unique epigenome of embryonic stem cells. Third, we need to develop bioinformatic data from cancer and stem cell studies that help to discriminate

risky alterations from benign ones, and define how much culture adaptation is too much. This data is being collected for tumor cells and somatic tissues, and as we shall see, the human genome contains some surprises. It is likely that the tools being developed for cancer studies will also yield safety benefits for cell therapy.

CYTOGENETIC METHODS

Recent years have brought rapid growth in the number and capabilities of methods for cytogenetic analysis. An excellent summary of the technical details and development of these assays is by Speicher and Carter (43). The techniques fall into two general categories: whole chromosome methods, which analyze the intact chromosomes of mitotic or interphase nuclei; and molecular cytogenetic methods, where the extracted or amplified DNA is analyzed by examining specific target sequences (Table 10.2).

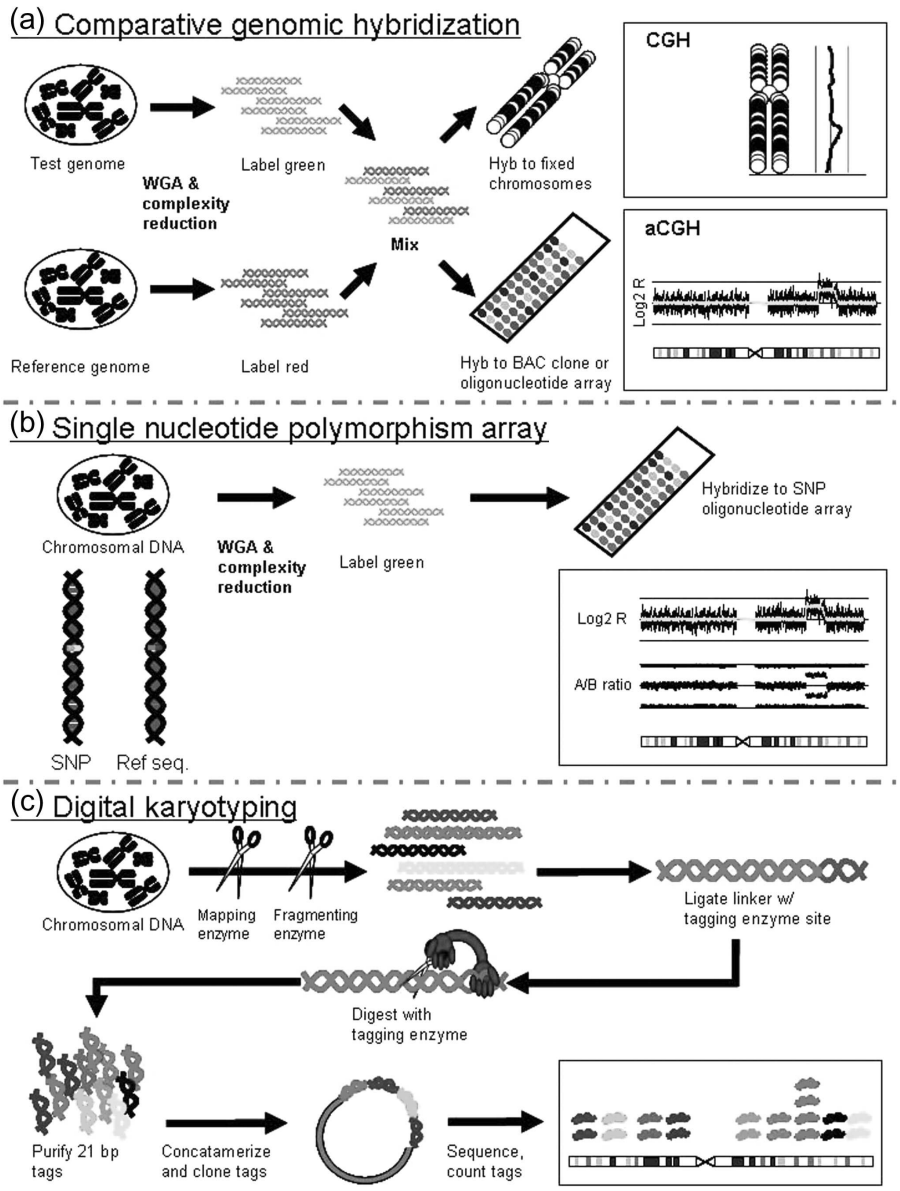
By far the most commonly used cytogenetic method in the hESC field is the classic G-banded karyotyping. This is still the most powerful, able to detect aneuploidies, insertions, deletions, inversions, and translocations with single-cell sensitivity. The spatial resolution is not as fine as with molecular methods, and is mainly dependent on the expertise of the cytogeneticist in the art of producing metaphase spreads. The requirement for actively dividing cells poses a difficulty for researchers who must ship cultures to be spread and analyzed elsewhere. When hESC numbers are limited, such as at thawing or during manual passaging, it can be difficult to spare cells for karyotyping; to avoid sampling error, cytogeneticists generally prefer to analyze cells in 20 to 50 independent colonies per line.

Several improvements have been made to classical cytogenetics by the use of fluorescently labeled probes to identify sequences within the intact chromosomes. Fluorescence *in situ* hybridization (FISH) is typically used to query interphase nuclei for sequences corresponding to a particular chromosome. The only whole chromosome method frequently performed on single cells, it is used in PGD to determine the copy number of specific chromosomes; current methods do not yet allow counting of all 23 chromosome pairs in a single cell. FISH was the first method to detect microdeletions, defined as deletions too small to observe by microscope. With careful probe choice and methods to pull chromatids out to a line (fiber FISH), it can be used to map rearrangement breakpoints to very high resolution (44).

For simultaneous analysis of all the chromosomes, a variety of techniques have been developed for chromosome painting. In these methods, a combination of FISH probes is interpreted by computer to algorithmically paint each chromosome a different color. The various techniques are known as Multiplex-FISH (M-FISH) (45), Spectral Karyotyping (46), or Combined Binary Ratio Labelling (47). These methods are especially valuable for the detection of small translocations. However, position along the chromosome is difficult to

TABLE 10.2. Common Cytogenetic Methods for Whole Genome Analysis

Method	Detects	Resolution	Starting Material	Notes
Whole Chromosome Methods				
G-banded karyotype	Deletion Amplification Translocation Inversion Marker Chromosomes	10–20 Mb	Dividing cells in metaphase	Best detection of abnormal subpopulations
FISH	Aneuploidy (few chromosomes tested) Translocation Microdeletion syndromes	Interphase: 50–100 kb Fiber FISH: 1–5 kb	Interphase nuclei	
Chromosome painting	Aneuploidy Translocation Marker Chromosomes	2–3 Mb	Dividing cells in metaphase	Labor intensive
Molecular Cytogenetics				
Metaphase CGH	Aneuploidy Amplification Deletion	5–10 Mb for deletions; 1 Mb for amplification	Genomic DNA WGA	Labor intensive
aCGH	Aneuploidy Amplification Microdeletion CNP	32 kb	Genomic DNA WGA	Strictly copy number (no LOH)
SNP array	Aneuploidy Amplification Microdeletion LOH Copy-neutral LOH Uniparental disomy	~25 kb	Genomic DNA WGA	
Digital karyotyping	Aneuploidy Amplification Microdeletion CNP	Single gain or loss: 4 Mb 100 000 tags <0.6 Mb 1 000 000 tags	Genomic DNA WGA	Can detect nonhuman sequences



determine, so breakpoint mapping must be done by FISH or banding techniques. Chromosome painting is also relatively insensitive to changes in the overall length of chromosomes, so small intrachromosomal rearrangements such as inversions, microamplifications, or microdeletions are not detectable.

MOLECULAR CYTOGENETICS

Molecular cytogenetics begins with comparative genomic hybridization (CGH) (48) (Fig. 10.1a). In conventional or metaphase CGH, genomic DNA is extracted from the test sample as well as a normal diploid reference sample. The two samples are differentially labeled with fluorescent markers and hybridized to immobilized metaphase chromosomes. Any region of the fixed chromosome that is not equally labeled with both fluorescent tags indicates that the test sample bears a copy number variation in the corresponding genomic region. Thus, insertions, deletions, and whole chromosome copy number changes can be detected in isolated DNA, or in whole genome amplification (WGA) material amplified from a single cell (49). This method greatly increases the resolution of detection over G-band karyotypes, while also broadening the FISH methods so that abnormalities can be discovered anywhere in the genome.

Figure 10.1. Schematic diagram of methods for molecular cytogenetics. Three molecular biological approaches for assaying DNA copy number are presented in cartoon form; many details of each procedure have been omitted for clarity. The mapping of results across one chromosome is illustrated in boxes at the right of each figure. (a) In comparative genomic hybridization (CGH), DNA from the test cell is amplified without bias and fluorescently labeled, in parallel with DNA of a diploid reference cell, which is labeled with a different fluorophore. These labeled DNAs are mixed, then hybridized either to immobilized chromosomes on a slide or to an array of BAC clones or oligonucleotides. The ratio of the two labels varies significantly from 1:1 only where the test cell carries an amplification or deletion of the corresponding chromosomal fragment. (b) Single nucleotide polymorphism (SNP) array analysis is performed similarly to aCGH, except that the array carries oligonucleotides corresponding to both the major and minor alleles at each locus. The data generated gives both a fluorescence ratio to measure copy number (Log2 R) and the genotype at each locus (A/B ratio). (c) Digital karyotyping is performed by cutting genomic DNA into many fragments, then cutting a 21 bp tag from the end of each fragment after appending a special linker. Long chains of tags are cloned and sequenced; 21 bp are usually sufficient to map uniquely to the genome because the position relative to the recognition site for the mapping enzyme is known. The frequency of each tag among the sequenced DNA is proportional to copy number in the genome.
WGA = whole genome amplification; Hyb = hybridization of DNA fragments; aCGH = array comparative genomic hybridization; BAC = bacterial artificial chromosome. (See color insert.)

The transition from metaphase CGH to purely molecular cytogenetics came when a library of cosmids or bacterial artificial chromosome (BAC) clones was first used to represent the genome, in place of the immobilized metaphase chromosomes (array CGH) (50). These clones were spotted in defined places on an array, so that abnormal copy number anywhere in the genome was detected as a spot unevenly labeled by test and reference probes. With improvements in the manufacturing of oligonucleotide arrays, the 150–200 kb BAC clones can be replaced by oligos 25–85 bases in length (51). This allows resolution to be limited only by the number of features that can be placed on an array, currently well over half a million. Array CGH is now commonly performed with WGA material from as little as a single cell.

A modification of array CGH increases the amount of information gathered to assess genetic variation along with copy number (52). A single nucleotide polymorphism (SNP) is a one base pair variation occurring anywhere in the genome; the majority of the population shares a common sequence at this site, but at least 0.1% has the same variant base or "minor allele." Most SNP array technologies detect the target sequence with oligonucleotides just like array CGH, but additionally discriminate between two alleles, termed A and B (Fig. 10.1b). Thus, SNP arrays offer copy number detection with resolution similar to array CGH, but can also detect allelotype differences such as LOH. These arrays are most commonly used in human genetics to map disease loci by association, with SNP markers spaced as little as a few kilobases apart. They are also used in cancer research for discovery of novel anti-oncogenes, which are frequently uncovered by LOH during tumor progression. Also similar to array CGH, WGA material can be genotyped by this method. The limitation of array CGH and SNP arrays is that certain karyotypic abnormalities seen by whole chromosome methods, such as balanced translocations and inversions, cannot be detected. Another important point for both array methods is that the signal-to-noise ratio for detection of single copy number changes can be greatly increased by averaging of results over several adjacent loci, at the expense of resolution. The noise for single copy detection by SNP arrays is also greatly decreased by paired sample analysis with genetically matched samples (53).

The third molecular cytogenetic method is a complete departure from hybridization protocols. Digital karyotyping (Fig. 10.1c) instead works on the same principle as serial analysis of gene expression (SAGE) (54, 55). The difference is that genomic DNA, and not cDNA, is digested with a restriction enzyme and ligated to an oligonucleotide bearing the site for a second restriction enzyme which cuts at an offset; this yields a tag of defined size, such as 21 base pairs, which can be isolated and cloned in tandem with other tags. When sequenced, the representation of any tag within the total sequence is proportional to the copy number of the corresponding locus. The resolution of this method is limited only by the frequency of the first restriction site in the genome, and by how many tags can be economically sequenced. Although digital karyotyping is intended only to detect copy number variations, allelic

variations are sure to be present in some of the tags; LOH could presumably be detected, but at much lower resolution than copy number changes. Also, this is the only molecular method that returns a sample of whatever DNA is in the sample, not just preselected probes; therefore contamination by nonhuman DNA from mouse feeder cells, fungi, even bacteria or viruses possessing the correct restriction site, can be detected. However, low-level contamination would be indistinguishable from the background noise of sequencing errors.

One challenge for all these molecular cytogenetic methods is the complexity of the human genome. Digital karyotyping reduces complexity in a very straightforward way, by sequencing not the entire genome but only short sequences located next to a particular restriction site. The bulk of the DNA is discarded early on. The hybridization-dependent methods also have to deal with sequence complexity, or else nonspecific hybridization will swamp out the detection of the relatively rare target sequences. Metaphase CGH protocols often include unlabeled placental Cot-1 DNA or herring sperm DNA to absorb nonspecific binding of labeled DNA fragments. Oligonucleotide and BAC array methods for CGH and SNP genotyping typically require that the complexity of the genomic sample be reduced prior to probe labeling. This is done either by a restriction digest of the genome followed by size fractionation, or by various polymerase chain reaction (PCR) methods that simultaneously increase the amount of DNA for the analysis and reduce the genome to those regions flanked by primer sites and of a product size selected by the PCR conditions. However, oligonucleotide arrays have been used for analysis of total genomic DNA without a complexity reduction step (56). This simplifies the assay in cases where sufficient genomic DNA is available, but the quality of genomic DNA can greatly affect the reliability of molecular cytogenetics (57).

For certain samples, such as single blastomeres for PGD or preserved tissue biopsies, sufficient genomic DNA for a whole-genome analysis is not available. A key advantage of molecular cytogenetics is that the hybridization methods can be performed with amplified DNA from as little as a single cell. This was first demonstrated by metaphase CGH for single fetal cells (58) or single blastomeres (59). Several methods for whole-genome amplification of the test DNA have been developed, with the primary concern being representation, or the amplification of all sequences in proportion to their prevalence in the starting material. Techniques to minimize amplification bias include primer extension preamplification (60), degenerate oligonucleotide primed PCR (61), and multiple displacement amplification (MDA) (62). The MDA method has several advantages, including linear instead of logarithmic expansion with a highly processive bacteriophage phi29 DNA polymerase, which also offers high proofreading activity. Thus, sequence errors from misincorporation and degenerate primers can be avoided, but MDA does not perform well with DNA from fixed or degraded samples (63). SNP array experiments have been performed following amplification by several methods including MDA (64–68), but no SNP array analysis of single cells has been reported. Array CGH experiments have been successfully conducted with MDA products from a

single cell (65, 66). However, MDA from a single cell often fails to yield unbiased amplification, instead preferentially amplifying certain regions or one of two heterozygous alleles, whereas amplifications from 10 ng or more of genomic DNA have low rates of these errors (63, 67, 68).

CYTOGENETIC STUDIES OF EMBRYONIC STEM CELLS

Commercial SNP arrays from Affymetrix have been used to demonstrate karyotype changes in several hESC lines due to extended passaging, despite various culture methods and mechanical or nonenzymatic passaging (26). These range from duplication of entire chromosomes to amplification of as little as 2 Mb encompassing the *myc* proto-oncogene. These changes were confirmed via qPCR or FISH, and were also observable using a competing SNP array from Illumina, Inc. (26, 69). Both array hybridizations were conducted following WGA for complexity reduction. In addition to structural changes, SNP arrays allow detection of genetic variants such as LOH and disease-linked markers; these can be observed in hESC and other cell lines (57).

Both chromosome and array CGH methods primarily measure a ratio of hybridization intensities of two differently labeled probe sets, one from the test sample and one from the reference (Fig. 10.1a). In array CGH, the base 2 logarithm of this ratio is graphed over distance along the chromosome; a log2 close to zero indicates that both probe sets have the same intensity, so the copy number of the test is the same as that of the diploid reference DNA. Values above zero indicate copy number amplification in the test sample, and values below zero indicate loss; the change corresponding to a single copy loss is theoretically –1, but actually varies between platforms and this affects discrimination (70). SNP arrays measure copy number as well, but as the sum of intensities for two alleles, A and B.

Illumina SNP array data are presented as two variables mapped over the length of a chromosome (Fig. 10.1b). The first value, Log R ratio, is a comparison between two samples of the total signal intensity for that locus (53). A measurement of zero indicates that the test sample has the expected intensity, based on the reference sample or training set, and thus the copy number of the locus is unchanged. Values above zero mean that the test sample has a higher intensity than expected, indicating amplification, and values below zero point toward a deletion. There is typically enough noise in the measurement that the moving average over a number of loci, perhaps 3–10 SNPs, is the preferred indicator. The second value, B Allele Frequency, indicates the proportion of B calls at the locus out of the multiple measurements performed on each array. Thus a locus that is homozygous for the A allele is scored close to 0, a locus homozygous for B is scored near 1, and a heterozygous locus is scored near 0.5. As can be seen in Fig. 10.1, the B Allele Frequency not only detects genotype but is also helpful in interpreting copy number changes. A gain of a single copy pushes the B Allele Frequency for heterozygotes away

from 0.5, toward the new Mendelian ratios of 1/3 and 2/3. Greater increases in copy number produce more extreme heterozygote ratios. Loss of a single copy from a diploid line eliminates the heterozygote allele frequency altogether (LOH), and all loci within the deletion are scored as 0 or 1. Heterozygotes also disappear in uniparental disomy, where a portion of one sister chromatid is replaced by a copy of the other and the cell remains diploid. Thus uniparental disomy appears as a LOH region with a Log R ratio of zero but no heterozygous allele calls.

Like CGH, SNP genotyping requires a reference sample. The above referenced analyses were performed with paired hESC lines, comparing a late passage line to an earlier passage, or a known variant line with a karyotypically normal exemplar of the same line (26, 69). The origin of the paired lines from the same embryo is easily demonstrated in SNP arrays, because the allelic patterns are identical in the vast majority of loci. One caveat of paired analysis is that any karyotypic changes that occurred during establishment of the line, before the earliest passage tested, will not be detectable. To do so requires comparing the data to a training set of data compiled by analyzing 100–200 genetically diverse samples on the same type of array. Fortunately, data sets from normal adult tissue are sufficient because training sets from hundreds of normal hESC lines would be difficult to create. Lymphocyte cell line training data is also available, but should be avoided because of copy number polymorphisms that occur following immortalization with Epstein–Barr virus (71). Training sets from normal individuals are typically available from array manufacturers. The use of training sets allows SNP genotyping of a single unpaired sample, but the paired sample technique increases the signal to noise ratio (53).

Although the spatial resolution of CGH and SNP arrays is generally assumed to increase with the number of elements per array, the uneven spacing of elements within the genome can be a limiting factor. Coe et al. have recently introduced a useful new metric, functional resolution, which can be described as the size of a random amplification or deletion that would fall between detection elements in no more than 5% of all possible locations (72). This is further refined into three numbers: the theoretical sensitivity, which is the functional resolution for highly amplified elements, the single-copy sensitivity, which is the functional resolution for a loss or gain of a single copy, and the breakpoint precision with which the ends of copy number polymorphisms can be mapped. The most relevant of these numbers for detecting changes in hESC lines is likely the single-copy sensitivity, which is usually poorer than the theoretical sensitivity for oligonucleotide arrays because data from multiple adjacent elements must be pooled to allow detection of a single copy change. Using a moving average of hybridization intensity improves the signal to noise ratio for copy number measurement; in fact, none of the current oligonucleotide CGH or SNP arrays can make a definitive copy number call based on a single element (70). The theoretical and single copy sensitivity of the best available large-insert BAC arrays is approximately 50 kb, but the large clone size lowers

sensitivity to smaller alterations (72). Currently, the best oligonucleotide arrays are able to detect 95% of single copy changes of 36 kb or larger. See Table 10.2.

However, as technology improves our ability to detect genetic and epigenetic abnormalities, it also redefines what is normal. Regions of copy number polymorphism (CNP) are common in the human genome, and vary between individuals (73, 74). SNP arrays have been used to map the genomes of normal humans for regions of copy number variation (75), deletions (76), homozygosity (77), and both CNP and homozygosity (71). The typical person harbors numerous examples of each of these aberrations in their genome. The stretches of homozygosity in a large subset of the population could be due to frequent uniparental disomy, but are generally thought to reflect an unsuspected rate of parental consanguinity. There is as yet little evidence that CNP or homozygous regions affect the health of most individuals; the growing list of exceptions includes the uniparental disomies associated with Prader–Willi syndrome (matUPD15), Angelman syndrome (patUPD15), Silver–Russell syndrome (matUPD7), Beckwith–Wiedemann syndrome (patUPD11p), and pseudohypoparathyroidism (patUPD20q) (78). In an effort to document CNP in the human genome, and to absolve variations found in unaffected individuals, the Hospital for Sick Children has created the Database of Genomic Variants (79).

EPIGENETICS AND THE HISTONE CODE

Epigenesis is defined as heritable changes in gene function that are not encoded in the DNA sequence. Cell fate decisions made during development are "remembered" as epigenetic modifications. Epigenomics, then, is the assessment of epigenetic marks on a genome-wide scale. Epigenetic marks are of two types: covalent modifications of DNA by methylation of cytosine, and alterations in the chromatin structure determined by DNA-associated proteins. These modifications alter gene expression by controlling access of the transcriptional machinery to gene promoters, which can be located within open chromatin or inaccessible within condensed chromatin.

Most cytosine-guanine dinucleotides (CpG) in mammalian genomes are constitutively methylated on the cytosine. The exception is the CpG islands, CpG-rich sequences which overlap the promoters of about 40% of genes (80). The CpG dinucleotides in these islands are generally unmethylated, except in situations such as differentiation or cancer where the nearby gene has been epigenetically silenced. A methyl group is added to position 5 of cytosine within CpG by DNA methyltransferases (DNMTs). DNMTs are often divided into two functional categories: maintenance and *de novo* methyltransferases. DNMT1 prefers hemimethylated CpGs as a substrate, such as a CpG methylated on the parental DNA strand but not on the newly replicated strand,

thus maintaining a preexisting methylation pattern during DNA replication. DNMT3a and DNMT3b have a strong preference for unmethylated CpG, and are considered to be responsible for *de novo* methylation. However, both maintenance and *de novo* methyltransferases remain active in differentiated cells, keeping up a steady state balance between methylation and demethylation which is required for cellular memory (81).

The role of DNA methylation in cell type differentiation is illustrated by tissue-specific differentially methylated regions (T-DMRs) (82). T-DMRs are loci which are hypermethylated in some tissues but unmethylated in others. Methylation of promoters is correlated with gene silencing both in cancer and in normal tissues; most T-DMRs are located in gene promoter CpG islands, 5′ to genes which are transcriptionally silent in differentiated tissues (83). Methylated T-DMRs are predominately associated with silenced genes. They are recognized by methyl-CpG binding proteins, such as MECP2 and MBD2, that recruit corepressors including histone deacetylases (HDACs) and thereby link DNA methylation to histone modifications and chromatin condensation. In contrast, unmethylated CpG dinucleotides are specifically bound by CpG-binding protein, a transcriptional activator.

The second major category of epigenetic marks is histone modifications. Diverse developmental signals are integrated within the chromatin structure, whose accessibility to the transcriptional machinery governs cell type-specific gene expression. Local openness of chromatin is determined by discrete domains of altered nucleosomes. The N-terminal tails of the histone core proteins are the site of modifications including methylation, acetylation, phosphorylation, ubiquitination, sumoylation, and at least a dozen others. Activation of gene expression requires DNA-binding transcription factors, which in turn recruit multiprotein transcriptional coactivator complexes that have histone acetyltranferase (HAT) activity. HATs add an acetyl group to lysines in histone proteins 3 or 4 (H3ac or H4ac), and these acetylated histones are found mainly at the 5′ end of coding regions (84). Acetylated histones are in turn recognized by bromodomain proteins, which recruit further protein complexes to the local chromatin. Histone acetylation recruits SWI/SNF, NURF, and RSC ATP-dependent chromatin remodelling complexes that assemble/dissassemble and reposition nucleosomes, as well as p300/CREB binding protein (CBP), p300/CBP-associated factor (PCAF) and Spt-Ada-Gcn5 acetyltransferase (SAGA) complexes which contain HAT activities. HATs likely propagate the open chromatin structure across the gene, allowing room for binding and processivity of the RNA polymerase complex.

Chromatin structure is also influenced by methylation of the histone tails. Trimethylation of lysine 4 on histone 3 (H3K4me3) is found at most active promoters in the genome (85). H3K4me3 mainly marks discrete regions at the 5′ end of active genes, especially those with 5′ CpG islands (86). H3K4 methylation is catalyzed by trithorax group proteins (trxG), a group of transcriptional antirepressors. The trxG family also includes the chromatin remodeling

SWI/SNF and NURF enzymes. The H3K4me3 mark is recognized by the chromodomain protein Chd1, which recruits the SAGA complex that catalyzes histone acetylation and leads to chromatin remodeling (87).

In contrast, transcription is repressed by trimethylation of lysine 27 on histone 3 (H3K27me3) or di- or trimethylation of lysine 20 on histone 4 (H4K20me2/3). H3K27me3 often marks large chromosomal domains hundreds of kilobases long, such as the HOX complexes in embryonic stem cells. H3K27 is methylated by polycomb group proteins (PcG), a group of transcriptional repressors mainly found in two complexes called Polycomb Repressive Complex 1 and 2 (PRC1 and PRC2). The enzyme EZH2 is a component of PRC2 that catalyzes the repressive methylation of H3K27, and also recruits DNMTs to target genes (88, 89). Subsequently, the methylated H3K27 is bound by Polycomb, a component of PRC1. PRC1 activates several pathways to repress transcription, including ubiquitylation of histone 2A at lysine 119 (H2AK119ub), preventing nucleosome remodeling by SWI/SNF, and directly blocking transcription initiation (90). In *Drosophila*, interactions between trxG and PcG proteins are required for maintenance of epigenetic states (91).

Many other histone modifications occur, with various effects on chromatin and transcription. One more example is methylation of histone 3 at lysine 9 (H3K9me), which is a signal for formation of constitutive heterochromatin within silenced domains such as pericentromeric DNA. H3K9me is the signal for binding of heterochromatin-associated protein-1 (HP1). The histone methyltransferase SUV39, which methylates H3K9, also recruits DNMTs to methylate the associated DNA (92).

This and other evidence points to cross talk between DNA methylation and chromatin structure that reinforces the epigenetic control of transcription. Just as methyl-CpG is bound by proteins that recruit histone methylases and histone deacetylases, condensed chromatin can recruit DNA methylases to specific locations. This may have a role in propagating open or closed chromatin structures along the DNA, but it is not yet clear why DNA and histone modifications overlap and which takes precedence during development. However, some studies have found that gene silencing can precede DNA methylation and is concurrent with histone modifications (81). Alternatively, the two layers may interact to prevent erasure of the established epigenetic state.

EPIGENOMIC METHODS

The same advances in microarrays and sequencing that are improving the resolution of genomics also enable diverse approaches in epigenomics. Many of these have been reviewed in detail by Callinan and Feinberg (93). In general, methods of detecting epigenetic marks such as methylation-sensitive restriction enzymes, bisulfite conversion, or antibody binding can be coupled

with whole-genome detection methods such as microarrays or SAGE. Because most of the epigenetic marks of interest in stem cell biology are concentrated at gene promoters, resolution is not such an issue as it is with cytogenomics. This section will describe several methods for whole-genome analysis of DNA methylation or histone modification with less emphasis on comparative metrics.

Methylated CpG dinucleotides can be detected in several ways (Fig. 10.2). Perhaps the simplest to apply to the whole genome is restriction enzyme digestion with CpG methylation-sensitive enzymes such as the frequent cutter *Hpa*II or the rare cutter *Not*I. Afterwards, methylation of individual CpG islands can be detected by PCR. Methylation-sensitive enzymes have been combined with restriction fragment length polymorphism analysis to simultaneously assay thousands of gene promoters; this procedure is called restriction landmark genome scanning (RLGS) (94). RLGS has been used to sample up to 10% of CpG islands in the human genome without any need to preselect for promoters (29).

The identity of methylation-specific restriction fragments can be determined by hybridization to CGH arrays. One approach is to hybridize *Not*I fragments to BAC arrays (95). The rare cutter *Not*I queries relatively few CpGs, but thanks to the GC-rich recognition site these are mainly in CpG islands. The frequent cutter *Hpa*II assays many more CpGs, as in the method called *Hpa*II tiny fragment enrichment by ligation-mediated PCR (HELP) (95). This method co-hybridizes *Hpa*II fragments and methylation-insensitive *Msp*I fragments onto a custom array.

A second method to detect methylated CpG is bisulfite conversion (Fig. 10.2b). A chemical reaction with bisulfite or metabisulfite converts unmethylated cytosine to uracil, while methylated cytosine is protected. Bisulfite conversion thus produces sequence changes which, unlike methylation, are maintained during PCR amplification. Individual CpG loci can be assayed by sequencing, or by PCR reactions which produce separate products for converted or unconverted sequences (methylation-specific PCR) (97). Thus, new high-throughput resequencing methods could greatly reduce the barriers to methylation detection. Bisulfite-created sequence changes can also be detected by hybridization to oligonucleotide arrays, just as single nucleotide polymorphisms are (98). This array has been used to assess methylation of 371 gene promoters in hESC cells and show a pattern of repression in hESC that is distinct from other cell lines, adult stem cells, and differentiated cell types (37).

Array hybridization can also be used to detect methylation loci via chromatin immunoprecipitation (ChIP). In this method, genomic DNA is fragmented enzymatically or physically, then precipitated with an antibody to methyl-CpG (Fig. 10.2c). The precipitated DNA fragments can be identified by hybridization to a CGH microarray, as in methylated DNA immunoprecipitation (MeDIP) (98). Methylation is not the only epigenetic alteration detectable by ChIP; DNA-binding proteins can be cross-linked to their binding sites prior to immunoprecipitation. Hybridization to CGH arrays allows

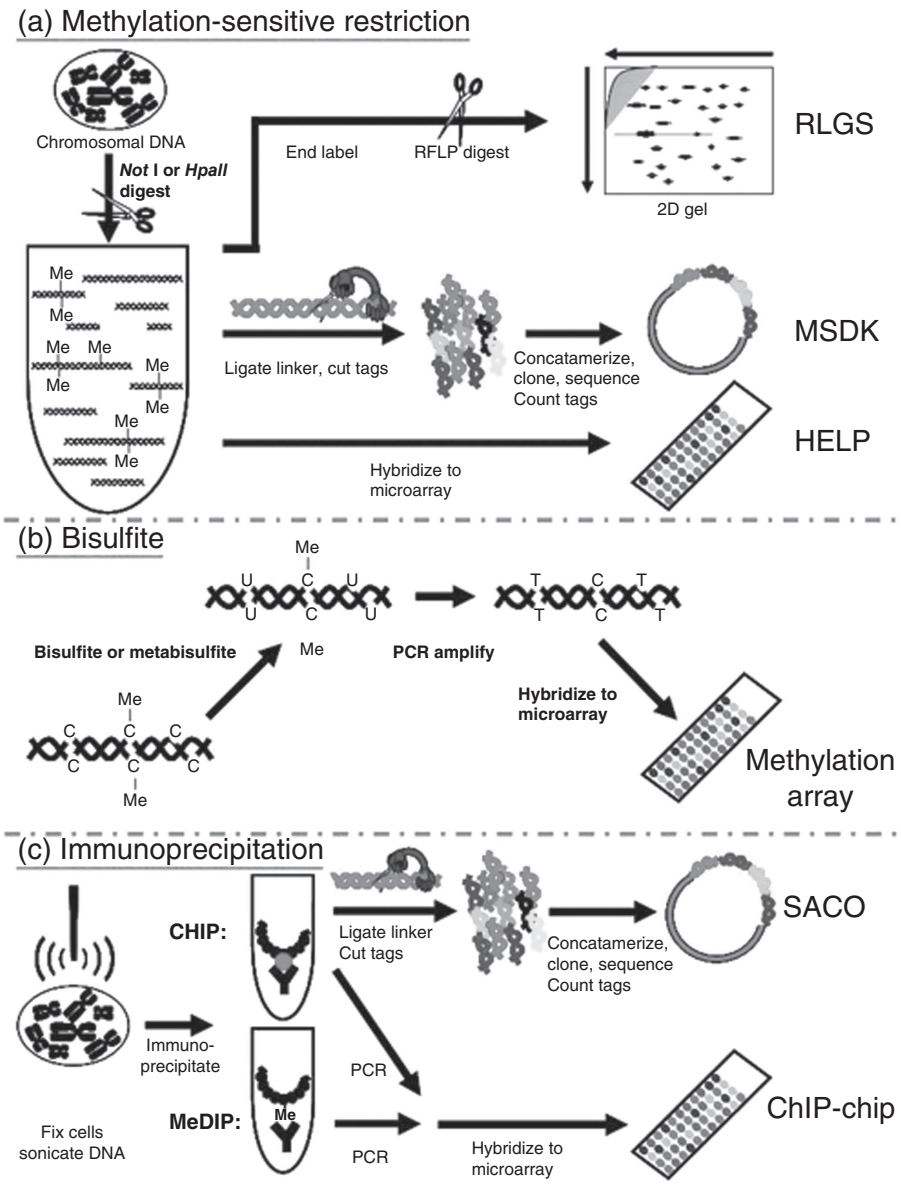


Figure 10.2. Schematic diagram of epigenomics methods. Three types of single-locus assays are typically used to assay methylation or histone modification at a site on the DNA. (a) Methylation-sensitive restriction digests the genome with restriction enzymes which will not cut if their recognition site is methylated at CpG. The resulting fragments can be compared to reference DNA or other cells on 2D gels (RLGS), by microarray (HELP), or by digital karyotyping (MSDK). (b) Bisulfite conversion converts unmethylated cytosines to uracil, creating sequence differences which can be mapped by hybridization to a microarray. (c) Immunoprecipitation of modifications can be performed with antibodies that recognize methylated CpG, modified histones, or other DNA-associated proteins. The antibody-bound DNA fragments can be amplified and hybridized to a microarray (ChIP-chip), or identified by tag sequencing as in digital karyotyping (SACO). Note that details of each procedure have been omitted for clarity.
RFLP = restriction fragment length polymorphism; RLGS = restriction landmark genome scanning; MSDK = methylation sensitive digital karyotyping; HELP = *Hpa*II tiny fragment enrichment by ligation-mediated PCR; ChIP = chromatin immunoprecipitation; MeDIP = methylated DNA immunoprecipitation; SACO = serial analysis of chromatin occupancy. (See color insert.)

whole-genome detection of promoters bound by specific transcription factors or modified histones. This method has been dubbed ChIP-chip (100). One caveat of all ChIP methods is that in mammalian genomes, relatively large amounts of repeat DNA can be nonspecifically precipitated. Another is that extensive amplification of precipitated targets may be required prior to hybridization, which can introduce bias.

Three types of arrays have been used for ChIP-chip: high-density oligonucleotide arrays, promoter or CpG island arrays, and genomic tiling-path arrays. For investigating epigenetic regulation of gene expression, high-resolution sampling of the genome may not be as important as representing the largest possible set of gene promoters.

Another tool for assessing higher-order chromatin structure at defined loci across the genome is called chromatin array (101). In this method, chromatin is fractionated according to its condensation state, either by solubility or by sensitivity to DNase I digestion, then labeled and hybridized (alongside reference genomic DNA) to aCGH arrays. In this way, areas of condensed or open chromatin can be mapped across the genome.

However, microarrays are not always required for whole-genome detection of epigenetic marks. Several methods exist to generate short tags from gene promoters and identify these through high-throughput sequencing methods, just as digital karyotyping illustrated for copy number detection. In methylation-specific digital karyotyping, genomic DNA is digested with methylcytosine-sensitive restriction enzymes (102, 103). This is followed by linker ligation

and digestion with an offset enzyme to generate a short tag sequence of 18–22 basepairs. Large numbers of tags are concatenated, sequenced, and counted just as in SAGE. DNA-binding proteins and modified histones have also been mapped using tag sequencing, and these assays are variously called serial analysis of chromatin occupancy (SACO) (104), genome-wide mapping technique (GMAT) (105), chromatin immunoprecipitation paired-end ditags (ChIP-PET) (106), or ChIP enrichment by sequencing (ChIP-Seq) (107). These vary mainly in how tags are generated and the length of tags (21–22 bp GMAT; 21 bp SACO; 2×18 bp ChIP-PET; 27–36 bp ChIP-Seq). All of these methods can assess epigenetic marks across the genome by sequencing only a small fraction of it; recent improvements in automated sequencing methods provide the necessary power to read millions of tags per assay.

EPIGENOMIC STUDIES OF EMBRYONIC STEM CELLS

It has long been hypothesized that the chromatin of stem cells might be peculiar in some way, so that stem cells are allowed a choice of multiple fates while keeping all differentiation pathways temporarily in check. This is partly based upon the distribution of heterochromatin in the stem cell nucleus. Stem cells have a higher ratio of euchromatin to heterochromatin than differentiated cells, and more diffuse heterochromatin centers (15). Biochemical assays have detected looser DNA binding of chromatin components such as HP1 and histone H1 (108). This suggests that most genes may be open to transcription in stem cell chromatin. There have been suggestions of promiscuous low-level expression of multilineage differentiated markers in multipotent cells such as HSC (109, 110).

Epigenomic studies have attempted to map active and inactive chromatin in stem cells and their progeny. Comparison of mouse ES and germ cells with differentiated cells by RLGS reveals a large number of T-DMRs, which indicates that many CpG islands throughout the genome become methylated and silenced during differentiation (81). Undifferentiated hESC can be distinguished from other cell lines and from differentiated cells by methylation array analysis of hundreds of CpG islands (37). In ES cells, large numbers of gene promoters are bound by the pluripotency transcription factors Oct-4 and Nanog; using ChIP-PET, Loh et al. mapped Oct-4 and Nanog binding in mouse ES cells to over 1000 and 3000 loci, respectively (111). However, RNAi inhibition demonstrates that Oct-4 and Nanog can activate or repress target gene transcription. In fact, inhibition of differentiation pathways seems to be a major part of their roles in maintaining pluripotency. Gene knockout studies have shown that Oct-4 represses the trophectoderm fate in ES cells and early embryos, while Nanog represses the primitive endoderm fate (112–114).

These two master regulators are themselves regulated epigenetically. In embryonic stem cells, the promoters of the pluripotency genes Oct-4 and Nanog are marked by acetylation of histones 3 and 4, plus H3K4 methylation

(115–117). In contrast, trophoblast stem cells, the earliest lineage to split off from the totipotent mammalian embryo, repress Oct-4 and Nanog, and hypermethylate their promoter DNA (115, 116). The promoters of both Oct-4 and Nanog, but not several other pluripotency-associated genes, are hypermethylated upon differentiation of hESC (118).

Thanks to epigenomics, we now have a better understanding of what makes stem cell chromatin unique and how the differentiation/pluripotency balance is maintained. Single-target epigenetic assays showed initially that the promoters of developmentally important genes not expressed in ES cells, including *Sox1, Nkx2-2, Msx1, Irx3*, and *Pax3*, carried both activating and repressive histone modifications in stem cell chromatin (119). By whole-genome ChIP-on-chip methods, Bernstein et al. have identified a general pattern in mouse ES cells where many repressed genes have a promoter marked by the activating mark H3K4me, within a broader repressive domain of H3K27me3 marks (120). During differentiation, such regions resolve to have solely repressive or solely activating marks. This "bivalent chromatin" structure marks numerous lineage-controlling transcription factor families such as the HOX, Sox, Pax, and POU groups. The structure is hypothesized to repress multiple differentiation pathways while keeping them poised to respond to developmental signals. Oct-4, Sox2, and Nanog occupy a significant number of these bivalent gene promoters, but not all (121).

However, recent epigenetic studies find that bivalent domains are not restricted to pluripotent cells; they can also be found in mouse embryo fibroblasts and neural progenitors, and T cells (107, 122). Histone methylation has now been mapped across the whole genome of hESC by ChIP-chip and by ChIP-PET (123, 124). These studies confirm that bivalent domains of H3K27me3 and H3K4me3 exist; in fact, H3K27me3 in promoter regions of hESC almost always colocalizes with H3K4me3. A second category of repressed genes carries neither of these histone marks; this is approximately a third of all genes in hESC. Many of these genes can also be induced during differentiation of hESC, which casts doubt upon the notion that bivalent chromatin primes silenced genes for expression (125). However, the set of genes marked by bivalent chromatin is quite consistent across studies and largely represents different functional categories than genes with H3K4me3 alone or with neither mark. Genes without either mark are often required for mature physiological functions such as sensory perception and the immune response, while genes with H3K27me3/H3K4me3 include more developmental regulators such as HOX transcription factors (123, 124). This could one day provide benefits by narrowing the set of genes that must be assayed epigenomically to gauge the openness of an hESC line to multilineage differentiation.

Efforts to define the structure of the hESC epigenome are paralleled by work examining how it is established. The balance of open and closed chromatin appears to be regulated by the Polycomb group (PcG) and Trithorax group (trxG) proteins. Epigenomics methods can be used to map DNA-binding proteins across the genome of ES cells. ChIP-chip assays have shown that each

of the transcription factors Oct-4, Nanog, and Sox2 binds to more than a thousand promoters in hESC and all three bind in common to about 350 promoters (121). Intriguingly, about half of these are promoters of genes not expressed in hESC. A similar ChIP-chip mapping of binding sites for SUZ12, a PRC2 component, identified many repressed developmental control genes among the targets (126). The promoters were marked by the repressive signal H3K27me3, and many were identical to the silenced genes found in the Oct-4, Nanog, and Sox2 screen. These targets of PcG repression include large numbers of homeodomain-containing transcription factors, other developmental transcription factors, and components of signal-transducing pathways involved in early lineage differentiation (90, 112, 126). This suggests that PRC2 maintains the repression of lineage specification pathways that is established by Oct-4, Nanog, and Sox2.

Genetic evidence also argues that some PcG proteins are essential for establishment of pluripotency. Targeted disruption in mice of the PRC2 components EZH2 or EED, or the PRC1 member RNF2, results in early embryonic lethality. ES lines can be established from EED mutant embryos, but not lines deficient in EZH2 (90). Other stem cell types also depend on PcG proteins for maintenance or self-renewal; adult mice lacking the PRC1 component *Bmi-1* maintain neither hematopoietic stem cells nor neural stem cells (127).

The effectiveness of PcG proteins in maintaining stem cell self-renewal may also be a factor in cancer. Genes, including anti-oncogenes, which are specifically hypermethylated in tumors are more likely to be occupied by PRC2 in ES cells (42). This raises the possibility that aberrant methylation at PRC-bound sites in tissue resident stem cells could stabilize the stem cell phenotype and lead to overproliferation. On the other hand, PcG members are generally overexpressed in tumors; they may be capable of inducing a stem cell-like state of self-renewal in precancerous cells (128).

THE FUTURE OF ESC GENOMICS AND EPIGENOMICS

It is clear that genomics and epigenomics offer the ability to collect vastly more information about an hESC line than can be obtained from the usual biomarkers. But antibodies have an advantage over reams of whole-genome data: There are recognized markers of undifferentiated hESC which can be detected by antibodies. The "-omics" fields are still working out what a pluripotent cell should look like. We need to develop a whole-genome benchmark to indicate pluripotent cells with no predisposition to oncogenesis. Alternatively, it may be possible to establish a set of specific biomarker loci that provide this information, such as the epigenetic state of anti-oncogenes.

Another area of importance to stem cell researchers that is not hotly pursued in genomics and epigenomics is the detection of variant subpopulations within a culture. As detailed above, most of the molecular assays begin by homogenizing a population of cells; a few abnormal cells could easily be

disregarded as noise. Fortunately, the trend of increasing assay resolution also decreases noise and makes smaller subpopulations detectable. Combined with improving prices for array-based assays and sequencing, these two trends may make genomics and epigenomics affordable for the routine characterization and quality control of hESC.

Since the first hESC derivation, there have been many descriptive studies attempting to define pluripotency in terms of gene or protein expression. These data sets are in general agreement but do vary between hESC lines (129). Other recent work has attempted to define pluripotency as a unique epigenetic state. The histone modification pattern called bivalent chromatin was initially thought to fit this description, but it has also been found in some differentiated cell types (107, 122). Nevertheless, the distribution of bivalent chromatin on developmental regulatory genes may yet be found to be diagnostic of pluripotency. In contrast, either hypermethylated promoters or suppressive histone marks on subsets of these genes could potentially indicate that a line has limitations on its capacity for multilineage differentiation.

Like a billion years of evolution or a lifetime of smoking, relatively brief periods *in vitro* leave their mark upon human chromosomes. Thanks to new technologies for genomics and epigenomics, we are learning to detect the whole set of subtle changes that can occur during culture adaptation. These marks constitute the cell's memory of its environment, and bias its fate. Our challenge is to interpret the marks so as to predict outcomes of differentiation and avoid problems that may manifest years after transplantation into a patient. This effort will rely heavily on databases of changes observed in cancer. The recent successes in reprogramming adult cells have moved hESC products much closer to clinical use; we should expend every effort to be sure they are ready.

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11

DIRECTED DIFFERENTIATION OF EMBRYONIC STEM CELLS

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INTRODUCTION

Human embryonic stem cells (hESCs) can be derived from the inner cell mass (ICM) of the blastocyst stage embryo (1, 2). The hESC lines generated retain their pluripotent features of early embryonic epiblast cells. This pluripotent ability enables the *in vitro* differentiation of these cells to be directed toward many cell types found in the adult human body. Thus, these cells have the potential to become sources of all adult differentiated cells, raising the hope for future cell replacement therapies in humans. However, this will only be possible if reliable protocols for directed differentiation have been established. In order to use hESCs to their optimum potential, it will be essential to understand their signaling systems and the transcription factors that drive their differentiation. Further, there will be a need to characterize and isolate precursor cells and establish methods to improve the homogeneity of differentiated cells. In addition, there is an urgent need to discover the factors that allow serum-free (and feeder cell-free) expansion of hESCs.

Little is known about human development, especially during the first few weeks of the human embryo, due to lack of experimental material. The current understanding is that during the first stages of embryogenesis, the ICM differentiates through the epiblast stage to generate three embryonic layers—the endoderm, mesoderm, and ectoderm. This process is known as gastrulation,

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and it is believed that cells migrate through the area known as the primitive streak to form the intraembryonic layers of mesoderm and endoderm. Outside the embryo (extraembryonic development), the trophectoderm, primitive endoderm, and extraembryonic mesoderm emerge. The use of *in vitro*-directed differentiation of hESCs should enable scientists to unleash the exact requirements and stages for these developmental pathways to occur *in vitro* and facilitate the understanding of the various stages of human development, which to date, remain an enigma.

Both mouse and hESCs exhibit a remarkable capacity to become virtually any differentiated tissue after appropriate manipulation in culture. This ability may be used to generate hESCs that are competent to repopulate damaged tissue in the adult host. *In vitro*, ESCs can either spontaneously differentiate into a multitude of tissues, or can partially be directed by the addition of growth factors. The ability to manipulate the cells *in vitro* is a powerful tool for the study of early developmental processes and will enable the study of otherwise inaccessible processes of early human development.

While many studies have been completed on gene expression in hESCs, we are only beginning to know which genes control the "stemness" or the self-renewal character of the cells. Over 75% of the mouse ESC (mESC) genes are expressed in hESC and vice versa, attesting to the underlying similarity of mESCs and hESCs (3). However, despite this remarkable similarity, the molecular mechanisms governing self-renewal are quite different with the self-renewal of mESCs dependent on the addition of leukemia inhibitory factor (LIF) and bone morphogenic protein (BMP) 4, while these types of factors are still elusive in the human system. Research into the transcriptional profiling of genes in hESCs has validated the pluripotential ability of hESCs with expression of "stemness" genes involved in transcription, such as OCT-4 [Pou5f1] (3–6), NANOG (3, 6), SOX2 (5), REX1 (5, 6), as well as novel genes such as SLAIN1 (3). Recent work on hESCs overexpressing NANOG enabled the propagation and multiple passaging of the cells while maintaining hESCs' self-renewal capacity in the absence of feeders and conditioned media (7). hESCs also express genes for multiple cytokine receptors, such as the fibroblast growth factor (FGF) receptor family and the BMP receptor family, making the cells receptive to cytokine stimulation (4, 6, 8). Yet, a complete understanding of the regulatory mechanisms responsible for maintaining hESCs in their pluripotent state using growth factors is still inaccessible. Even with the knowledge accrued so far, one of the most challenging and crucial, yet unanswered, questions regarding hESCs is how they can be maintained in a pluripotent state *in vitro* without feeders, animal product remains, or genetic manipulation.

Another interesting finding in hESCs is that their telomerase activity is very high and similar to the activity seen in cancer cells (1, 9–11). However, this activity decreases as the cells differentiate and mature cell populations emerge (10). Telomerase, a ribonucleoprotein that adds telomere repeats to chromosome ends, is responsible for the maintenance of chromosome length and is

instrumental in the indefinite replication capacity of cells. Telomerase expression is highly correlated with immortality in human cell lines (1, 12). The high level of telomerase activity expressed by the hESC lines, therefore, suggests that their replicative life span exceeds that of somatic cells (1) and enables long-term maintenance of cells in cell culture conditions.

Furthermore, if hESCs are to be used in the future, the cells must lose their ability to produce tumorigenic growth *in vivo*, such as forming teratomas when transplanted into immuno-suppressed mice. It appears that directing their differentiation and removing hESCs' totipotent capabilities eliminates this characteristic. Thus far, various groups have implanted differentiated hESCs into mice and found no teratoma growth occurring, including cells differentiated into neurons (13, 14), skin (15), and liver (16).

It has been shown with mESCs that in order for the cells to differentiate, the cells must pass through an aggregation step, that gives rise to cell clusters named embryoid bodies (EBs), which stimulates a stage called gastrulation. Without this step, most cells spontaneously differentiate to extraembryonic tissues (17). More recently, hESCs have been induced to differentiate in suspension and reproducibly generate, *in vitro*, into EBs comprising the three embryonic germ layers (Fig. 11.1) (18). Forced aggregation of hESCs into EBs seems to initiate a better and more robust directed differentiation of hESCs (19). Other research has shown that the addition of a variety of growth factors, including FGF2, Activin, and/or BMP4, directed the differentiation of hESCs toward certain lineages (Fig. 11.1). Unlike mESCs, hESCs can be induced to differentiate in a monolayer with the addition of BMP4 and a conditioned medium from mouse embryonic fibroblasts (MEFs) feeders and generate extraembryonic tissue, such as trophoblasts (cells of the placenta) (20), and embryonic tissue, such as neurons (14, 21, 22), skin (22), and mesenchymal stem cells (MSC) (23) (Fig. 11.2).

The family of transforming growth factor (TGF)β includes about 40 polypeptidic growth factors that share similarities in their structure and are classified into two groups: The first group includes 20 types of BMP and 15 diverse growth differentiation factors (GDFs), and the second one comprises 3 isoforms of TGFβ, activin, and nodal. All these factors, whose expression and function have been conserved in many species throughout evolution, play a key role during embryonic development in processes of cell differentiation, many of which have been tested, *in vitro*, to direct the differentiation of hESCs. However, many variables have affected the conclusions drawn so far and have made it difficult to delineate the exact roles the cytokines play in development. These variables include concentration of cytokine, time of addition, type of hESC line, and presence of serum and/or feeders. Furthermore, there could be more proteins, growth factors, adhesion molecules, and cell-to-cell interactions that will be necessary for differentiation of hESCs yet to be discovered.

Some of these variables and results will be addressed below. This section will cover the specific area of directed and spontaneous differentiation of human embryonic stem cells toward various cell lineages. The aim is to give

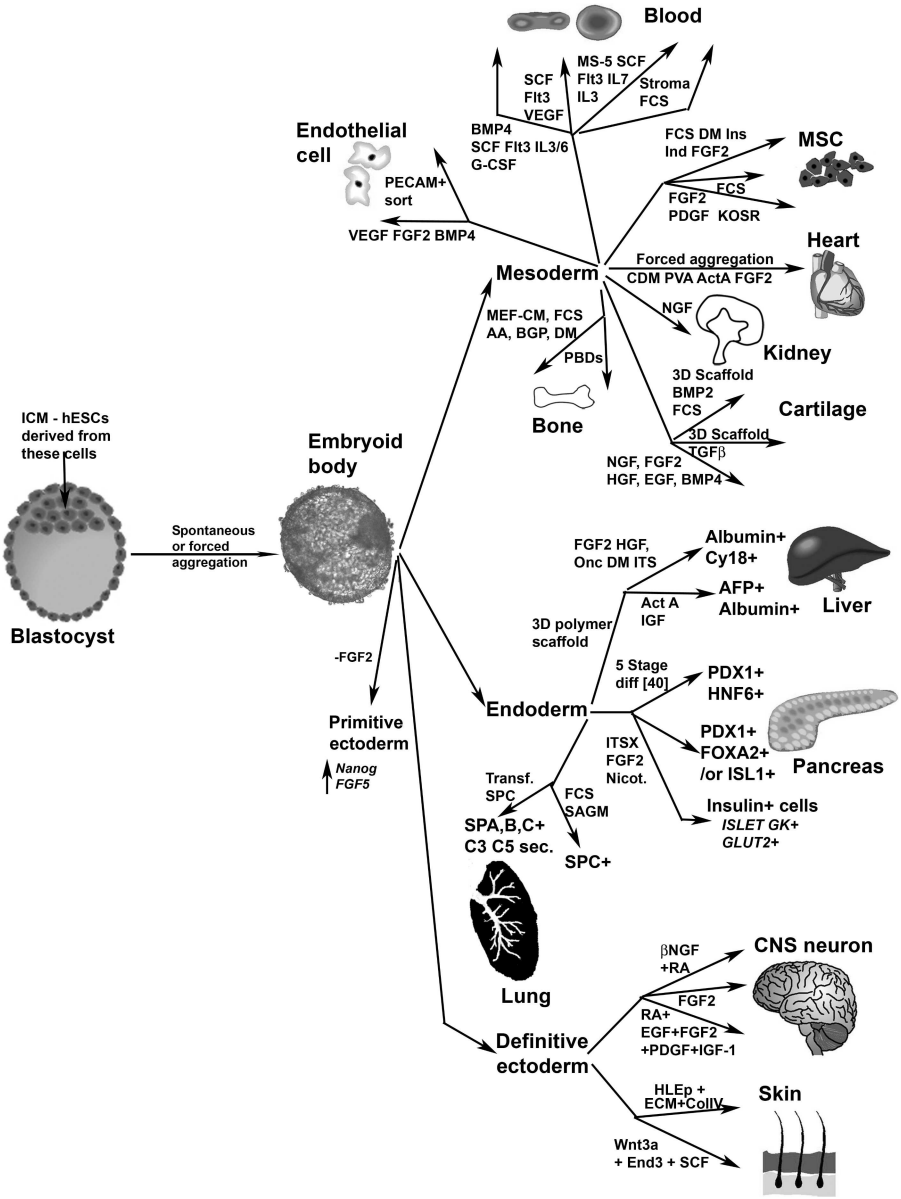

Figure 11.1. Scheme of all cell types generated from differentiation of human embryonic stem cells (hESCs) via embryoid body formation. Embryoid body formation can occur spontaneously in suspension if nonadherent conditions are used. Single cells aggregate and form round balls of growth. hESCs can also be forced into aggregates by placing cells in a round bottom well and spun in a centrifuge. Various conditions that have been used to differentiate cells can be seen by the diverse and extensive figure. VEGF = vascular endothelial growth factor; FGF2 = fibroblast growth factor 2; BMP4 = bone morphogenic protein 4; SCF = stem cell factor; IL = interleukin; G-CSF = granulocyte colony stimulating factor; FCS = fetal calf serum; DM = dexamethasone; Ins = insulin; Ind = indomethacin; PDGF = platelet-derived growth factor; KOSR = knockout serum replacer; CDM = chemically defined medium; PVA = poly vinyl alcohol; ActA = activin A; MEF-CM = mouse embryonic feeders conditioned medium; AA = ascorbic acid; BGP = beta glycerophosphate; PBDs = primary bone-derived cells; NGF = nerve growth factor; TGFβ = transforming growth factor beta; HGF = hepatocyte growth factor; EGF = epidermal growth factor; Onc = oncostatin; ITS = insulin transferrin selenium; IGF = insulin growth factor; Nico. = nicotinamide; SAGM = small airway growth medium; Trans = transfected; HLEp = human limbal epithelium; ECM = extracellular matrix; Coll IV = collagen IV; End3 = endothelin-3.

an overall understanding of the current state of this research where this area of research stands and what lies ahead for regenerative medicine to be successful. This review will raise scientific issues that need to be resolved and summarize highlights of research that have been published to date.

DIRECTED DIFFERENTIATION TOWARD ECTODERM

The Primitive Ectoderm

In mammalian development, the blastocyst contains two extraembryonic lineages, trophectoderm and primitive endoderm, with the embryo itself comprising a pool of pluripotent cells called the ICM. As development proceeds, the pluripotent cells of the ICM differentiate to form a second pluripotent cell population, the primitive ectoderm, which further differentiates to form visceral and parietal endoderm, the precursors of extraembryonic lineages, including the cells of the embryo proper (24). During gastrulation, the primitive ectoderm gives rise to the primary germ layers of the embryo proper, mainly, the mesoderm, endoderm, ectoderm, and the extraembryonic mesoderm. The generation of primitive ectoderm cells would enable a better understanding of how the embryonic germ layers are formed.

The primitive ectoderm can be distinguished from the ICM by its inability to form primitive endoderm. However, molecular characterization of these postulated populations has not been possible because few markers delineating this time in embryogenesis have been described. In an *in vitro* mouse model

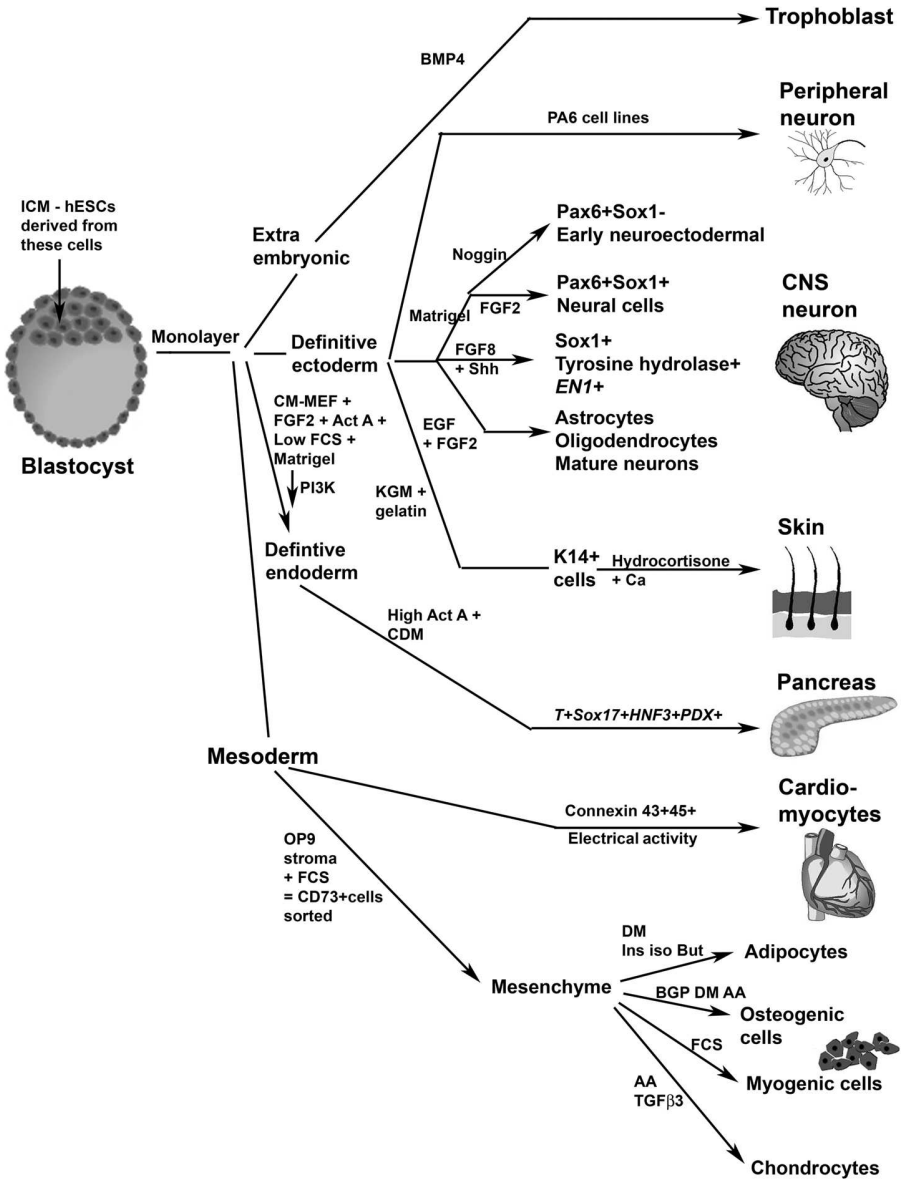

Figure 11.2. Scheme of all cell types generated from differentiation of human embryonic stem cells (hESCs) from a monolayer. hESCs cells can be differentiated to various cell types in a monolayer. This monolayer of cells is grown feeder-free. Once feeders are removed, the cells no longer maintain the pluripotent state but begin to spontaneously differentiate. The differentiation can be directed by the addition of growth factors or serum. BMP4 = bone morphogenic protein 4; FGF8 = fibroblast growth factor 8; Shh = sonic hedgehog; EGF = epidermal growth factor; Act A = activin A; CDM = chemically defined medium; FCS = fetal calf serum; DM = dexamethasone; Ins = insulin; IsoBut = isobutylxanthine; BGP = beta glycerophosphate; AA = ascorbic acid; TGFβ3 = transforming growth factor beta 3; KGM = keratinocyte growth media; OP9 = name of stromal cell line.

inducing primitive ectoderm formation, four novel genes were identified and believed to be markers of primitive ectoderm, and were differently expressed during pluripotent cell progression: *peri-implantation stem cell 1 (Psc1), Cp2-related transcriptional repressor-1 (CRTR-1), protein related to cut1/ESP1 (PRCE)*, and *FGF5* (25). More recent work with hESCs overexpressing NANOG demonstrated that its expression in hESCs is upregulated during early differentiation, and expression patterns of genes seen in the differentiated hESCs are similar to those of primitive ectoderm cells with the down-regulation of *REX1* and *GBX2* upregulation of *FGF5* but no change in *CRTR-1* and *PRCE* and expression (7). However, no other reports on generation of the existence of a primitive ectoderm population in hESCs have been published, and therefore true knowledge of the existence of these cells in human embryogenesis is still elusive.

Definitive Ectoderm

In mouse development the ectoderm becomes the tissue that covers the body surfaces and is known as epithelia. This covering encloses the mesoderm from which all the skeletal elements, such as cartilage and muscle, develop (26) and emerges first and forms from the outermost germ layers. Additionally, within the embryo, the ectodermal lineage differentiates from anteriodistal primitive ectoderm and is formed by epibolic expansion of the preexisting progenitor population in the embryonic ectoderm. Differentiation of these cells results in the formation of neuroectoderm and differentiation potential consistent with embryonic neural tube.

Most success with differentiating hESCs *in vitro* toward the ectodermal lineages has been with the generation of neural and skin cells and using the methodology of spontaneously differentiating the cells in a monolayer using conditioned medium from MEFs and the addition of growth factors. The unfortunate outcome of this type of differentiating, however, is that the resulting cultures can contain many other cell types, and the conditioned medium contains many unknown factors.

Neurons. During normal development, nerve production is the first stage of organ development and thus the first cells to appear when hESC's spontaneously differentiated are neural in origin. Therefore, much study has focused on differentiating cells along the neuronal lineage and has enabled the development of an *in vitro* model of early human neurogenesis. Generating neural derivatives from hESCs will facilitate further understanding of neurodegenerative disorder models, enabling the identification of novel genes, growth, and differentiation factors, as well as becoming a potential source of neural cells for transplantation to man.

Most directed differentiation of hESCs toward the neural lineage has been performed by generating a monolayer of hESCs and allowing spontaneous differentiation to occur. One of the first published studies added inactivated

MEFs as a feeder layer to generate the neural precursors, then recultured with media supplemented with cytokines epidermal growth factor (EGF) and FGF2 to further direct the differentiation (14). Cells were then plated on poly-d-lysine and laminin-coated dishes to further differentiate the cells into the three neural lineages—astrocytes, oligodendrocytes, and mature neurons (14). These cells were then transplanted into the ventricles of newborn mouse brains where they were incorporated in large numbers of sites in the host brain (14).

More recently, hESCs were differentiated in a monolayer by the addition of FGF8 and sonic hedgehog (Shh) to the media until $Sox1^+$ neuroepithelial cells appeared. The $Sox1^+$ neuroepithelial cells differentiated to tyrosine hydroxylase $(TH)^+$ neurons that mostly coexpressed γ-aminobutyric acid bipolar cells that lacked the midbrain marker, engrailed 1 (En1), expression (21). However, during the addition of FGF8 to the precursor cells before Sox1 expression, differentiation led to the generation of a similar proportion of TH^+ neurons that were characteristic of midbrain dopamine (DA) neurons with large cell bodies, complex processes, and coexpression of En1 (21). In this study, adding the cytokine at the right time was critical for generating a specific cell type. Another recent study cultured hESCs on Matrigel-coated tissue culture plates in chemically defined conditions supplemented with Noggin to induce neural differentiation (27). Cells within rosette-like structures immunostained positive for $Pax6^+Sox1$—suggesting that they are early neuroectoderm cells (27). When cells at this stage were trypsinized and replated into single cells with FGF2 but without Noggin, they became $Pax6^+Sox1^+$ neural cells that further differentiated into neurons as evidenced by positive III-tubulin and Map2ab staining as well as characteristic neuronal morphologies (27). This later data suggests that hESCs can be readily differentiated into cells of the neural lineage and that Noggin can suppress the nonneural differentiation as well as induce selective neural specification of hESCs (27).

Conversely, EBs have been spontaneously differentiated from hESCs to generate functional neurons. Media were supplemented with either high concentrations of retinoic acid (RA), nerve growth factor (βNGF) or TGFβ1 (28). RA and βNGF were found to be potent enhancers of neuronal differentiation, while TGFβ1 downregulated these responses (28). The addition of RA and βNGF increased mRNA levels of neurofilament heavy chain (NF-H), dopamine receptor D1, serotonin 2A and 5A and dopa decarboxylase, and enhanced formation of neuronal processes from the EBs (28). Immunostaining of these cells reveals NF-H protein, found only in mature neurons (28). In another study, EBs cultured in fibronectin-coated plates and medium containing RA, EGF, FGF2, platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1), and neurobasal medium, generated a highly enriched, almost pure population, of functional neuronal cells that responded to neurotransmitters, and an exhibited action potential was produced (13). Another group took differentiating EBs and in the presence of FGF2 alone and generated neural precursors. These cells were further purified on the basis of differential adhesion and then differentiated into neurons, astrocytes, and oligodendrocytes or

transplanted into the neonatal mouse brain where the cells were incorporated into a variety of brain regions to test their functional abilities (29).

A major study published to generate peripheral neurons used a monolayer of hESCs with a coculture of the mouse stromal line, PA6, to induce the production of cells that either coexpress peripherin and Brn3a or peripherin and tyrosine hydroxylase, combinations characteristic of peripheral sensory and sympathetic neurons, respectively (30). The potential of this system is to study molecular and cellular events of human neural development, as well as to produce normal human peripheral neurons for developing therapies for diseases.

Epithelial Cells (Skin). Keratinocytes are the major cell type of the epidermis of the skin. In the absence of sufficient autologous skin in a patient with excessive burns, or major deformation, cultured autologous and/or allogeneic human keratinocytes are emerging as a viable option for treatment. hESCs differentiated into keratinocytes could provide suitable alternative sources of epithelial cells for this type of treatment.

To generate keratinocytes from hESCs, supplementing the culture with defined serum-free keratinocyte growth medium on a gelatin matrix enabled the generation of keratin 14 (K14) expressing cells with an epithelial morphology (22). These $K14^+$ cells were then subcultured in medium supplemented with hydrocortisone and calcium to induce stratification and expression of early (p63, K14) and late (involucrin, filaggrin) terminally differentiated epithelial markers (22). Coculturing hESCs together with human limbal epithelium on extracellular matrix (ECM) pretreated with collagen IV enabled the differentiation of hESCs into corneal epithelial-like cells. Induction of genes, such as p63 and cytokeratin (CK)3, and their expression in fixed cells proved these cells to be of keratinocyte origin (31).

Melanocytes are pigment-producing epidermal cells in the skin, eye, or hair that determine color. Melanocytes originate from the neural crest (via the ectoderm) and undergo a complex process of fate-specification, proliferation, migration, survival, and differentiation before finally residing in the epidermis. To induce differentiation of hESCs into a melanocyte population, EBs were generated and Wnt3a, endothelin-3, and stem cell factor (SCF) were supplemented to the culture (15). The hESC-derived melanocytes expressed melanocyte markers, developed melanosomes, and produced melanin (15). They were able to incorporate into human reconstructed skin and home to the appropriate location along the basement membrane (15). Moreover, the cells were able to maintain a stable nonmalignant phenotype even after grafting of the constructs into severe combined immunodeficient (SCID) mice (15). The ability of these differentiated cells to engraft and function as normal is crucial for their use in medical settings. Thus, the generation of epithelia could provide a source of epidermal cells for skin tissue engineering applications.

All the protocols reviewed represent preliminary stages of directed differentiation of hESCs. Widespread use of standardized protocols will be essential

to provide the embryonic stem cell research world with a model to study early human ectoderm development, *in vitro*, and enable the growth factors regimes and differentiation protocols to be resolved. The use of differentiated hESCs as potential sources of both neural and epithelial cells for use in patients is foreseeable in the future. However, even with the knowledge at hand, protocols are diverse and measurements of success are variable. An additional problem remains as to which markers are to be used to determine if the cell type generated is of the correct phenotype; questions still remain as to what are the correct genes that need to be induced, the right protein to be found on the surface of the cells, and the accurate functional assay that needs to be performed to determine if the culture has generated the correct cell type.

DIRECTED DIFFERENTIATION TOWARD ENDODERM

During murine gastrulation, the process of definitive endoderm formation begins with the migration of endoderm precursor cells through the anterior region of the primitive streak (32). Nodal signaling is central to the generation of mesoderm and endoderm precursor cells and for the subsequent specification of definitive endoderm (32). As a result, cells that are produced are capable of forming mesoderm or definitive endoderm (32). The selection toward mesoderm or endoderm is believed to be dependent on the length and amount of Nodal signaling (33, 34). Recent evidence based on murine ESC differentiation suggests that the mesendoderm cell fate decision is based on the ability of a bipotential precursor to react to different levels of Activin/Nodal signaling. When there are high levels of Activin/Nodal signaling, endoderm is generated; when there are low levels, mesoderm is generated (35). With this knowledge at hand, protocols have been designed to generate definitive endoderm, the precursor cell type that gives rise to all endoderm-derived cell lineages, including those of the pancreas, lung, and liver.

Recently, a method for generating definitive endoderm from a monolayer of hESCs using conditioned medium from MEFs, FGF2, activin A, and low levels of fetal calf serum (FCS) and plates coated with Matrigel was published (36). It was found that reducing insulin/IGF signaling, via inhibition of the PI3K pathway, was critical for cell fate commitment into definitive endoderm (36). A year later, further research refined this work by stating that high concentrations of activin A in chemically defined medium and feeder-free conditions induced definitive endoderm and pancreatic differentiation (27). Immunocytochemical analysis of activin A-treated hESCs over 9 days revealed that the differentiating hESCs pass through a mesendoderm stage (Brachyury$^+$) toward definitive endoderm (Brachyury$^+$Sox17$^+$HNF3$^+$) (27). In addition, cells that immunostained positive for PDX1 could be observed on day 9 of activin A-treated cultures, suggesting that such conditions may also permit further pancreatic differentiation (27).

Primitive Gut

Pancreas. Pancreas transplantation has become a viable option for patients with insulin-dependent diabetes mellitus. However, it is associated with a higher morbidity and is not a long-term solution since the implant has a limited life span. Additionally, there is a limited supply of cadavers from which the pancreas can be harvested from, thus an alternative source of these cells must be sought.

The path from definitive endoderm to the mature hormone-producing islet cell types is complex and involves sequential cell fate decisions, including formation of pancreatic endoderm, endocrine progenitors, and hormone-producing islet cell types. There are also essential dissimilarities between human and mouse pancreas development, suggesting that not all aspects of pancreatic organogenesis and islet formation are conserved between the species (15). These include the physical structure and the pattern and timing of embryonic gene expression during pancreatic development (37). Therefore, the production of insulin-producing β-islet cells from pluripotent ESCs will need to be studied specifically in the human system. It is important to mention that all studies to generate insulin-producing cells must exclude insulin in the culture conditions to accurately characterize these cell types and enable better assessment of the protocols (38). This information will be crucial to assess the feasibility of using hESCs as potential sources for β-cell replacement therapy (38).

Initial attempts to differentiate hESCs toward the insulin-producing islet involved placing hESCs in suspension, resulting in an induction of synchronous differentiation characterized by initial formation of small aggregates and then EB formation. The cells spontaneously differentiated into an array of cell phenotypes including insulin$^+$ cells (38). These cultures also secreted higher than control concentrations of insulin into the media and induction of genes such as *INSULIN, ISLET GK*, and *GLUT2* detected via reverse transcriptase–polymerase chain reaction (RT-PCR) analysis (38). A couple of years later, the protocol was modified to include supplements to the media such as insulin-transferrin-selenium-fibronectin with FGF2, which was later withdrawn and exchanged for nicotinamide (39).

Spontaneous differentiation of hESCs toward the definitive endoderm and pancreatic progenitor cells stage using an EB formation step showed that differentiated cells expressed the definitive endoderm and pancreatic progenitor markers, *FOXA2, SOX17*, and *PDX1*, with some cells expressing islet endocrine hormones (37). Increased expression of endoderm markers during and after EB formation also correlated strongly with the size of cell clusters used to start EBs but no measurement of insulin secretion was reported (37).

However, insufficient characterization of the origin and phenotype of these insulin$^+$ cells together with recent observations that the majority of the insulin$^+$ cells do not synthesize *de novo* insulin, lack PDX1, a key transcription factor, and accumulate insulin from the medium, suggesting that this progenitor is

not a reproducible source for bona fide β cells and needs to be investigated further. In a different study, spontaneous differentiation of hESCs into pancreatic progenitors and their mature exocrine and endocrine descendents produced $PDX1^+/FOXA2^+$ pancreatic progenitors and $PDX1^+/ISL1^+$ endocrine progenitors but no insulin-producing cells (40). The differentiated hESCs were then transplanted into the dorsal pancreas of mouse embryos and resulted in differentiation of β cell-like cell clusters; however, no functional studies were performed (40).

Most recently, a sophisticated five-step protocol for differentiation of hESCs to pancreatic hormone expressing cells was established (41). Stage 1 generated definitive endoderm (DE) via differentiation through the mesendoderm layer using a high concentration of activin A in the presence of low FCS with the addition of Wnt3a during the first days of activin exposure (41). In stage 2, activin A was removed, which was essential for the transition of definitive endoderm to a stage resembling the primitive gut tube, and FGF10 and the hedgehog-signaling inhibitor, KAAD-cyclopamine, was added to the culture (41). In stage 3, the gut-tube endoderm is exposed to RA together with KAAD-cyclopamine and FGF10 (41). Upon addition of RA, the cells rapidly began to express high levels of *PDX1* and *HNF6* (41). At stage 4, the *PDX1*-expressing posterior foregut endoderm cells were recruited to the pancreatic and endocrine lineages by the addition of DAPI, an γ-secretase inhibitor, and exendin-4 (Ex4) (41). At stage 5, Ex4, IGF1, and hepatocyte growth factor (HGF) were supplemented to the cultures and by around day 15 of differentiation, endocrine cells expressing the pancreatic hormones insulin, glucagon, somatostatin, pancreatic polypeptide, and ghrelin were produced (41). This protocol shows the complexity needed to differentiate hESCs toward a particular lineage. The derivation of such protocols seems laborious and time-consuming but will become the nature of protocols required for the future of this field.

Liver. Generating cells of the liver from hESC will be critical for the medical field since hepatocyte cell transplants have already been shown to be a plausible treatment for human metabolic liver diseases. However, primary cultures of adult hepatocytes do not replicate sufficiently *in vitro* to produce the number of cells necessary for transplantation. The totipotential of hESCs could allow their differentiation toward liver cell precursors and thus become an alternative source of cells for cell replacement therapies.

The liver too is distinctly different in structure, development, and gene expression between mice and men. Therefore, it is important to consider protocols specific to human development in regulating differentiation of hESCs toward the generation of liver cells. The protocols published to date are very similar in their approaches; all of them are utilizing three-dimensional (3D) polymer as a scaffold. hESC differentiation and organization can be influenced by the scaffold and directed by growth factors such as activin A and IGF to induce differentiation of cells with the characteristics of the developing liver

(16). Immunostaining of tissue sections taken from the differentiating EBs taken off the 3D scaffold showed high levels of alpha fetal protein (AFP) and albumin (ALB) (16). When transplanted into SCID mice, the cells continued to express specific human proteins such as AFP in defined differentiated structures and appeared to incorporate with the host vasculature (16). In another study, five-day-old EBs were inserted into a collagen scaffold 3D culture system with and without stimulation of differentiation with exogenous growth factors to induce hepatic histogenesis (42). Although robust level of hepatocyte-like cells were generated in spontaneous differentiation systems, the addition of exogenous growth factors significantly enhanced their numbers. Immunofluorescence analysis revealed the expression of several characteristics of hepatocytes, including albumin and CK18, while quantitative real-time RT-PCR analysis revealed the expression of mRNA of genes such as CK8 and CK19, glucose-6-phosphatase, cytochrome P450 subunits 7a1, and secretion of AFP and ALB (42). Electron microscopy of differentiated hESCs showed hepatocyte-like ultrastructure, including glycogon granules, well-developed Golgi apparatuses, rough and smooth endoplasmic reticuli, and intercellular canaliculi (42).

In order to isolate the hepatic-like cells, one can introduce a reporter gene regulated under a hepatocyte-specific promoter, for example, ALB minimal promoter sequence (43). Clones of hESCs can then be isolated on their expression of enhanced green fluorescent protein (GFP), upon *in vitro* differentiation of hESCs (43). These GFP^+ cells coexpressed albumin and AFP. Using a fluorescence-activated cell sorter, the GFP^+ cells were further differentiated and expanded (43). Clusters of hepatic-like cells are seen in 20-day-old EBs and in teratomas. In order to examine the secreted factors involved in the induction of hepatic differentiation, hESCs were grown in the presence of various growth factors, demonstrating the potential involvement of acidic FGF in the differentiation (43).

Lungs. Lung transplantations have been performed for many years, but the accessibility of donor lungs is scarce, and most patients die before transplantation is possible. Thus, other forms of treatment have been sought, one of which is to create a gas-exchange unit with viable components and thus carry out the functions of a real lung (44). Alveoli are the major lung units that allow gas exchanges to occur across their membrane. Generating alveolar tissue *in vitro* would therefore be critical for a plausible gas-exchange unit. Lung alveoli generated from hESCs could become renewable sources of the alveolar epithelium.

The epithelium of the alveolus is an endoderm-derived tissue and is composed of two main cell types: type I and type II pneumocytes. If type I cells are damaged, type II cells differentiate into type I cells. Therefore, to generate type II cells *in vitro* would be very useful. However, very little work has been performed to generate cells of the bronchia partly because very little is known regarding the growth of lung cells *in vitro*. Recently, two articles that have started unraveling the pneumocytic pathways of differentiation were pub-

lished. The first group used a previously published protocol established in the mESC (44); hESC were induced to form EBs spontaneously in media containing FCS and FGF2 (44). FGF2 was removed from the culture after 2 d and at 10 d the media was changed to a media specific for generating pneumocyte growth (44). The generated cells showed the cobblestone-like morphology of epithelium and the presence of surfactant protein C (SPC), a specific marker of type II pneumocytes (44). Electron microscopy revealed cells with the typical ultrastructure of type II pneumocytes (44). The second group used a very similar protocol, but to achieve a pure population of type II pneumocytes, a neomycin-containing vector was transfected into the hESC under the human SPC promoter, a protein uniquely synthesized by type II pneumocytes. After 6 d in culture the cells were selected (45). The cells generated after selection were then characterized and were able to form lamellar bodies, expressed SPA, SPB, and, SPC, and synthesized and secreted complement proteins C3 and C5 (45).

This latter study and another cited earlier illustrate a new trend in directed differentiation of hESCs that is using molecular techniques and reporter genes to enable clearer and more decisive result in determining protocol success, and that will dominate the protocols of the future.

DIRECTED DIFFERENTIATION TOWARD MESODERM

Following gastrulation where the three germ layers—the mesoderm, endoderm, and ectoderm are generated, the mesoderm layer differentiates further into five cells types: 1) the chordamesoderm forms the notochord, a transient organ that defines the anterior-posterior axis of the embryo and induces differentiation of adjacent tissues; 2) the head mesenchyme, which differentiates into cells that will be incorporated into the skull and face of the embryo, including parts of the eye; 3) the dorsal mesoderm, or paraxial area, which synchronously segments into the area that forms the somites and later forms the skeleton, muscles, and connective tissue; 4) the intermediate mesoderm, which becomes the kidney and the gonads; and 5) the lateral mesoderm which develops into the heart and lungs, and later the circulatory system which includes the blood, blood vessels, and mesenchyme.

Very little investigation or generation of differentiated cells of the notochord and head mesenchyme have been reported but that latter part of the chapter will be dedicated to describing differentiating cells toward dorsal, intermediate, and later mesoderm lineages.

Dorsal

Bone. Osteoblasts, which are derived from the mesoderm, are cells that specialize in the production of extracellular matrix (mainly composed of type I colla-

gen) and the mineralization process. Bone matrix production and mineralization involves sophisticated mechanisms, including the initial formation of an organic extracellular matrix into which inorganic hydroxyapatite crystals are later deposited. hESCs offer a strong advantage in fundamental research by permitting the study of osteoblast and osteoclast differentiation from the earliest differentiation state of the cell. Furthermore, they are the best models to study the differentiation into mesenchymal cells that give rise to osteoblasts.

In one of the first studies published, hESCs spontaneously formed EBs in culture containing MEF-CM and FCS. The formed EBs were replated in media containing osteogenic supplements such as ascorbic acid phosphate, β-glycerophosphate, and dexamethasone to induce osteogenic differentiation (46). The cells expressed markers of osteogenesis such as osteocalcin, parathyroid hormone receptor, and collagen 1 (46). Moreover, mineralized nodules obtained were composed of hydroxyapatite similar to that found in osteoblasts in bone (46). More recently, again using a similar protocol, a quantitative assay of osteocalcin secretion demonstrated a rapid and sharp increase in osteocalcin expression on day 12 of *in vitro* hESC differentiation, which could suggest the appearance of differentiated osteoblasts already from day 12 onward (47). Another group implanted cells, on a 3D scaffold, generated from hESCs using a similar method of differentiation into SCID mice. The cells had the capacity to give rise to mineralized tissue *in vivo* which could be identified within the scaffold to be of human osteocalcin origin (48).

Another method was published recently for osteoblastic differentiation of hESCs cocultured with primary bone-derived cells (PBDs) (23). Shortly after EB formation, cells were allowed to adhere to a culture surface where arrested PBDs were preplated (23). As early as 14 days, mineralization and formation of nodule-like structures in cocultured EBs were prominent and expression of osteoblast-specific markers including bone sialoprotein, alkaline phosphates, and collagen 1 were detected by RT-PCR (23). In addition, flow cytometric analysis revealed that over 19% of the differentiated cells expressed osteocalcin. These results suggest that PBDs not only have osteogenic effects, releasing osteogenic factors as BMP2 and BMP4, but also have exerted other unknown effects, whether chemical or physical, for the differentiation of hESCs (23).

Another method compared hESCs differentiation via EB formation to differentiation of hESCs in a monolayer (49). In the presence of osteogenic supplements, similar to those published previously, osteoblasts could be generated efficiently via both methods (49). Characterization of the osteogenic phenotype revealed detection of a main osteoblastic marker *RUNX2* (49). Osterix, bone sialoprotein, and osteocalcin were also upregulated in these cells (49). Alizarin Red S staining demonstrated the formation of bone-like nodules, and bone sialoprotein and osteocalcin were localized to these foci by immunohistochemistry (49). However, cells differentiated in monolayer conditions exhibited greater osteogenic potential compared to those from EB-derived cells (49).

Cartilage. Results published on the ability to differentiate hESCs, via EB formation, toward cartilage lineage showed that cytokines such as NGF, FGF2, HGF, EGF, and BMP4 were not necessary to induce the expression of cartilage matrix proteins, but even so the yield of cartilage type cells were low (50). A few years later, with the introduction of supportive 3D polymer scaffolds and the addition of TGFβ, hESCs could be differentiated efficiently to cells that secrete cartilage-like glycosaminoglycan extracellular matrix (16).

A recent study demonstrated the potential of hESC-derived EBs to undergo well-defined full-span chondrogenesis from chondrogenic induction to hypertrophic maturation (51). Results showed that cell-to-cell contact and BMP2 treatment, in addition to the FCS presence, enhanced chondrocyte differentiation, resulting in the formation of cartilaginous matrix rich in collagens and proteoglycans, and that a high-density 3D microenvironment at the beginning of differentiation was critical in driving chondrogenesis (51). Induction of genes specific for chondrocytes, such as *Col 1, Col 2*, and *Col 10*, was detected, and the deposition of ECM proteins, such proteoglycans, and collagen II and collagen X demonstrated that the *in vivo* progression of chondrocyte maturation is recapitulated in the hESC-derived EB model system established (51).

Intermediate

Kidney and urogenital tract: Very little has been reported to date on directed differentiation of either mESC or hESC toward the intermediate mesoderm lineage and cells of the renal or urogenital system. One article mentioned that adding NGF to cultures of EBs induced the expression of genes such as *renin* and *kallikrein*, markers of kidney, but under no conditions were genes of the urogenital system induced (50).

Lateral

Heart. The regenerative capacity of adult heart tissue is limited, and therefore, after a myocardial infarction (heart attack) there is substantial cell death and major scar tissue formation that makes full recovery of heart function to normal impossible. Implanting myocardial precursors to regenerate the damaged heart, therefore lessening scar formation, is crucial. Using differentiated hESCs as possible sources of regenerative tissue has much potential. Here we discuss some methods present in the literature to generate these cells.

In the mouse, the heart is one of the first organs to form during embryogenesis. Myocardial precursors are present as early as in the posterior lateral region of the epiblast. They still express the transcription factor OCT-3/4 at early gastrulation (52) and in early mesodermal cardiogenic fate (53). Then, at gastrulation, following ingression of the epiblast at the primitive streak, cardiac progenitors migrate from the anterior region of the primitive streak into the mesoderm. The whole morphogenetic process is under the control of growth factors, including those of the TGFβ superfamily. Members of the

TGFβ superfamily have been involved in induction of the mesoderm in *Xenopus*, zebrafish, chicken, and mouse (54). It has been published that adding Nodal, TGFβ, and BMP2 to cultures of ESCs trigger the expression of mesodermal and cardiac-specific genes in mouse (53, 55) and human (50) ESCs.

Generation of cardiomyocytes has been performed by spontaneously differentiating hESCs on gelatin-coated culture dishes until the appearance of contracting cells was observed. The contracting cells expressed both connexin-43 and 45 that in many cases colocalized to the same gap junctions: a phenomenon common to embryonic cardiomyocytes. Electrical activity was detected when electromechanical testing was performed on the cells generated *in vitro*. Differentiated cells were also transplanted into the heart of swine with a slow heart rate. The transplanted cells were able to bring the heart rate of the swine to normal as assessed by a detailed 3D electrophysiological map and histopathological examination (56). Later, the same group used a similar protocol to increase the number of beating cells generated and to follow early human heart development by timing the expression of specific candidate and cardiac gene expression during cardiogenesis (57). Using histology, immunostaining, and RT-PCR, these spontaneously contracting cells were observed to possess morphological and molecular characteristics consistent with cardiomyocytic phenotypes (57). GATA-4 was the first gene to be expressed, then alpha cardiac actin and atrial natriuretic factor were expressed in older EBs, while light chain ventricular myosin was expressed only in EB with beating areas and its expression increased with time (57).

In another study, four-day-old undifferentiated hESC colonies were cultured on Matrigel-coated plates in feeder-free N2/B27 chemically defined medium (CDM) plus FGF2, activin A, and BMP4, induced marker expression associated with cardiac muscle lineage (27). These results are consistent with the model that transient activin A treatment induces mesendoderm differentiation of hESCs and BMPs play essential roles in cardiac muscle differentiation (27). Immunocytochemistry revealed that a high percentage of these differentiated cells were positive for specific cardiomyocyte markers, including cardiac troponin I (cTnI), MEF-2, and GATA-4, and RT-PCR analysis also confirmed the expression of these markers (27).

In a recent article, hESCs were forced to aggregate into EBs by placing a specific number of cells into round bottom wells and then applying centrifugal force. The cells were incubated with a chemically defined medium supplemented with polyvinyl alcohol supplemented with activin A and FGF2, enabling uniform production of EBs (58). At day 6 of differentiation, FCS was added to the media and cells were differentiated for a further 3 weeks (58). As high as 28% of EBs generated contained beating cells (58).

Mesenchyme. MSCs are multipotent progenitors that can be found in many connective tissues including fat, bone, cartilage, and muscle. Because of this differentiation potential, MSCs are generally considered to have a large therapeutic promise, particularly in the areas of cell therapy and regenerative and

reconstructive medicine. MSCs have been isolated from the adult bone marrow, adipose tissue, dermis, and other connective tissues. Adult tissue-derived MSCs have demonstrated therapeutic efficacy in treating diseases or repairing damaged tissues. Harvesting them from these sources requires invasive procedures and the availability of a suitable donor. The number of MSCs that can be obtained from a single donor is limited, and the capacity of these cells for long-term proliferation is rather poor. Generating MSCs from hESCs could provide an unlimited supply of specialized cells.

Since no unique marker of MSCs has been identified, investigators have relied on a series of functional and morphological criteria to identify them. These criteria include growth on plastic, resistance to trypsin, presence of specific cell surface antigens, and potential to differentiate into the various cell types. This has made it difficult to adapt and determine differentiation protocols for the hESCs. Here I report a few studies that have succeeded in generating MSC from hESCs.

In one study, mesenchymal differentiation from hESCs was induced by coculturing the cells on a monolayer of OP9 cells in the presence of FCS in alpha medium (59). After 40 days, cells expressing CD73 were sorted, labeled as hESC-derived mesenchymal precursor cell (hESMPC) and then further differentiated according to the following protocols in the presence of FCS: adipocytic—hESMPCs are grown to confluency followed by exposure to dexamethasone, insulin, and isobutylxanthine in medium for at least 4 weeks; chondrocytic—differentiation of hESMPCs was induced by the addition of TGF-β3 and ascorbic acid in medium for at least 4 weeks; osteogenic—hESMPCs were plated on tissue-culture-treated dishes in the presence of β-glycerol phosphate, dexamethasone, and ascorbic acid in medium for up to 4 weeks; myogenic—confluent hESMPCs were maintained for 3 weeks in medium (59).

Another report spontaneously differentiated hESCs into adipocytes using only FCS in the medium (60). Differentiated cells were observed to display the key features of adipocytes, that is, expression of specific molecular markers, such as peroxisome proliferator-activated receptor 2, adipocyte fatty acid binding protein and adiponectin, the secretion of leptin, and the accumulation of lipid droplets in cytoplasm (60). Taken together, the results demonstrate that adipocytes derived from hESC *in vitro* can provide a novel model system to study human adipogenesis (60).

hESCs maintained in culture frequently differentiate around the colony edges. These are still multipotent and have been induced to differentiate toward the MSC lineage by plating in medium containing FCS for at least 4 weeks until a thick multiplayer of epithelium appears (61). To induce osteogenic differentiation, cells were seeded at a low density in medium containing FCS. At 50%–70% confluency, growth medium was supplemented with dexamethasone, ascorbic acid-2-phosphate, and glycerophosphate (61). The medium was replaced every 3–4 days for 21 days. For adipogenic differentiation, cells were seeded at a high density (61). At confluency, cells were either put in medium supplemented with dexamethasone, indomethacin, insulin, and

3-isobutyl-1-methyl-xanthine or cells were put in medium containing knockout serum and FGF2 and incubated into partial hypoxia (61). In both cases, the medium was replaced every 3–4 days for 21 days (61). The cells obtained with this procedure are morphologically similar to BM MSCs (61). The hESC-differentiated MSC are contact inhibited, can be grown in culture for about 20–25 passages, have an immunophenotype similar to BM MSCs, can differentiate into osteocytes and adipocytes, and can be used as feeder cells to support the growth of undifferentiated hESCs (61).

Recently, a method was described that was most clinically relevant and in a reproducible manner generated MSC from hESCs (62). Trypsinization and propagation of hESCs in feeder- and serum-free selection supplemented with serum replacement medium, FGF2, and PDGF generated three polyclonal, karyotypically stable, and phenotypically MSC-like cultures that did not express pluripotency-associated markers but displayed MSC-like surface antigens and gene expression profile (62). The cells were then sorted using fluorescence-activated cell sorter based on their expression of CD105 and differentiated into adipocytes, osteocytes, and chondrocytes (62). MSC from primary BM and adipose-derived MSCs, from two biological replicates, were compared in their gene expression profile and were found to be identical to each other with a high correlation (62).

The ability to produce MSCs from hESCs should prove useful to produce large amounts of genetically identical and genetically modifiable MSCs that can be used to study the biology of MSCs and for therapeutic applications.

Blood. *De novo* human hematopoietic development has been studied quite extensively due to the availability of samples from human embryo at 23–50 days of development (i.e., postconception) immediately after voluntary terminations of pregnancy. Most of this data were contributed by the work of Bruno Péault and his group (63–68). Differentiated cells of the hematopoietic and endothelial lineages arise from the mesodermal layer and emerge during gastrulation (7.5 dpc in mouse (69), day 16 in human (70)), when cells recruited from the epiblast (embryonic ectoderm) migrate through the primitive streak and become organized into the embryonic and extraembryonic regions of the mesoderm (71). The extraembryonic region of the mesoderm produces a local but transient wave of primitive hematopoiesis primarily composed of nucleated red blood cells (72). The yolk sac was long considered to be the original and only provider of self-renewing definitive stem cells for lifelong hematopoiesis (73), but later studies found that the para-aortic splanchnopleura (p-SP) and derived aorta-gonad-mesonephros (AGM) territory additionally harbored pluripotential hematopoietic cells before the onset of fetal liver colonization (70, 74). Finally, the last stage of hematopoietic development, where the generation of definitive lympho-myeloid stem cells occurs, has been found to be in the human embryo proper, not its yolk sac (65).

The derivation of functional, multipotent, and engraftable hematopoietic stem cells (HSCs) from hESCs will alleviate the need to search for compatible bone marrow donors and the generation of terminal differentiated blood cells,

such as platelets and RBCs, could represent a potential alternative supply of blood cells and blood products, which are currently sourced from an increasingly restricted pool of eligible blood donors. Investigating the genetic pathways specifying blood formation will lay the foundation for hematopoietic cell replacement therapies based on engineered hESCs (75). Although significant progress has been made in achieving hematopoietic differentiation and directed differentiation of hESCs toward hematopoietic cell lineages is the most well studied, we have only a primitive understanding of the underlying mechanisms that specify hematopoietic cell fate, and a very limited capacity to direct the differentiation of the definitive HSC that would be suitable for clinical engraftment studies (75). The ultimate medical goal is to create patient-specific and/or generic ES cell lines that can be expanded *in vitro*, genetically altered, and differentiated into cell types that can be used to treat hematopoietic diseases (76). In order to capitalize on the potential of hESCs to generate blood cells for future human cell therapies, it will be essential to recapitulate the key embryological stages *in vitro* in a defined and reproducible fashion.

Previous protocols for the generation of hematopoietic cells from hESCs have used serum-containing media and/or have involved coculture with inducing stromal cell layers. Here are the results of some of these studies. The ability of the murine bone marrow cell line, S17, to generate HSC was compared to the yolk sac endothelial cell line C166 (77). S17 lines were much more efficient in generating hematopoietic cells that expressed CD34 and colonies of myeloid, erythroid, and megakaryocyte origin than C166 (77). The cells generated expressed the hematopoietic transcription factors TAL-1, LMO-2, and GATA-2, and sorted $CD34^+$ cells derived from hESCs were enriched for hematopoietic colony-forming cells (CFCs) (77). In another study, a human fetal liver-derived cell line and S17 were used as stroma to induce the differentiation of hESCs into hematopoietic cells (78). Results revealed that the fraction of $CD34^+$ cells and the number of clonogenic progenitors were higher from cells differentiated on the human fetal liver-derived cell line than with S17 (78). A much more efficient method for generating HSC using OP9 bone marrow stromal cells was established in the same year (79). The hESC-derived $CD34^+$ cells were highly enriched in CFCs, expressed hematopoietic associated genes GATA-1, GATA-2, SCL/ TAL1, and Flk-1, and retained clonogenic potential after *in vitro* expansion (79). These $CD34^+$ cells displayed the phenotype of primitive hematopoietic progenitors as defined by coexpression of CD90, CD117, and CD164, along with a lack of CD38 expression, and contained aldehyde dehydrogenase-positive cells as well as cells with verapamil-sensitive ability to efflux rhodamine 123 (79). When further cultured on MS-5 stromal cells in the presence of SCF, Flt3-L, interleukin (IL)-7, and IL-3, isolated $CD34^+$ cells differentiated into lymphoid (B and natural killer cells) as well as myeloid (macrophages and granulocytes) lineages (79).

Another article reporting directed differentiation of hESCs toward HSC treated the hESCs during Ebs' development with a combination of low dose hematopoietic cytokines, including SCF, Flt-3 ligand, and VEGF, on an under-

layer of human bone marrow stromal cells, which generated cell clusters that contained KDR^+ hemangioblasts, $CD34^+$ hematopoietic stem cells and $CD45^+$ mature hematopoietic cells, and expressed hematopoietic genes such as KDR, SCL/TAL, and RUNX1 (80). However, there is a problem with generating HSC on stromal lines or with FCS for clinical purposes due to the contamination of feeders cells and foreign animal protein in the product.

Other groups have generated HSC without the use of feeder layers and FCS and directing a more efficient differentiation with the addition of cytokines. Again there is a great advantage for groups working in this field since there are many cytokines that have been established in the adult hematopoietic system that are freely available. In one of the first studies, hESCs were induced to form EBs by overnight incubation in low-attachment plates containing medium supplemented with FCS (81). The next day, cultures were given fresh differentiation medium or were supplemented with the following treatments: 1) SCF, Flt-3 ligand, IL-3, IL-6, and granulocyte colony-stimulating factor (G-CSF); 2) cytokines with BMP4; 3) or BMP4 alone (81). The conclusion to this study found that the appearance of $CD45^+$ cells was influenced by cytokine treatment, with or without the addition of BMP4 with a small proportion of $CD45^+$ spontaneously differentiated (81). However, when quantitating the number of CFCs, the addition of BMP4 to the cytokines mix increased the progenitors produced (81). Adding vascular endothelial growth factor (VEGF) to the culture containing the cytokines and BMP4 selectively promoted erythropoietic development from hESCs (82) and was augmented by further addition of erythropoietin (EPO) (82). Treatment of human EBs with VEGF increased the frequency of cells coexpressing CD34 and KDR, as well as cells expressing erythroid markers (82). In addition, VEGF induced the expression of embryonic zeta and epsilon globins, and the hematopoietic transcription factor *SCL/TAL1* and promoted the self-renewal potential of primitive hematopoietic cells capable of erythroid progenitor capacity (82).

Using the above differentiation protocol, the hematopoietic potential of cells comprised in the EBs prior to the onset of hematopoietic commitment at day 11 was assessed. A population of cells that were VE-$cadherin^+PECAM1^+$ were sorted from day 10 EBs and were found to coexpress CD34 and Flk1, do not express CD45, but could uptake low-density lipoprotein (LDL) (83). When these cells were assessed for their ability to generate HSC by culturing them in hematopoietic conditions, almost all the cells became CD45+ and generated hematopoietic colonies (83). When these same cells were cultured for 7 d in endothelial culture conditions, the cells became mature endothelial cells (83). These sorted cells also had a gene profile consistent with both endothelial and hematopoietic potential and therefore, both human hematopoiesis and endothelial maturation originate from a subset of ESCs that possess hemangioblastic properties (83).

Recently, a group tried to elucidate the cellular and molecular kinetics with the use of the differentiation of hESCs *in vitro* from primitive to definitive erythro-myelopoiesis using human EBs (68). Spontaneously generated hESC

clumps were cultured in nonadherent plates in media containing methylcellulose medium supplemented with FCS, ascorbic acid, cholesterol/lipoprotein, and PFHM-II. Semisolid hEB cultures were harvested after 4 days and formed EBs were collected and replated in ultra nonadherent plates in media consisting of serum-free expansion medium (SFEM) supplemented with FCS, ascorbic acid, EX-CYTE, insulin/transferrin/selenium supplements, and protein-free hybridoma media (PFHM)-II. Ebs' liquid cultures were fed or passaged (68). During the differentiation, a first wave of hematopoiesis was observed, which was predominated primitive erythropoiesis, characterized by a brilliant red hemoglobinization, CD71/CD325a (glycophorin A) expression, and almost exclusive embryonic/fetal hemoglobin expression (68). After which, a second wave of definitive-type hematopoiesis generating erythroid-burst forming units, erythroid- and granulocyte/macrophage CFCs, and multilineage CFCs followed from hEB progenitors (68). These stages of hematopoiesis proceed spontaneously from hEB-derived cells without the requirement for supplemental growth factors during hEB differentiation (68). Gene expression analysis of differentiating EBs revealed that initiation of hematopoiesis correlated with increased levels of *SCL/TAL1, GATA1, GATA2, CD34, CD31*, and the homeobox gene-regulating factor *CDX4* (68). These data indicate that hematopoietic differentiation of hESCs models the earliest events of embryonic and definitive hematopoiesis in a manner resembling human yolk sac development.

The hardest thing for all directed differentiation protocols is the correct identification of the cell type. Most of the work published on markers of hematopoiesis is targeted at adult and cord blood cell and it is difficult to superimpose the information accrued from the adult system to the developing embryo. For example, the marker CD45 is known to be expressed on all hematopoietic cells from stem to most mature cells found in the peripheral blood and on HSC is associated with CD34 expression. CD45 in hESC is expressed much later in differentiation than CD34 yet the $CD45^{-}CD34^{+}$ cells can generate hematopoietic colonies. Recently, a new marker to differentiate between endothelial committed $CD34^{+}$ and hematopoietic $CD34^{+}$ was found. CD43, leukosialin, a marker unknown to the adult hematopoietic world was found to be expressed earlier than CD45 in hESC differentiation and allowed an earlier delineation of endothelial and hematopoietic cells to be made in the embryonic system (84).

Recently I published a clean chemically defined, serum- and feeder-free differentiation method using a defined number of hESCs per EB (85). Using BMP4, VEGF, SCF, and bFGF, an efficient robust levels of $CD34^{+}$, $CD45^{+}$, and $CD33^{+}$ CFCs that expressed transcription factors such as *SCL, RUNX1*, and *GATA2* were generated (85). This study was approached systematically, where the role of each cytokine supplemented to the culture was defined and varying doses of each cytokine was tested and only those published were found to significantly enhance the direct differentiation of hESCs toward dorsal mesoderm and then hematopoiesis (85).

Endothelial Cells. The developmental process to generate blood vessels in humans has been well studied and begins at about day 18 in the embryo. During this process, the underlying endoderm secretes factors that cause some cells of the splanchnopleuric mesoderm to undergo *de novo* differentiation into endothelial progenitors. Small vesicular structures are then created, which fuse to form networks of vessels that unite, grow, and vascularize the embryo with the participation of an additional cell type, the periendothelial smooth muscle cell. Endothelial cells can be detected in a human vascular system in the yolk sac and embryo at 23 days of gestation, and by day 35 day of gestation, CD34 is uniformly expressed at the luminal portion of the endothelial cells in developing intraembryonic blood vessels (63).

Manufacturing endothelial cells *in vitro* from hESCs will help treat patients with vascular disease and augmenting vessel growth to areas of ischemic tissue. The first work published on generating endothelial cells induced EB formation spontaneously from hESCs with no growth factors added (86). $PECAM1^+$ cells were generated and were sorted at day 13 of differentiation, grown on plates coated with gelatin, and supplemented with endothelial growth medium (EGM-2), or Matrigel to access their ability to form vessels (86). $PECAM1^+$ cells could uptake ac-LDL, expressed viniculin and actin, and expressed transcription factor *GATA-2* (86). To analyze the therapeutic potential of hESC-derived endothelial cells, the cells were seeded on highly porous biodegradable polymer scaffolds, and implanted subcutaneously in SCID mice (86). Implants became encapsulated by fibrous connective tissue that was permeated by mouse blood vessels and histological examination showed microvessels that were immunoreactive with human PECAM1 and CD34 (86).

Another group investigated the molecular mechanisms involved in vessel formation by using differentiation of hESCs as a model (87). They demonstrated that stromal cell-derived factor-1 (SDF-1) and CXCR4 were expressed concurrently with hESC-derived embryonic endothelial differentiation (87). Blocking of CXCR4 signaling abolished capillary-like structures induced by SDF-1 (87). Inhibition of the SDF-1/CXCR4 signaling pathway disrupted the endothelial sprouting outgrowth from human EBs, suggesting that the SDF-1/CXCR4 axis plays a critical role in regulating initial vessel formation, and may function as a morphogen during human embryonic vascular development (87).

Described recently was a differentiation protocol for generating endothelial cells from hESCs using a scalable two-dimensional method that avoids an EB intermediate and differentiated in FCS for 10 days supplemented with VEGF, FGF2, and BMP4 (88). $CD34^+$ cells (with the majority of cells beings $CD45^-CD31^+$) were purified and enriched using magnetic beads (88). Using GFP transfected hESCs, the differentiated cells were transplanted into SCID mice, the differentiated cells contributed to newly formed blood vessels that integrated into the host circulatory system and served as blood channels for 150 d (88).

A new methodology was proposed to enhance the vascular differentiation of hESCs by encapsulation in a bioactive hydrogel (89). The fraction of cells expressing VEGF receptor KDR/Flk-1 increased up to 20-fold, as compared to spontaneously differentiated EBs (89). When the cells were removed from these networks and cultured in media conditions conducive for further vascular differentiation, the number of vascular cells was higher than the number obtained through EBs, using the same media conditions (89).

MAJOR HISTOCOMPATIBILITY ANTIGENS ON HUMAN EMBRYONIC STEM CELLS

Immune rejection by the host immune system has been considered to be one of the greatest hurdles for cellular transplantation (90). hESCs express low levels of major histocompatibility complex (MHC)-I antigens, such as HLA-1, lack expression of MHC-II antigens and costimulatory molecules, are not recognized by natural killer cells, and inhibit T-cell induced-stimulation by third-party antigen-presenting cells (91). Expression of (MHC-1) HLA-1 increases with differentiation and the addition of interferon (IFN)-γ, not IFN-α or IFN-β (91). Upon injection into immunocompetent mice, hESCs are unable to induce an immune response as demonstrated by their inability to induce an inflammatory response (90).

hESCs have a great potential in cell transplantation therapy. However, due to the above results, recipients of such allogeneic transplants will probably need treatment with immunosuppressants to dampen an immune response to the transplanted differentiated hESCs or a bank of hESC lines will need to be generated encompassing the majority of the MHC classes known in humans. The number of hESC lines needed to achieve varying degrees of HLA match was estimated as follows: a bank of 150 donors would provide a full match at HLA-A, HLA-B, and HLA-DR for a minority of recipients (<20%); a beneficial match (defined as one HLA-A or one HLA-B mismatch only) or better for 37.9% (range 27.9–47.5); and an HLA-DR match or better for 84.9% (range 77.5–90.0) (92). Extending the number of donors beyond 150 conferred only a very gradual incremental benefit with respect to HLA matching (92).

CONCLUSION

hESCs can, in theory, produce all cell types of living organisms while renewing themselves with a stable genetic background (93). These unique features make hESCs a favorable tool for biomedical research as well as a potential source for therapeutic application (93). hESCs can be directed to differentiate to cells of interest, either by aggregation of cells to form EBs (Fig. 11.1) or in monolayer (Fig. 11.2). Directing the differentiation can be further enhanced in some cases with the addition of FCS or recombinant cytokines. However, directed

differentiation of hESCs into specific tissue types poses a formidable challenge.

A number of obstacles do remain even once the directed differentiation of hESC toward a progenitor fate is achieved. The development of cell isolation techniques will be required to ensure progenitor purity, thereby overcoming the possibility of teratoma formation by residual undifferentiated hESCs. Furthermore, are the cells that have been produced functionally active? To date, most transplantation studies in mice have shown early engraftment of cells, but long-term engraftment is still a problem. Without somatic cell nuclear transfer to generate autologous hESCs that are tailored for individual patients, an hESC-based therapy is likely to require immunosuppression, although the data showing the immunologic protection of embryonic material may mean that immune rejection is less of an issue than expected. Conversely, there is considerable scientific debate over the ability to derive hESCs safely using somatic cell nuclear transfer because of our lack of understanding and inability to reprogram genomic imprinting.

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12

IDENTIFICATION OF SIGNALING PATHWAYS INVOLVED DURING DIFFERENTIATION

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INTRODUCTION

Embryonic stem (ES) cells uniquely are endowed with the capacity of self-renewal and the potential to give rise to all possible cell types. ES cells are important sources of stem cells in regenerative medicine, and much remains unknown about their molecular characteristics. To develop a detailed transcriptional profile during differentiation in ES cells, transcriptomes of an undifferentiated ES cell and its differentiated ES cell were compared by massively parallel signature sequencing (MPSS). Over 2 million signature tags from ES cells and their differentiating embryoid bodies (EBs) were sequenced. Major differences between ES cells and their differentiating EBs were identified by MPSS. This large-scale transcriptome characterization at various stages of ES cell differentiation has implicated important molecules and signaling pathways as central players in stem cell biology. This chapter shows that MPSS could identify the signaling pathways that exist between cell cycle progression and the regulation of pluripotency and differentiation in ES cells, suggesting that the usage of MPSS data to characterize ES cells might enhance the

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understanding of pluripotency mechanisms and be useful for the small molecule discovery process that can activate the ES cell state.

THE USAGE OF LARGE-SCALE TRANSCRIPTOME ANALYSIS TO IDENTIFY TRANSCRIPTS FROM ES CELLS

Analysis in ES cells has suggested that pluripotent cells express a multilineage transcriptosome (1–4). Most genes (including tissue-specific genes) are maintained at low/undetectable levels of transcription. However, in undifferentiated ES cells, most genes of ES cell-specific genes (such as Oct3/4, DNMT3, Sox-2, Nanog, ERas, Ehox) are expressed at high levels of specific transcription (5–11). Maintenance of the ES cell state likely requires multiple regulatory networks to maintain the expression of ES cell-specific genes and likely includes both the presence of inhibitory factors that prevent the transcription/translation of cell type-specific genes and the absence of specific positive regulators (reviewed in [12–14]). The overall state of ES cell can be assessed by a variety of methods, including microarray analysis (15–17), expressed sequence tag (EST) enumeration (2, 18), serial analysis of gene expression (SAGE) (3), and MPSS (19, 20).

MPSS have been chosen to examine gene expression in ES cells from the undifferentiated ES cells and EBs derived from the same cell lines. MPSS has allowed the assessment of the complexity of ES cells and EBs and the generation of a far more exhaustive list of differences between ES cells and EBs than has previously been reported. Furthermore, MPSS has the ability to detect novel transcripts. The usefulness and power of developing such a comprehensive expression database is highlighted by the ability not only to identify putative signaling or biochemical pathways active in ES cells but also to assess the integrity of these pathways from receptors to signaling intermediates and then target substrates at the transcript level.

MODULATION OF CELL CYCLE AND P53 DURING DIFFERENTIATION IN ES CELLS

The MPSS data were used to provide clues about the role of p53 signaling in ES cells. The protein p53 is a tumor suppressor that normally responds to DNA damage by inducing apoptosis and thereby preventing cell transformation (21, 22). It is also a critical regulator of the cell growth response to various types of cellular signals, including telomere shortening, DNA damage, oncogene activation, and overexpressed tumor suppressor genes (23–28). Besides playing an important role in controlling cellular life span and proliferation, p53 has also been implicated in regulating cellular differentiation (reviewed in [29]). In addition, dominant-negative mutants of p53 inhibit hematopoietic and muscle differentiation, and the forced expression of p53 results in a differenti-

ated phenotype (30). Likewise, several other reports have shown that endogenous p53 levels and activities are modulated upon differentiation. p53 expression is upregulated during maturation of human hematopoietic cells (83). A role of p53 during cellular differentiation indicates an alternative mechanism for p53 to maintain genetic stability in ES cells (31). In addition, p53 has also been reported to be downregulated at the protein and RNA levels during embryogenesis (32). These results suggest that p53 is a key integrator of diverse signaling pathways and, by determining whether cells will self-renew, differentiate, or die, its regulation plays an important role during early embryonic development.

The regulation of p53 does not appear to be critical for ES cells. ES cells, in contrast to all other somatic cells (including somatic stem cells), do not undergo cell cycle arrest under conditions such as ribonucleotide depletion and DNA damage. Stimuli that would normally activate the p53-mediated cell cycle arrest pathway in somatic cells appear unable to activate this pathway, despite the fact that mouse ES cells express high levels of p53. It appears that the p53-mediated response is inactive because of cytoplasmic sequestration of p53 and low efficiency of p53 translation to the nucleus (33, 34). Moreover, it has been shown that the p53-mediated cell cycle arrest response is restored upon differentiation of ES cells (33). Similar results are seen in EC cells. High levels of normal p53 are present in EC cells (35), and the expression of p53 is downregulated upon differentiation of EC cells. p53 can induce differentiation of mouse ES cells by suppressing Nanog expression (36). Likewise, p53 appears critical only during late stages of normal embryogenesis (32, 37, 38).

Although p53-null mice are viable (39, 40), mice lacking mdm2, which is a negative regulator of p53, die at an early embryo stage (41, 42). Collectively, the data suggest that p53 is important during differentiation and, while it is expressed in ES cells, its activity is inhibited and not required for ES cell self-renewal or early embryogenesis. Consistent with these observations, it has been possible to derive ES cell lines from p53-null animals. The gene expression profile of p53 pathway in the human ES cells and EBs by MPSS (19, 43) were generated. Interestingly, p53 was mainly present in human ES cells. In addition, levels of INK4a locus gene (p16 and p19) and p21 are low in ES cells and induced as cells differentiate (Fig. 12.1a). These p53 regulated genes have been found in cells undergoing replicative senescence along with p53 and p21Cip proteins (44). Thus, ES cells might not also require p53 for self-renewal, and its absence or lack of activity may be important in permitting ES cells to renew indefinitely.

How p53 is maintained in an inactive state remains to be determined and may be a promising line of inquiry in defining key regulators of cell proliferation. Mdm2 or a related family member may be important, as may be the ubiquitinylation pathway that regulates p53 activity (45, 46). The MPSS data suggest that the p53 pathway, which is a critical modulator of cell growth, is inactive in human ES cells as well as rodent ES cells, and that this inactivity is correlated with the ability of cells to self-renew indefinitely. Additionally,

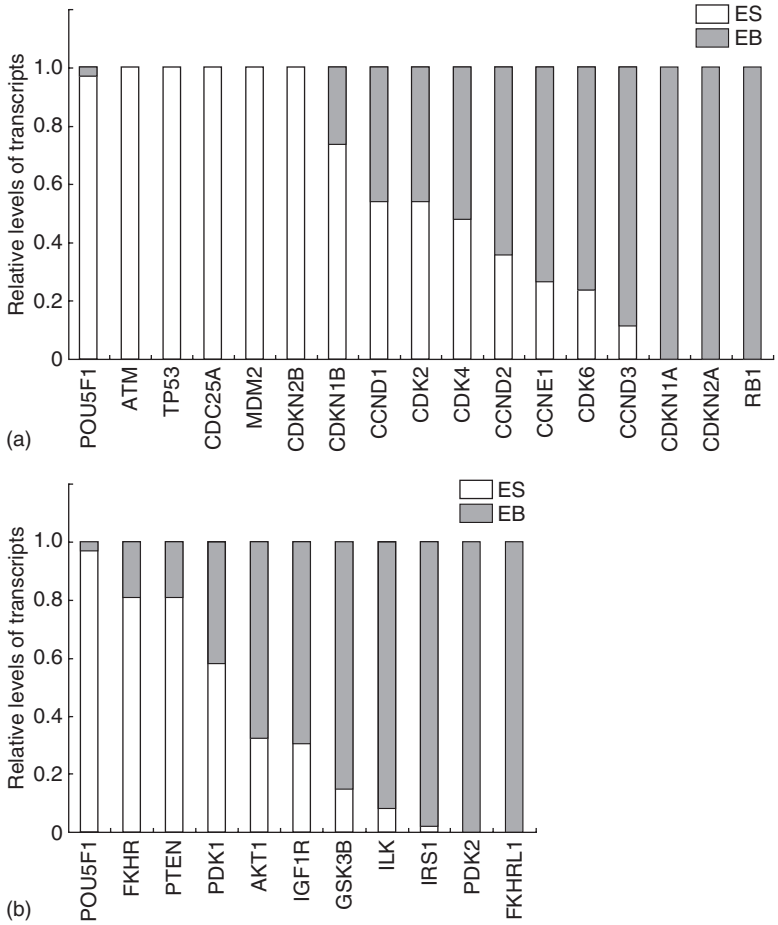

Figure 12.1. MPSS data of genes of the cell cycle regulatory pathway predicted to be upregulated or downregulated. EBs were prepared as previously reported (Miura et al., 2004) and harvested 14 d after replating. Expression levels in the EB samples are compared relative to the expression levels in undifferentiated hES cells, 1 being relative total expression levels combined gene expressions in the hES cells and EBs. All genes are differentially regulated. Representative genes in p53 and pRb pathway (a) and IGF-1/Akt pathway (b) were selected from MPSS data list (19). POU5F1, which is a known marker for undifferentiated hES cells, is shown for comparison.

Donehower and colleagues have recently generated a p53 mutant mouse that appears to display hypermorphic p53 activity. This mutant mouse, the p53+/m mouse, shows reduced longevity and an early onset of phenotypes associated with aging, which include organ atrophy and a reduced regenerative response (47), suggesting that stem cell function could be an important component of

these phenotypes. Thus, increased p53 activity results in inhibition of stem cell proliferation, apoptosis, or differentiation, suggesting that maintaining p53 in an inactive state is required for prolonged self-renewal.

EFFECTS OF THE RETINOBLASTOMA (RB)/E2F PATHWAY ON DIFFERENTIATION IN ES CELLS

A survey of the Rb/E2F pathway was performed by MPSS. Similar to p53, Rb is an important cellular life span regulator and interacts with p53. Rb and p53 are typically activated during senescence, and enforced expression of either protein induces growth arrest in some cell types (48, 49). The Rb/E2F pathway also plays a key role in controlling cell growth by integrating multiple mitogenic and antimitogenic stimuli. The components of this pathway are gene families with a high level of structural and functional redundancy and are expressed in an overlapping fashion in most tissues and cell types. Evidence from a variety of studies has suggested that cell cycle activation is coordinated by D-type cyclins that are rate limiting and essential for the progression through the G1 phase of the cell cycle. D-type cyclins bind to and activate the cyclin-dependent kinases Cdk4 and Cdk6, which in turn phosphorylate their downstream target, Rb. Upon Rb phosphorylation, the E2F transcription factors activate the expression of S-phase genes and thereby induce cell cycle progression. The rise of cyclin D levels in early G1 also serves to titrate p27Kip1/p21Cip1 proteins away from cyclinE/Cdk2 complexes, further accelerating cell cycle progression (reviewed in [50, 51]).

Intriguingly, Rb and the cell cycle appears to be differently regulated in ES cells when compared to all other somatic cells, raising the possibility that this difference may underlie the ability of ES cells to self-renew indefinitely. Cell division of ES cells is driven by unusually high Cdk2-cyclinA/E activity that is constitutively active throughout the cell cycle (52, 53). Hence, cell cycle regulation in ES cells resembles the mode of regulation described for many tumor cell types (54). These activities account for the abundance of hyperphosphorylated pRb (55) and the absence of cell cycle-regulated E2F transcriptional activity (52). The current view is that ES cells are deficient in cyclin D-associated Cdk activity (12, 56, 57) and rely on Cdk2 activity to drive the G1-S transition (52). This overall scenario indicates that mitogenic signaling is not coupled to the cell cycle machinery and the pRb/E2F pathway is inactive in ES cells (see below and [12]). As a consequence, ES cells have a very short G1 phase during which hypophosphorylated Rb protein cannot be observed (55). This absence of hypophosphorylated Rb in ES cells may trigger DNA replication right after exit from mitosis and is likely critical for prolonged self-renewal and distinguishes ES cells from all other differentiated cells. Consistent with this hypothesis which suggests that expression of the Rb family of proteins is low or absent, triple knockout ES cells, where all three Rb family genes

are knocked out, show normal growth rates, and DNA damage does not lead to arrest at G1 as is typical of other cells. Thus, lack of Rb regulation of the cell cycle may be relevant to the high proliferation rate, lack of growth arrest of ES cells.

The E2F family of transcription factors controls cell proliferation by regulating entry into the cell cycle as well as the G1-S phase transition. E2F proteins bind pocket proteins of the Rb family, consisting of Rb, p107, and p130 (58). The MPSS data showed that regulators of D-type cyclins are expressed at relatively higher levels in EB than in ES cells (Fig. 12.1a). Therefore, p16INK4a/cyclin D/Rb pathway may also be inactive in human ES cells, consistent with observations in mouse ES cells (12, 56, 57). Altogether, these data support the idea that signaling directed by the pRb pathway is not active in ES cells.

Intriguingly, Rb and p53 are regulated differently in mouse and in human cells. Ginis et al. examined the expression of cell cycle and cell death pathway gene by microarray and showed that while the overall regulation was similar, different members of the same family or different pathways were often used to regulate activity (18). Thus, while both populations show indefinite self-renewal and the absence of growth arrest, the mechanisms by which they achieve this differs. This is reminiscent of the differences in telomerase biology between rodents and humans (reviewed in [59–61]). Taken together, these findings strongly suggest that the cell cycle regulatory pathways in undifferentiated ES cells differ significantly from those in differentiated somatic cells and resemble those in transformed cells. This method of regulating cell cycle may be important in ES cells avoiding growth arrest as Rb regulation is induced when cells begin to undergo differentiation and lose their ability to bypass growth arrest. Both mouse and human cells appear similar in their regulation of Rb although important differences in how Rb is inactivated are observed. Further experiments are required to verify how critical this pathway is in regulating long-term self-renewal although the current results provide a framework in which to design such experiments.

ROLE OF THE PI3K/AKT PATHWAY IN THE MAINTENANCE OF ES CELL PLURIPOTENCY AND VIABILITY

As discussed above, the viability in ES cells is associated with many molecular mechanisms, including p19ARF/p53 and p16INK4a/Rb pathways. An additional key regulator of cell proliferation and organism aging is the IGF/Akt pathway. The importance of this pathway was convincingly demonstrated in mutational analysis in flies and worms (62, 63), and the relative importance of different components of this signaling pathway were established (reviewed in [64]).

Recent genetic analyses have shown that the signaling pathway of insulin/insulin-like growth factor-1 (IGF-1)/phosphatidylinocitol-3-kinase (PI3K)/Akt (also known as protein kinase B) dramatically extends the life span of the

nematode *C. elegans* (65–67). Likewise, in mammalian cells, activation of Akt has been reported to induce proliferation, enhance cell survival, and increase replicative life span. Overactivity of this pathway may promote tumorigenesis (68–70). Indeed, overexpression of Akt can transform NIH3T3 cells (71), while introduction of Akt antisense RNA inhibits the tumorigenic phenotype of cancer cells expressing high levels of Akt (72). The IGF/Akt pathway is likely also important in regulating cell size, as suggested by the phenotypes of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (a phosphatase that inhibits the Akt pathway) (reviewed in [73]) and thus plays a critical role in regulating long-term proliferation. Furthermore, its expression is correlated with enhanced life span.

IGF and insulin are ubiquitously expressed and appear to be important in early development of many tissues. Cardiac development and growth depend upon activation of Akt (74). Hepatic and pancreatic development requires IGF signaling and aberrant signaling such as those in PTEN mutants leads to early embryonic abnormalities. Thus, Akt may play an important role in regulating cellular life span and cell death in ES cells. Recent studies have also shown that IGF-1 plays an important role in preventing apoptosis in the early development of the embryo, as well as in the progressive regulation of organ development (66). IGF-1 signaling is known to be mediated via IGF-1 receptor (75–77), which is expressed during early blastocyst development.

The PI3 kinase/Akt signaling pathway has been implicated in ES cell self-renewal through studies of ES cells lacking PTEN (78, 79). These PTEN knockout ES cells have both increased viability and an increased rate of cell proliferation. Importantly, *Pten*-/- MEF (mouse embryo fibroblasts) also upregulated and activated the Akt-controlled anti-cell death pathway (79). However, the increase in the rate of cell division is more marked in *Pten*-/- ES cells than in *Pten*-/- MEF cells, highlighting the importance of this pathway in ES cell self-renewal. These findings suggest that the PI3K/Akt pathway might influence ES cell growth through a pathway that is dependent on IGF-1. However, it still remains unclear whether the IGF/PI3K/Akt pathway plays an important role in ES cell self-renewal. Examining expression of this pathway in human ES cells and comparing levels with expression in EBs by MPSS data (Fig. 12.1b) suggests that this pathway is active in both ES cells and EB, and somewhat surprisingly, that the gene expressions of the IGF/PI3K/Akt pathway were relatively high in EB compared with ES cells. Consistent with this result, PTEN is expressed at higher levels in ES cells than in EB (Fig. 12.1b). Indeed, it has been also reported that FGF signaling through PI3K/Akt is required for mouse embryoid body differentiation (80). Other investigators have also showed that activation of Akt signaling sufficiently maintains pluripotency in ES cells (81). Moreover, cell cycle regulatory proteins in ES cells are acting their cell proliferation via the PI3K/Akt and MAPKs pathways (82). Thus, this MPSS data suggest that IGF/PI3K/Akt pathway genes may be differently regulated in mouse and in human cells, and these differences may be important in understanding the differences in ES cell cycle and self-renewal.

CONCLUSIONS

This chapter discusses signaling pathways of cell cycle progression and viability in ES cells. It is suggested that while the usage of transcriptome analysis remains speculative, this chapter provides a framework to test specific hypotheses and select appropriate readouts. ES cell lines offer a useful model to study *in vivo* developmental processes at cell line level. Importantly, ES cells are capable of self-renewal as well as of differentiation into any cell types given an appropriate signal. Since proliferation and differentiation could be on the opposite sides of a coin, pluripotency of ES cells may be intimately linked to their growth properties. The most unique feature of cell cycle progression of undifferentiated mouse ES cells is very short gap phases (G1 and G2) and a high proportion of cells in S-phase (52, 55). This is associated with a unique mechanism of cell cycle regulation. As ES cells differentiate, their cell cycle structure changes dramatically so as to incorporate a significantly longer G1 phase, and their mechanism of cell cycle regulation changes to that typically seen in other mammalian cells. The unique cell cycle structure and mechanism of cell cycle control indicates that the cell cycle machinery plays a role in maintenance or cell immortalization of the stem cell state. Therefore, it is believed that antagonizing the growth arrest pathway in ES cells may be one key factor required to achieve constitutive proliferation. These findings indicate that further studies of signaling pathways related to cell cycle regulation in ES cells will provide useful clues for identifying cell cycle genes critical for understanding pluripotency mechanisms as well as small molecule drug screening for generation of induced pluripotent stem cells.

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PART IV

13

MEDIA AND EXTRA CELLULAR MATRIX REQUIREMENTS FOR LARGE-SCALE ESC GROWTH

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INTRODUCTION

Human embryonic stem cells (hESCs) are a unique cell type isolated from the inner cell mass of preimplantation blastocysts (1, 2). Theoretically, hESCs are capable of unlimited self-renewal and can differentiate to every somatic cell type in the body. In addition they may provide a system to study early human development *in vitro*, and in the future, differentiated populations may be used for cell replacement therapies. There are many scientific and regulatory hurdles to overcome if these applications are to become a reality. One aim of Novocell is the use of hESC-derived pancreatic cells to treat type I diabetes. Transplantation treatments for this disease are aimed at restoring glucose regulation by reintroducing cells capable of glucose sensing and a metered response in insulin secretion. Current successful therapies include whole organ transplantation, and cadaver-derived islet transplantation into the portal vein, termed the "Edmonton protocol" (3). However, the number of patients that can be treated is limited, as only about 50% of the ~12,000 pancreases that are donated in the United States every year are actually recovered (4). Because of their capacity for self-renewal and differentiation to multiple cell types, hESCs are capable of generating virtually unlimited numbers of cells for transplantation. Recent groundbreaking work has shown that hESCs can differentiate to pancreatic

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endodermal lineages that may be of eventual clinical significance for type I diabetes (5, 6). The demonstration that pluripotent cells can be directed to differentiate in a stepwise and sequential fashion to mesendoderm, definitive endoderm, foregut endoderm, posterior foregut, pancreatic endoderm and endocrine precursors, and finally to single hormone expressing pancreatic endocrine cells, is a powerful demonstration of this strategy. Diabetes may be one of the first therapeutic applications for hESC-derived cells because of the success of the Edmonton protocol, and the conceptual ability to encapsulate the transplantable population. Encapsulation may enable transplantation across histocompatibility barriers while avoiding the need for long-term immune suppression, which can cause substantial undesirable side effects such as mouth ulceration and anemia (7). Novocell are currently conducting a phase I/II clinical trial using a novel polyethylene glycol (PEG)-based technology (8, 9) to encapsulate cadaver-islets without long-term immune suppression (Novocell, http://www.novocell.com/ [ClinicalTrials.gov identifier: NCT00260234]).

Some of the objectives to be achieved prior to the clinical application of hESC-derived islet cells include the demonstration of functional recovery in animal models of diabetes, production of transplantable cells under current good manufacturing practice (cGMP), and demonstration of safety. Another current issue is the development of techniques to scale the expansion of hESCs. The most efficient differentiation regimes developed thus far generate roughly equivalent numbers of single hormone-producing endocrine cells to starting hESCs (6). While most laboratories grow hESCs on a very limited scale, in the order of 5×10^6 cells per 60 mm dish, approximately 10^9 cells are currently used to treat individual patients with islet transplantations. While it may be possible to isolate and expand committed progenitor populations during pancreatic differentiation, this has not yet been demonstrated. Developing systems that enable the expansion of $10^{10–12}$ hESCs is therefore one approach to generate the cell numbers required for actual transplantation. Such systems have not yet been described.

Many researchers use methods for propagation of hESCs that involve coculture with feeder layers, or the use of feeder-conditioned medium, serum, or undefined serum substitutes (10). While these conditions enable the maintenance of undifferentiated cells, their undefined nature leads to a lack of consistency in the quality of cultures and poor control over spontaneous differentiation. While human feeder-based systems can be qualified and have already been used to isolate hESC lines under cGMP (CyT49 cells, Novocell unpublished data; ES Cell International [Nature, http://www.nature.com/, News, July 27, 2006]), these conditions are not likely to be sufficiently robust to enable simple large-scale propagation of hESCs. Another substantial hurdle has been the observation by several groups that aneuploidies can occur in hESC cultures (11, 12). Additional copies of hChr12, 17, 17q, or X are the most common alterations observed (13), suggesting that the selective pressure that drives this phenomenon can exist under multiple culture conditions. While it

has been suggested that aneuploidies are primarily associated with single cell passaging using agents such as trypsin, it is also possible that poor survival after plating single cells when these complex culture conditions are used (14) provides selection pressure leading to the accumulation of aneuploidies. Another possibility is that this selection pressure is influenced by a component of serum replacer, via induction of CD30/NFκB signaling (Chung, T.-L. et al., 2007 ISSCR Annual Meeting, Poster WED-225). Whatever the actual cause of this selection pressure, if it can be avoided, the simplest approach to scale hESCs would involve fully defined media and single cell disaggregation.

There have now been several reports of defined media formulations for hESCs (15–18). Most of these formulations are based upon commercially available base media and supplements, and overlapping combinations of growth factors. In one case a fully defined medium supported the isolation of new hESC lines, although aneuploidies were observed at later passages (17). While these formulations were all effective in maintaining hESC cultures, large-scale expansion of undifferentiated cultures has not been described. Furthermore, these reports have not contributed to our understanding of the cell surface receptors and signaling pathways that promote self-renewal and limit differentiation. One example is the widespread use of fibroblast growth factor 2 (FGF2) in hESC culture conditions. Several reports have demonstrated that FGF2 impacts self-renewal of undifferentiated cells, and inhibition of FGF receptors with SU5402 induces differentiation (18–20). However, in simplified or fully defined media backgrounds, FGF2 only appears to promote self-renewal of hESCs at >10–20-fold higher concentrations than required for other FGF-responsive cell types, such as neural progenitors (21, 22). The need for supraphysiological levels of FGF2 has been attributed to instability of the protein in culture media (22), but another interpretation is that exogenously supplied FGF2 has an indirect role in self-renewal. It has recently been suggested that FGF2 acts upon differentiated cells within heterogeneous cultures to induce production of paracrine factors that support undifferentiated cells (23). Additionally, a careful examination of the activation of FGF receptors and downstream signaling in hESCs in response to FGF2 has not yet been described. Therefore we are still some way from a full understanding of the pathways that drive self-renewal and limit differentiation, and the extracellular ligands that can impact this efficiently.

PROPERTIES OF AN OPTIMIZED HESC CULTURE SYSTEM

We aimed to gain a more thorough understanding of receptors and downstream signaling in hESCs and use this information to develop a defined medium for the propagation of hESCs. We felt this approach would enable us to meet strict criteria in the overall quality of hESC cultures and utility of the medium:

1. The formulation would be fully defined and feeder free.
2. The choice of ligands would be based on a clear understanding, or demonstration, of biological relevance to self-renewal, such as activation of receptor/signaling pathways.
3. The medium would enable proliferation of hESCs in the absence of overt spontaneous differentiation, or generation of undesirable "autogenic feeder" cells.
4. The medium would enable propagation of cells without the requirement to constantly manually select undifferentiated cells or remove overtly differentiated regions.
5. The medium would enable single cell passaging of hESCs without overgrowth of aneuploid cells, enabling scaled expansion of cultures.

While it is obviously desirable to develop a media free of animal-derived components for clinical applications, this greatly increases the cost and is not required for research applications. In fact, Food and Drug Administration (FDA) approval of the final clinical product is the relevant standard for a cell-based biologic. This is achieved in part by compliance with cGMP, and animal derived components can be qualified within this system. For example, some companies produce bovine serum albumin (BSA) using cGMP principles in FDA-licensed facilities. This includes full traceability, stability data, screening for adventitious agents, and certificates of suitability. Thousands of tons of BSA are used yearly in the production of clinical products such as Remicade, a monoclonal antibody targeting TNFα. Furthermore, products that contain BSA have been approved by the FDA for direct use in patients, including BioGlue, a surgical adhesive that consists of 45% BSA and 10% glutaraldehyde. We therefore elected to develop an affordable research grade medium containing BSA and bovine transferrin as the only animal-derived components. The BSA was sourced from Celliance (Millipore) and manufactured in an FDA-licensed cGMP facility. More expensive human transferrin could also be substituted seamlessly for bovine-derived transferrin (not shown). Therefore, the use of these components does not preclude the ability to comply with cGMP if desired. Indeed, BSA was a component of the culture medium used to isolate Novocell's cGMP compliant hESC line. The choice of extracellular matrix (ECM) components used to mediate cell adhesion was also largely dictated by cost. Matrigel is a mixture of ECM proteins purified from the Engelbreth–Holm–Swarm mouse sarcoma (24). It consists primarily of laminin, collagen IV, heparan sulfate proteoglycans, and entactin, but it also contains trace levels of other factors, such as TGFβ, FGF's, and tissue plasminogen activator. Matrigel has been used widely for hESC propagation (25) and was therefore considered suitable for inclusion in a research-grade culture system. Clinical conditions will require a cGMP source of ECM, so various individual ECM proteins were subsequently tested for supporting hESC plating and growth.

RECEPTORS, SIGNALING, AND SELF-RENEWAL

Key observations that were made in our investigation of self-renewal that contributed to the development of a defined medium included:

1. PI3 Kinase signaling is a central pathway in self-renewal and inhibition of key members of this pathway, including PI3K, AKT, or FRAP1 (mTOR), induces differentiation of hESCs (26–29).
2. Insulin-like growth factor 1 (IGF1) and high concentrations of insulin provide strong signaling through PI3K via binding to the insulin and IGF1 receptors (26). Inhibition of IGF1R with the A12 monoclonal antibody or shRNA inhibits proliferation and induces differentiation of hESCs (26). Widely used media components such as serum replacer, N2 and B27 contain μg/mL concentrations of insulin, driving IR and IGF1R signaling in a wide range of hESC culture conditions.
3. The EGF receptor-family members ERBB2 and ERBB3 are highly expressed by hESCs (26, 30) and reside on hChr17 and 12, respectively. ERBB2 and ERBB3 are associated with the transformation of breast and other epithelial tissues through over expression, gene duplication, or trisomies (31–34). Inhibition of ERBB2 phosphorylation with AG825 inhibits self-renewal and promotes apoptosis in hESCs (26). Heregulin1β (HRG) is an EGF-family member that activates signaling of the ERBB2/3 heterodimer via high affinity binding to ERBB3 (34). Mouse embryonic fibroblasts (MEFs) produce a soluble ERBB2/3-activating ligand, and HRG promotes self-renewal and proliferation of hESCs via activation of the ERBB2/3 heterodimer and PI3K signaling (26). Conversely, EGF-family ligands that do not activate ERBB2/3, such as EGF, TGFα, and heparin binding EGF, do not support self-renewal (26).
4. Inhibition of TGFβ family signaling with SB431542, or FGF receptor signaling with SU5402 inhibits self-renewal and causes differentiation (18, 26).

DEVELOPMENT OF DC-HAIF MEDIUM

From these observations we assembled a fully defined media, termed DC-HAIF (26), containing HRG, an IGF1 analogue (LR^3-IGF1), and low concentrations of FGF2 and Activin A. LR^3-IGF1 does not bind IGF binding proteins, is made to cGMP, and is currently used in clinical-grade manufacturing processes. DC-HAIF conditions enabled long-term self-renewal of multiple euploid hESC lines, with negligible levels of spontaneous differentiation (26). Extensive analyses by comparison with cells maintained using traditional approaches demonstrated the retention of pluripotency. This included maintenance of classical marker expression by immunofluorescence, reverse

transcriptase–polymerase chain reaction (RT-PCR) and microarray comparison, pluripotent differentiation in teratomas, and directed endodermal differentiation *in vitro* (26). Under these conditions, self-renewal appeared to be driven largely by IGF1 and HRG mediated signaling (26). Low concentrations of FGF2 and Activin A were included as inhibition of their receptors led to overt differentiation. Exogenously supplied FGF2 was subsequently found to be essentially dispensable, and high-quality cultures could be maintained long-term without added FGF2 (Schulz and Robins, unpublished data). These cultures also exhibited marker expression, transcriptional profile (data point shown in Fig. 13.2c), and differentiation potential in teratomas that was consistent with full retention of pluripotency (Schulz and Robins, unpublished data). Treatment of these cultures with SU5402 caused partial differentiation over 10 days, however, suggesting that hESCs produce an FGF ligand that is still required for FGF signaling in this context (Schulz and Robins, unpublished data). Initial experiments indicated that Activin A supplied a TGFβ signal that contributed to suppressing differentiation in the context of this defined medium. However, proliferation was not tightly dependant on this factor when HRG and LR^3-IGF1 were present, and Activin A could be periodically omitted, or used at 1–10 ng/mL. This was of significant practical consideration in attempting to scale hESC cultures. Bioactive Activin A is a homodimer that is generated by expression in Chinese hamster ovary (CHO) cells, and is therefore relatively expensive. For comparison, the list prices for the growth factors we used are: HRG ($7.50/μg), LR^3-IGF1 ($0.58/μg), FGF2 ($7.20/μg) and Activin A ($50/μg). While the standard DC-HAIF formulation contained 10 ng/mL HRG, 200 ng/mL LR^3-IGF1, 8 ng/mL FGF2 and 10 ng/mL Activin A, the medium used for scaling cells (described below) included Activin A at 1 ng/mL.

Several other general considerations and observations were made during the development of DC-HAIF. hESC colonies were passaged by partial disaggregation with Collagenase IV, and a split ratio of 1:3–4 was typical (26). The use of collagenase, or other enzymes such as dispase, is common in the field, and results in splitting colonies to clumps rather than single cells. The base medium selected was DMEM/F12, containing pyruvate and glutamax. Both bicarbonate and HEPES buffered versions were used successfully, although the former was mostly used. Fatty acid free-BSA, manufactured with heat-shock fractionation, was used throughout. Of all the components in DC-HAIF, the quality of the BSA was the single largest source of variation in the quality of cultures. Although hESCs could be maintained in most batches of this "biotech grade" BSA, poor lots resulted in reduced morphological quality and lower cell viability at passaging. Batch testing was required to assess lots of this reagent. BSA was used at a standard concentration of 2%, although for some applications such as generating samples for proteomics, cells could be maintained successfully in as little as 0.2% BSA, with final overnight cultures BSA-free. In an attempt to augment lipid metabolism, several different defined mixes of lipids were tested. Addition of lipids rapidly induced differentiation, even at extremely low concentrations, and large numbers of migratory cells

similar to "autogenic feeders" filled in the spaces between otherwise undifferentiated colonies. It may be that undefined lipid contaminants contribute to the uncontrolled background differentiation observed in some other hESC culture systems. The only source of lipids in DC-HAIF was therefore the linoleic and lipoic acid provided in the DMEM/F12 base medium. The medium was also supplemented with nonessential amino acids, ascorbic acid, and trace elements. These components were used at standard concentrations, and improvements are likely possible by titrating concentrations of individual components. While the initial ECM used in these studies was Matrigel diluted 1:30 (25), we determined that it could be diluted up to 1:1000 without impacting plating or growth characteristics of hESCs. A standard 1:200 dilution of Matrigel was used subsequently. Other ECMs such as human serum (18), fibronectin (11), or VitroGro (35) could also be used successfully for serial culture of hESCs. These human-derived ECMs could potentially be sourced easily to cGMP for clinical applications. This defined medium has been licensed to Invitrogen Corp. and has been released as "StemPro hESC SFM". A large advantage of commercial production is that batch-testing of components is performed by the manufacturer and large lots of equivalent reagent are therefore produced.

The defined medium system we developed is the basis of a sophisticated culture platform for hESCs (Fig. 13.1) that has enabled: (a) robust single cell passaging; (b) directed differentiation to endodermal lineages (26) using defined media conditions; (c) reliable small-scale culture in high-throughput formats for molecule screening applications; and (d) scaled propagation of hESCs.

ROBUST SINGLE CELL PASSAGING OF HESCS

In considering approaches for large-scale expansion of hESCs, it should be noted that splitting colonies as clumps generally provides sufficient viability when using traditional approaches involving feeders or conditioned medium. In contrast, single cell disaggregation with agents such as trypsin causes extremely low cell viability with these approaches (14). We reasoned that substantially higher viability might be possible in DC-HAIF medium and focused on identifying gentle disaggregation approaches that would enable this. The Accutase reagent consistently enabled high plating and survival efficiencies after splitting hESCs to single cells and plating in DC-HAIF medium (Fig. 13.2). Accutase is a proprietary formulation of protease and collagenase agents, as well as 0.5 mM EDTA, and is available from several distributors. After disaggregation, $2.5–5 \times 10^4$ single cells/cm^2 were plated (typically 0.5–1×10^6 cells/60 mm dish), and counting studies demonstrated that after 24 h the number of surviving cells was consistently greater than 85% of the seeding population. While this figure does not distinguish initial viability from subsequent proliferation or apoptosis, the high survival rate indicates the robust

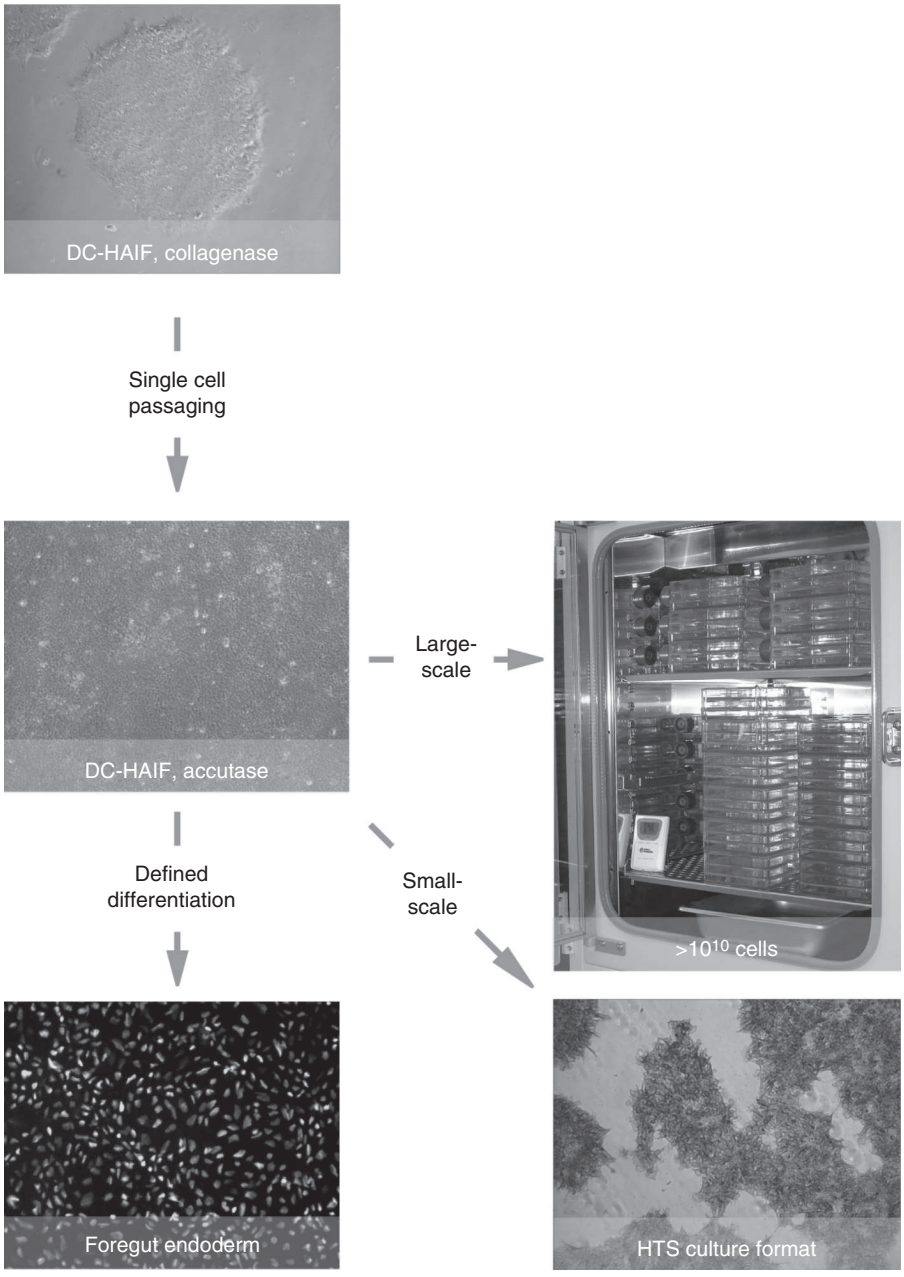


Figure 13.1. An advanced culture system for hESCs. DC-HAIF medium supported the propagation of undifferentiated euploid hESCs, including cultures that were split to single cells at passaging. Single cell suspensions could be plated at defined densities with high viability and maintained without manual selection. This enabled the directed differentiation of hESCs using defined media ($HNF1\beta^+$ foregut endoderm is shown in green), reliable culture in high throughput screening culture formats (alkaline $phosphatase^+$ hESCs in a 384-well tray are shown), and scaled expansion to $>10^{10}$ cells. (See color insert.)

nature of this approach. While other reagents such as Versene and TrypLE (Invitrogen) were also similarly effective and enabled long-term passaging in DC-HAIF, the remaining studies described here used Accutase.

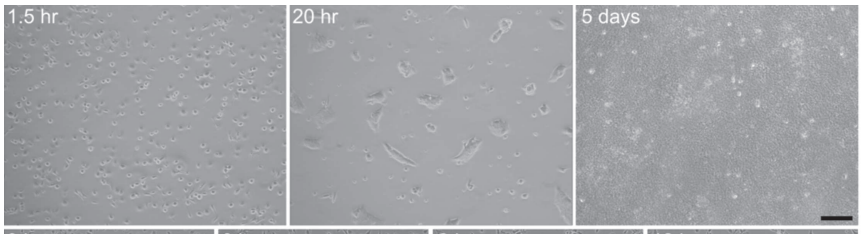
Upon passaging with Accutase, single hESCs attached to the culture dish rapidly and flattened within 3 hours. Individual cells were highly migratory and coalesced to form micro-colonies of 20–50 cells within ~20 h (Fig. 13.2a). Time-lapse microscopy revealed a highly dynamic process, with cells extending and contracting lamellipodia-like extensions within 30 min periods, and some cells migrating a distance of >25 cell diameters within 12 h (Fig. 13.2b). Occasional mitotic or apoptotic cells were also observed. Individual cell movement was reduced once colonies were formed, although cells on the edges of colonies still exhibited dynamic extensions. Over the course of 5–6 d, such cultures would proliferate, fill in the spaces between the initial colonies, and form a confluent monolayer of undifferentiated $OCT4^{+}$ cells. While these experiments were initially performed with cells already growing in DC-HAIF, we subsequently showed that cells could be passaged directly from MEFs or MEF-CM/Matrigel to these conditions with comparable efficiency and without the requirement for a "transition" period. Accutase could be used to serially passage hESCs over long-term culture (>6 months), without the requirement for manual selection prior to splitting. These cultures remained undifferentiated, as demonstrated by morphology (Fig. 13.2a,b), marker expression (not shown), microarray comparison to other conditions and cell lines (Fig. 13.2c), and pluripotent differentiation *in vivo* (not shown). These cultures displayed several characteristics of polarized epithelia (manuscript submitted), a feature of hESCs also recently reported by others (36). This included expansion to a uniform confluent monolayer, formation of tight junctions as demonstrated by organized localization of ZO1 and occludin, and localization of ERBB3 (manuscript submitted). G-banding and copy number enumeration of hChr12, 17, and X by fluorescence in situ hybridization (FISH) demonstrated that cultures remained euploid over long periods (Fig. 13.2d,e), indicating that these conditions did not induce the generation of characteristic trisomies. However, DC-HAIF did not appear to alter the comparatively faster expansion of aneuploid cells, as overgrowth of cells containing an additional hChr17 could still occur from "parental" MEF-CM cultured cells harboring low levels of this trisomy (<10% by FISH), or when 5% BG01v cells (49XXY, +12, +17) were spiked into an otherwise euploid BG01 culture.

It should be noted that all the data reviewed here describes hESCs plated and grown at densities suitable for serial propagation. While DC-HAIF medium supports the survival of single cells, the cells appear to require cell–cell contact within the first 24–48 h after plating. Hence, hESCs plated at clonal density still appear migratory, but apparently lose viability over time (not shown). It is possible that antiapoptotic factors, such as ROCK inhibitors (37), may enable efficient single cell cloning in a fully defined medium. Regardless, single cell passaging and plating at propagation density was robust, and enabled a range of culture vessels to be utilized. This included 96- and 384- well formats,

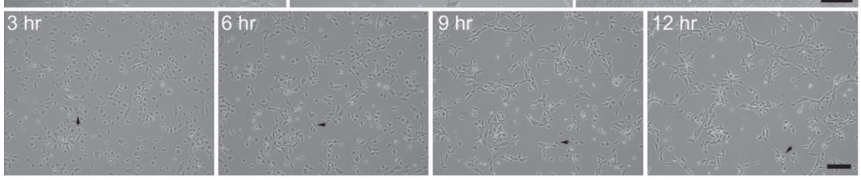
where reliable quantitative data could be generated in drug screening applications (not shown).

The ECM requirements of hESCs passaged with Accutase appeared to be very similar to cells passaged as clumps. Matrigel was also used at a dilution of 1:200, and human serum, fibronectin, or VitroGro, could be used in this culture format. While others have used purified human ECM proteins to enable attachment and growth of hESCs (17, 21), this is expensive and unnecessary for most research applications. While coating plates with human serum was relatively cost effective, there were drawbacks associated with this approach. Serum is undefined and appeared to cause a higher level of spontaneous differentiation than purified ECM substrates (data not shown). Therefore, while Matrigel is a suitable reagent for research purposes, there is a clear need for an affordable defined-, or cGMP grade-, ECM as large-scale hESC culture systems are developed.

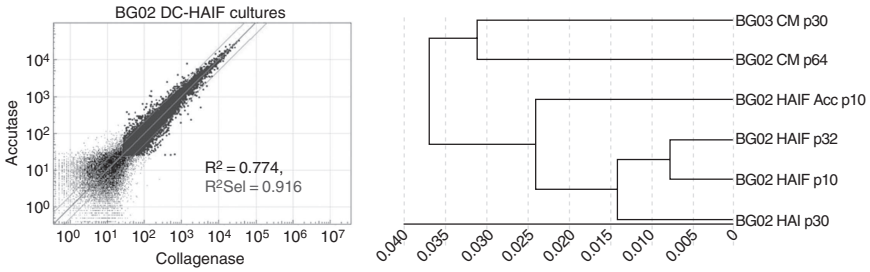
(a)



(b)



(c)



(d)

G-banding

BG01	HAIF	Acc p11	46,XY [19/20]
	HAIF	Acc p17	46,XY [19/20]
BG02	HAIF	Acc p5	46,XY [20/20]
	HAIF	Acc p5	46,XY [20/20]
	HAIF	Acc p5	46,XY [19/20]
	HAIF	Acc p18	46,XY [20/20]

(e)

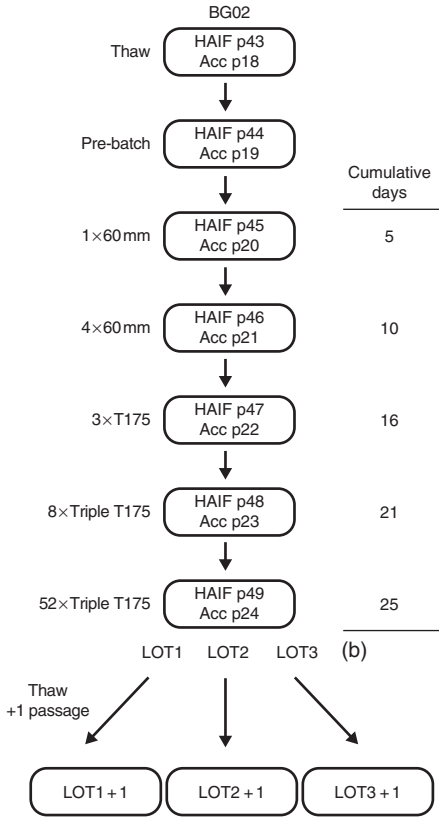
FISH

	% Normal hChr (n=)			
	12	17	X	Y
BG01 HAIF Acc p5	97 (512)	97 (922)	98 (516)	NA
BG02 HAIF Acc p49	96 (521)	96 (667)	98 (597)	97 (822)
CyT49 HAIF Acc p7	98 (602)	96 (542)	97 (572)	97 (533)

SCALED EXPANSION OF HESCS

Given the robust nature of single cell passaging in the context of DC-HAIF medium, we were interested in investigating the scalability of this system for the manufacture of hESCs for eventual clinical applications. We therefore expanded, harvested, and characterized a single batch of $>10^{10}$ BG02 hESCs (Fig. 13.3). We elected to use a simple approach to reach the surface area required for this expansion, rather than specialized equipment such as cell factories. We determined that triple-layered T175 flasks were suitable for serial propagation of hESCs using DC-HAIF and Accutase, and could be used to grow $>2 \times 10^8$ cells/vessel. A detailed method for single cell passaging in DC-HAIF and this scaled expansion, including cell densities and medium volumes for different flasks, is presented below. A frozen stock of BG02 cells was selected that had been maintained in DC-HAIF for extended periods (43 passages), with extensive single cell passaging (18 passages), and had previously been demonstrated to have a normal karyotype (20/20 normal metaphase spreads). To initiate the process, a single vial of 10^6 BG02 cells was thawed into DC-HAIF medium in a 60 mm dish coated with 1:200 Matrigel. The plate

Figure 13.2. Robust single cell passaging of hESCs. (a) hESCs were disaggregated to single cells with Accutase and plated in DC-HAIF on 1:200 Matrigel. Images taken 1.5 and 20 h after plating indicate that cells migrate to form micro-colonies. These cultures expand to form monolayers of undifferentiated cells after 5–6 d of growth. Scale = 100 µm. (b) Time-lapse imaging of the migration of hESCs during the period after plating. Virtually all cells display dynamic movement, coalescing to form small colonies within ~20 h. The movement of an individual cell that migrated >25 cell diameters within the first 12 h is indicated (arrowheads). Selected images are shown from a set that was captured every 30 min over 19 h. Scale = 100 µm. (c) Illumina bead microarray comparison of BG02 cells growing in DC-HAIF and maintained by passaging with collagenase or Accutase (left) was performed as described previously (26, 38). A large proportion of transcripts were within a two-fold range of expression. Correlation coefficients (R^2) were generated from all detected transcripts (all data points), or from transcripts with a high detection confidence level (>0.99, R^2Sel, blue data points). The red lines indicate the mean and the two-fold range of detection. A hierarchical comparison (right) was used to demonstrate the close clustering of samples from multiple different hESC lines, culture conditions and passaging techniques, examined with this platform. Global transcription was not substantially different across these samples. The samples included BG02 and BG03 cells maintained in MEF-conditioned medium (CM); Early (p10) and later (p32) passage BG02 cells maintained in DC-HAIF with collagenase passaging; BG02 cells maintained with collagenase passaging in defined medium without exogenous FGF2 (BG02 HAI p30); and BG02 cells propagated in DC-HAIF with Accutase passaging (Acc). Previous analyses showed that differentiated populations such as embryoid bodies or fibroblasts were well separated from this cluster (~0.0875 and ~0.160, respectively) (38). (d) Accutase passaged BG01 and BG02 cells retained a normal 46,XY karyotype. (e) FISH analyses confirmed the maintenance of euploidy for chromosomes 12, 17, X, and Y in the BG01, BG02, and CyT49 cell lines. (See color insert.)



(a)

Cumulative days	Medium (L)/day	Medium per stage (L)
5	0.004	0.02
10	0.016	0.08
16	0.105	0.525
21	0.8	3.2
25	5.2	15.6

(b)

	$
Accutase	156.00
PBS	20.64
Dishes	1.05
T175	7.51
Triple T175	498.79
Cryotubes	242.06
Cryotube inserts	18.62
Freezing media	370.01
BSA wash	103.49
DC-HAIF (ActA 1 ng/ml)	3222.36
Matrigel	393.72
DMEM:F12	133.09
Total cost ($)	5167.34

(c)

Figure 13.3. Scaled expansion of BG02 hESCs. (a) Schematic representation of the batch of BG02 hESCs. A vial of 10^6 cells was thawed, split to "pre-batch" cultures and qualified by FISH and G-banding. This culture was split to generate the initiating 1×60 mm dish, which was expanded over five additional passages. The final 52× triple T175 stage was harvested in three lots. Thaws from each lot were designated as LOT1 + 1, 2 + 1, and 3 + 1. (b) A summary of the timing and usage of medium at each stage is indicated. (c) A summary of the actual costs of consumables for the scaled expansion. Along with only using 1 ng/mL Activin A, the cost of the DC-HAIF medium takes into account discounts for large orders that were negotiated with the suppliers of the growth factors.

was passaged, generating "pre-batch" cultures in 60 mm dishes, which were qualified by karyotyping and FISH (Fig. 13.4a,b) and shown to be normal. To initiate the large-scale expansion, 10^6 cells were passaged to a 1×60 mm dish. This culture was then expanded over four passages and 25 total days, to sequentially generate 4×60 mm dishes, 3× T175 flasks, 8× triple layer T175, and finally 52× triple layer T175 flasks (Fig. 13.3a). The 52× triple T175 flask batch was harvested as three lots to simplify processing. While it was only

possible to monitor the lowest layer of a triple T175 flask with an inverted microscope, all layers could be visualized with a dissecting microscope. The cultures remained morphologically undifferentiated, and no manual selection or removal of differentiating regions was conducted during the expansion.

In terms of project design, a single lab member performed the scaled expansion to 52× triple T175s (Fig. 13.3a), while five lab members were required to harvest, prepare, and dispense cells to vials on the final day. A total of ~19.5 L of media was used for the five expansion steps, 15.6 L of which was for the final stage (Fig. 13.3b). As outlined above, the media contained 1 ng/mL Activin A, and including discounts negotiated with the suppliers, the cost of the media was ~$3200 out of a total reagent/consumables cost of ~$5200 (Fig. 13.3c).

The outcomes of the batch were:

1. An average of 2.14×10^8 cells per final triple T175;
2. 73 sq. meter total surface area at final stage;
3. 1.112×10^{10} total harvested cells;
4. 1200 cryopreserved vials of 10^6 cells/vial; and
5. 100 frozen cell pellets of 10^8 cells.

BATCH QUALITY-ASSURANCE TESTING

This batch of cells was characterized and qualified using multiple approaches. For these analyses, cells were either sampled directly from the batch (FISH, RT-PCR, mycoplasma), were from cultures 1–2 passages downstream of the batch (cytometry, teratomas), or were from thawed vials (thaw recovery, karyotyping). To examine recovery of cultures from frozen vials containing 10^6 cells, representatives from each lot were thawed. 8×10^5, 5×10^5, and 4×10^5 cells were counted 24 h after thawing and plating from LOT1, 2, and 3, respectively. Parallel representative thaws were expanded to 6.3×10^6, 7.8×10^6, and 2.7×10^6 cells after 5 d, respectively. These cultures could all be serially passaged (termed LOT1 + 1, 2 + 1, or 3 + 1), demonstrating the capacity to bank viable hESCs on this scale. Testing for mycoplasma contamination demonstrated that the batch was negative (not shown).

FISH analysis was used to follow the copy number status of hChr12, 17, X, and Y during the expansion process, and was performed at the pre-batch, the 1× triple T175, and 52× triple T175 stages. Obvious aneuploidy was not observed, with a high percentage of normal nuclei counted for each chromosome (Fig. 13.4a). The percentage of normal nuclei counted at the 52× triple T175 stage was: 98% hChr12 ($n = 1076$), 98% hChr17 ($n = 1151$), 95% hChrX ($n = 1188$), 98% hChrY ($n = 1120$). This approach was highly valuable in providing near real-time monitoring of the expansion and adverse results would have lead to early termination of the batch to minimize expenditure. The cultures were also karyotyped by standard G-banding at the pre-batch stage as described, as were

(a)

FISH

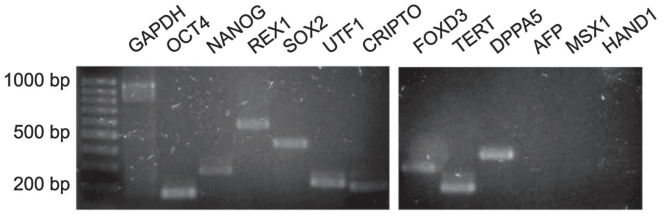
	% Normal nuclei (n=)			
hChr	12	17	X	Y
Pre-batch	98 (523)	98 (577)	98 (578)	97 (605)
1x Triple T175	95 (1108)	98 (1063)	93 (1222)	99 (1049)
52x Triple T175	98 (1076)	98 (1151)	95 (1188)	98 (1120)

(b)

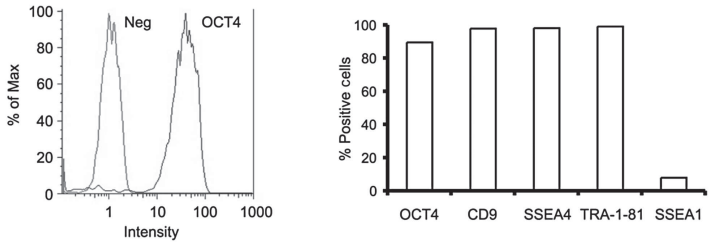
G-banding

	Primary analysis	Secondary analysis
Pre-batch	46,XY [19/19]	46,XY [31/32]; 46,XY del(11)(q13q21) [1/32]
LOT1 +1	46,XY [20/20]	46,XY [50/52]; 46,XY del(11)(q13q21) [2/52]
LOT2 +1	46,XY [19/19]	46,XY [45/47]; 46,XY del(11)(q13q21) [2/47]
LOT3 +1	46,XY [15/19] 44~46,XY del(11)(q13q21) [4/19]	

(c)



(d)



(e)

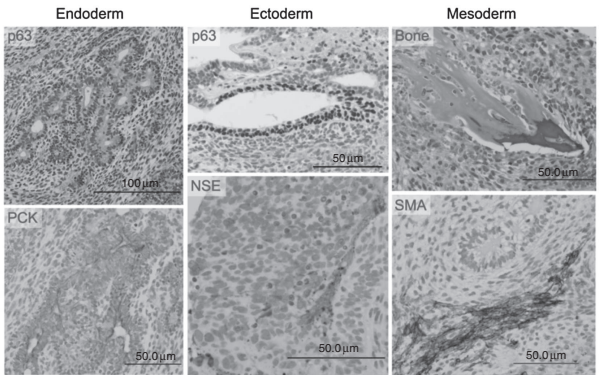


Figure 13.4. Characterization of the scaled batch of BG02 cells. (a) Enumeration of chromosome copy number by FISH. Chromosomes 12, 17, X, and Y were examined at the pre-batch, 1× Triple T175, and 52× Triple T175 stages. (b) Karyotyping by G-banding was performed on the pre-batch and LOT1 + 1, 2 + 1, and 3 + 1 cultures. A secondary analysis was performed because 4/19 metaphase spreads from the LOT3 + 1 culture indicated the presence of a deletion on chromosome 11 (q13–q21). The numbers indicated in the secondary analysis are the totals of both karyotypes. (c) End point RT-PCR demonstrated retention of expression of markers of pluripotency, and lack of expression of markers of differentiated lineages. Primer sequences were as described previously (11). (d) Flow cytometric analysis of markers of pluripotency including OCT4 (left) and OCT4, CD9, SSEA4, TRA-1-81 (right). Only a minority of cells exhibited detectable SSEA-1 (right), which is not expressed by undifferentiated hESCs. The antibodies used were OCT4 (Santa Cruz sc-5279), CD9 (Chemicon CBL162), SSEA4 (Chemicon MAB44304), TRA-1-81 (Chemicon MAB4381), and SSEA1 (Chemicon MAB4301). (e) Immunohistochemical analysis of teratomas generated from cells one passage downstream of the 52× Triple T175 stage. Teratomas were generated in SCID/beige mice and analyzed as described previously (39). Lineages that were detected included endodermal epithelia (left: $p63^-$ and pancytokeratin $[PCK]^+$); $p63^+$ ectodermal epithelia and neuron specific enolase $(NSE)^+$ neural lineages (middle); and bone and smooth muscle actin $(SMA)^+$ mesodermal lineages (right). Scale bars are indicated. (See color insert.)

LOT1 + 1, 2 + 1, and 3 + 1 cultures (Fig. 13.4b). Initial analyses indicated that the pre-batch (19/19 metaphase), LOT1 + 1 (20/20), and LOT2 + 1 (19/19) cultures were normal. Most LOT3 + 1 nuclei were normal (15/19), but 4/19 metaphase spreads exhibited random fragmentation, along with a recurring deletion on chromosome 11 (q13q21). A more detailed secondary G-banding analysis was therefore performed to determine if this deletion had occurred during this expansion process. The same deletion was observed in the pre-batch culture (1/32 total metaphase), as well as in the LOT1 + 1 (2/52 total metaphase) and LOT2 + 1 (2/47 total metaphase) cultures, although at frequencies below that detected by routine karyotyping. While these analyses demonstrated that the most common aneuploidies reported in hESCs were not observed, careful quality control monitoring of euploidy is still likely to be required in manufacturing scale batches of hESCs. The observation that the rare hChr11 deletion did not accumulate markedly through the course of the expansion indicated that it did not confer growth advantages.

Expression of transcriptional and cell surface markers was also examined. RT-PCR analysis of batch cells (Fig. 13.4c) demonstrated maintenance of expression of markers of pluirpotency including OCT4, NANOG, REX1, SOX2, UTF1, CRIPTO, FOXD3, TERT, and DPPA5. Markers of differentiated lineages, α-fetoprotein (AFP), MSX1, HAND1 were not detected. Flow cytometry (Fig. 13.4d) was used to examine the proportion of cells expressing OCT4 protein (89.7%), and surface markers of undifferentiated cells including CD9 (98%), SSEA4 (98%), and TRA-1-81 (99%). Only ~8% of cells exhibited

detectable SSEA-1 expression. These analyses confirmed that the batch consisted primarily of undifferentiated hESCs. Demonstration of the retention of the capacity for pluripotent differentiation was illustrated by the formation of complex teratomas *in vivo*, containing representatives of ectoderm, endoderm, and mesoderm (Fig. 13.4e).

SUMMARY

Pluripotent hESCs offer the promise of revolutionary cellular therapies that were inconceivable 20 years ago. The theoretical capacity for unlimited expansion and directed differentiation to specific functional and transplantable cell types suggests that some therapies could target large cohorts of patients. In the case of diabetes, it is possible that hESC derived β islet cells could be encapsulated for transplantation to nonmatched recipients without immune suppression. Such an approach would enable a cell population to be made as a product, rather than a patient-specific treatment such as autologous bone marrow stem cell transplantation. This approach will require a suite of new hESC technologies and infrastructure, including the ability to scale hESCs, direct differentiation precisely, purify the target cell population, all in a cGMP-compliant and industrial-scale setting. We have established a reliable platform for the application of hESC technology based on an increased understanding of the growth factor and receptor signals that are required for self-renewal. A simple defined medium containing heregulin, LR^3-IGF1, FGF2, and Activin A was developed that enabled hESCs to be maintained long term without loss of pluripotency. Importantly, these conditions also supported robust single cell passaging of euploid hESC cultures. By manipulating the timing and exposure to specific growth factors, hESCs could be directed to differentiate to endodermal lineages in fully defined medium. hESCs could also be plated uniformly in 96- and 384-well trays, enabling the identification of compounds that impact proliferation or apoptosis in drug screening assays. This approach could be used to identify small molecule agonists of the receptors that drive self-renewal, potentially reducing the cost of large-scale hESC culture. In a proof-of-principle demonstration of the scalability of this culture system, a single batch of $>10^{10}$ BG02 hESCs was grown in a 25 d period, harvested, banked, and qualified. This demonstrated the actual practical capacity to expand hESCs to clinically relevant numbers. Future improvements to these approaches are likely to include the identification of a cost-effective and cGMP-compliant ECM substrate, use of systems such as cell factories that greatly increase the potential surface area available for expansion, or automated systems to manage feeding, harvesting, and handling of mass cultures. While we successfully propagated $>10^{10}$ hESCs, current estimates suggest that 10^{13} cells would be required to differentiate enough islet cells to treat 10 000 patients with type 1 diabetes. A potential way to achieve this is through high-density suspension culture in bioreactors. In this scenario, hESCs could be propagated on microcarrier

beads coated with a suitable ECM, or given appropriate conditions, as cellular aggregates of undifferentiated cells (Schulz and Robins, unpublished data). These advances will be critical as hESC technology proceeds toward meaningful clinical reality.

METHODS FOR SINGLE CELL PASSAGING AND SCALED EXPANSION OF hESCs

DC-HAIF Medium (Components in Table 13.1)

- DMEM/F12, 2% fatty acid free-BSA, 1× nonessential amino acids, 1× Trace Elements –A, –B, and –C, 50 μg/mL Ascorbic Acid, 10 μg/mL Transferrin, 0.1 mM β-Mecaptoethanol, 1× Penicillin/Streptomycin (optional).

  Growth factors (added at time of use): 10 ng/mL Heregulin-1β, 200 ng/mL LR^3-IGF1, 8 ng/mL FGF2, 10 ng/mL Activin A (1 ng/mL Activin was sufficient in scaled culture, as described).

BSA Wash Medium

- DMEM/F12 with 0.2% BSA, 1× Penicillin/Streptomycin (optional).

METHODS

Preparation of Matrigel-coated Culture Vessels

- Thaw Matrigel slowly at 4 °C overnight to avoid gelling. Dilute 1:1 with cold DMEM/F12 on ice, mix and freeze aliquots at –20 °C.
- To coat culture vessels, thaw Matrigel aliquots for at least 2 hours at 4 °C. Add cold DMEM/F12 to a final concentration of 1:200. Add diluted Matrigel to vessels and coat for 1–2 h at room temperature. Remove the Matrigel solution immediately before use. Do not rinse the vessel.
- Appropriate volumes for coating different vessels are indicated (Table 13.2).

Single Cell and Scaled Passaging of Human ESCs with Accutase

A generic method for using multiple types of culture vessels is indicated. Specific volumes for each vessel are listed (Table 13.2). Sixty millimeter dishes are suitable for standard serial propagation. Specific instructions for triple T175 flasks are indicated.

- Warm an appropriate amount of DC-HAIF, BSA Wash medium and DMEM/F12 in a 37 °C water bath.

TABLE 13.1. Culture Components for Expansion of hESC: Storage Conditions are Listed on Reagent Specification Sheets

Reagent	Distributor	Catalogue Number	Stock Solution
Accutase	Innovative Cell Technologies	AT104	1×
DMEM/F12	Invitrogen	10565	1×
Fatty acid-free BSA	Celliance (Millipore)	82-047-3	20% stock in DMEM/F12. Store long-term at −80 °C. Requires batch testing. Do not substitute with lower grade.
Penicillin/Streptomycin	Invitrogen	15070-063	100×
Nonessential amino acids	Invitrogen	11140-050	100×
Trace elements A	Cellgro (Mediatech)	99-182-C1	1000×
Trace elements B	Cellgro (Mediatech)	99-176-C1	1000×
Trace elements C	Cellgro (Mediatech)	99-175-C1	1000x
Ascorbic acid	Sigma	A4034	50 mg/mL in H_2O
Bovine transferrin	Invitrogen	11107-018	20 mg/mL in PBS
Human transferrin (optional)	Invitrogen	0030124SA	4 mg/mL
β-Mercaptoethanol	Invitrogen	21985-023	55 mM
Heregulin-1β EGF domain	Peprotech	100-03	10 µg/mL in 0.1% BSA (PBS)
LR^3-IGF1	JRH Biosciences	85580	1 mg/mL in 10 mM HCl
Activin A	R&D Systems	338-AC	25 µg/mL in 0.2% BSA (PBS)
FGF2	Sigma	F0291	25 µg/mL in 1% BSA (DMEM/F12)
Growth factor-reduced Matrigel	Becton Dickinson	35 6231	1:1 in DMEM/F12
Nunc triple T175 flasks	Fisher	12-565-25	

TABLE 13.2. Cell Numbers and Volumes of Culture Media for Different Culture Vessels

Vessel	Surface Area (cm^2)	Matrigel Coating (mL)	Cells Seeded	DC-HAIF (mL)	Accutase (mL)	Cells Generated[b]
384-well[a]	0.08	0.05	10^3	0.05	0.05	~10^5
96-well[a]	0.3	0.1	10^4	0.1	0.1	~3.3×10^5
24-well	2	0.5	10^5	0.5	0.2	~1.6×10^6
12-well	4	0.5	2×10^5	1	0.5	~3×10^6
6-well/35 mm dish	10	1.5	5×10^5	2	0.5	~8×10^6
60 mm	20	2	10^6	4	1	$0.8–1.5 \times 10^7$
T25	25	2.5	10^6	5	1	$0.8–1.5 \times 10^7$
T75	75	7	3.75×10^6	15	3	$3–3.75 \times 10^7$
T175	175	20	8.75×10^6	35	5	$7–8.75 \times 10^7$
Triple T175	525	50	2.5×10^7	100	20	$2–2.5 \times 10^8$

[a]Not recommended for serial passaging.
[b]Cell line dependent, indicated numbers are based on counting BG02 cultures.

- Remove the medium from the culture to be passaged with an aspirating pipette, or a 5 mL pipette for large flasks, attached to vacuum hose.
  - For triple T175 flasks, flip the flasks over, and aspirate as the medium is poured slowly to the lip of the flask.
- Wash cells gently with PBS.
  - Use 50 mL PBS for triple T175 flasks.
- Add Accutase to cells, and rock the vessel gently to ensure all areas are covered. Leave 3–6 min and observe disaggregation of colonies under a microscope.
- Gently tap the sides of the vessel to dislodge cells, and remove with a pipette. Add to an appropriate excess of BSA wash medium (typically 1:5 cells : BSA wash buffer).
  - For triple T175 flasks, gently flip the flask over and pour the cells into a 50 mL tube. Add 15 mL of BSA wash medium to the flask and rinse. Add to the 50 mL tube. Repeat the wash with another 15 mL of BSA wash medium and add to the 50 mL tube.
- Spin cells at 1000 rpm (200 g) for 4 min at room temperature.
- Aspirate the medium and gently flick the tube to loosen the cell pellet.
- Gently resuspend the cells in an appropriate volume using a 5 mL pipette. Count viable cells using trypan blue exclusion.
- Make up the appropriate concentration of cells for the size and number of vessels. Mix gently and dispense to a Matrigel coated vessel.
  - For triple T175 flasks, add 2.5×10^7 cells per 100 mL of DC-HAIF medium in a container. Mix well and add 100 mL of cell suspension to each Matrigel coated triple T175 flask. Gently re-mix the cell suspension in between seeding each flask.
  - Let the suspension equilibrate in the chambers and then carefully tilt the flask to horizontal to evenly distribute cell suspension on each level.
- Place the vessels into an incubator set at 37 °C with 5% CO_2.
- Feed the cells daily with fresh DC-HAIF.
  - To reduce costs when expanding in triple T175 flasks, feed the cells on the second day after plating and daily thereafter.

Observe cells every day and passage by the above protocol whenever required (every 5–6 d).

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NOTE

While this manuscript was under review Novocell demonstrated the generation of hESC-derived pancreatic endoderm capable of differentiating to glucose-responsive insulin-secreting cells *in vivo* (40). Our description of the epithelial characteristics of hESCs was also published (41).

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14

AUTOMATED METHOD FOR EMBRYONIC STEM CELL CULTURE

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REPRODUCIBLE HESC CULTURE REQUIRES AUTOMATED METHODS

Human embryonic stem cell (hESC) culture as it is routinely performed today is an extremely labor-intensive and time-consuming chore. Even experience and immense zeal do not always suffice to avoid small variations in culture conditions and handling. In turn, small variations might result in major consequences in terms of alterations in growth rates, spontaneous differentiation levels and karyotypic aberrations. However, industrial and biomedical applications of hESC and hESC-derived somatic cells require the adoption of highly standardized and reproducible cell production processes. Taking into account both variability of manual cell culture per se and overlying interindividual variation, the required industry standard will hardly be met by manual procedures.

While automated liquid handling systems have become indispensable in genomics and proteomics, current cell culture techniques rely almost exclusively on manual intervention. Reasons for the hesitance to introduce automation into cell culture include the sensitivity of mammalian cells, the susceptibility of antibiotic-free cell cultures to contamination, and the need for a flexible response to cell culture condition and density. Coculture paradigms (1, 2) and

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TABLE 14.1. Comparative Analysis of Crucial Parameters Influenced by Manual Versus Automated Cell Culture

Parameter	Manual hESC Culture	Automated hESC Culture
Hands-on time	High	Low
Variability	High	Low
Compliance with industry standards	Low	High
Reproducibility	Medium	High
Traceability	Medium	High
Precision	Low	High
Initial costs	Low	High
Long-term costs due to personnel	High	Low
Reliance on experienced personnel	High	Medium
Susceptibility to contaminations due to inexperienced personnel	High	Low

complex differentiation protocols (3, 4) contribute to making hESC culture even harder to automate than conventional cell culture. On the other hand, extensive manual intervention and lack in standardization constitute a major bottleneck in hESC culture that only automation can overcome. Taking into account cell-type specific requirements, the development and the refinement of automated methods should result in a considerable reduction in hands-on time and a manifest advance in reproducible and standardized hESC production (Table 14.1).

THE CELL^{HOST} SYSTEM

In collaboration with Hamilton Life Science Robotics, we have devised an automated system (Cell^{host}), which performs crucial steps of ESC culture. The Cell^{host} is based on a Hamilton Microlab STAR® workstation which is contained in a sterile housing with laminar airflow and UV decontamination routine (Fig. 14.1a). The pipetting workstation is connected to a robot-accessible cell culture incubator (capacity of 153 cell culture plates) and to a CO_2-supplied refrigerator for storage of media, growth factors, and compounds (capacity of 20 media tubs or multiwell plates containing growth factors/compounds). Barcode readers integrated into both incubator and refrigerator ensure a correct sample tracking. The pipetting workstation is equipped with monitored air displacement technology, thereby rendering the use of system liquid, which is a major source of contamination, unnecessary. Several components of the system have been tailored to meet cell culture requirements. These include a plate lifter module, which allows complete removal of supernatant during media changes by tilting of the cell culture plate. Cell culture media

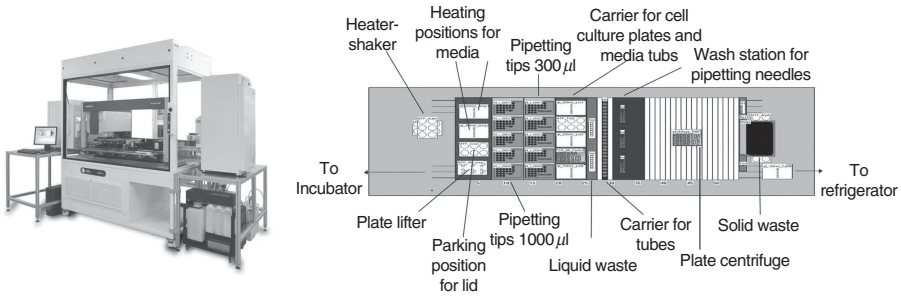

Figure 14.1. The Cell[host] system consists of a pipetting workstation contained in a laminar airflow cabinet. The cabinet is connected to two robot-accessible incubators for the storage of cell cultures and media (a). For efficient performance of cell culture tasks, a plate lifter for the complete removal of media from cell culture plates, heating positions for cell culture media, a centrifuge, and a heater-shaker module for enzyme-treatment of cell cultures have been integrated (b). Adapted from Ref. (5) with permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc. (See color insert.)

are prewarmed using Peltier-type heating elements. Furthermore, a heater-shaker device is employed for coating of plates and enzyme treatment of cell cultures. The system is equipped with a robot-accessible centrifuge which enables fully automated cell passaging. Homogeneous cell plating is achieved by simulation of typical manual movements with the robotic arm. Amplitude and number of repeats of the movement were adjusted for the plating of mouse and human ESC. With the aim of limiting costs for disposables, a washing station for reusable pipetting needles was directly coupled to a Millipore water purification system (Fig. 14.1b).

The specified configuration makes the Cell[host] system highly suitable for the automation of typical ESC culture processes. These include coating of cell culture plates with extracellular matrix components, plating, enzymatic passaging and media changes in feeder cell and murine ESC cultures, plating and media changes in human ESC cultures, and addition of growth factors and compounds (Fig. 14.2). Great importance has been attached to design the automated processes in a way that established culture methods could be adopted.

In a typical process, the user specifies one or several cell culture plates and media tubs. Subsequently, cell culture media are warmed up to 37 °C and the cell culture plate is transported from the incubator to the pipetting workstation. Employing liquid level detection, the cell culture system ensures that the media tub contains sufficient media for a media change of the complete plate. Only then is used media removed from the plate by means of the plate lifter and fresh media is added. While the cell culture plate and the media tub are

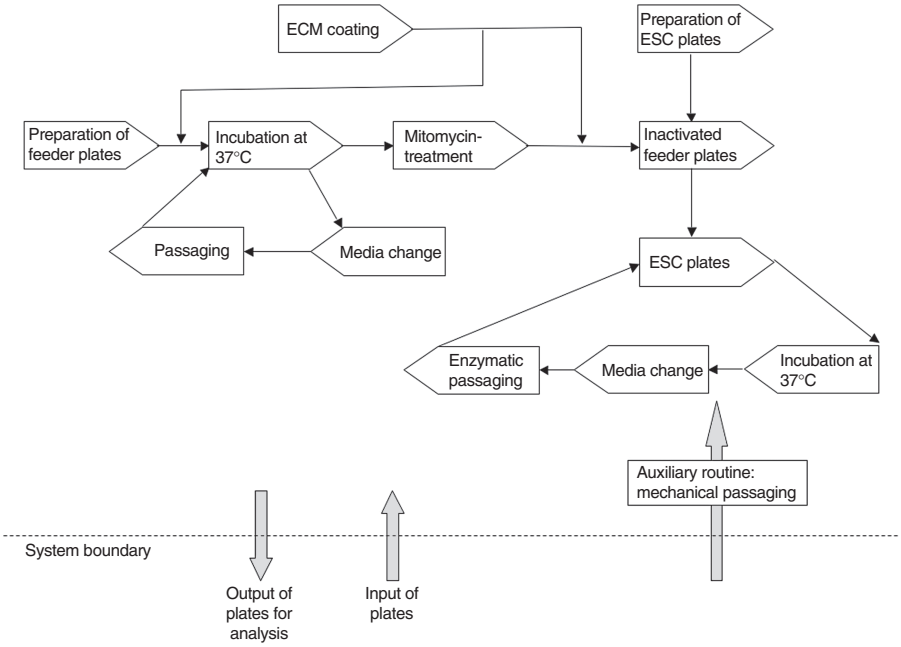

Figure 14.2. Crucial ESC culture processes are automated by the Cell^{host} system. These include ECM coating of cell culture plates, plating and passaging of feeder cells, media changes, and mitotic inactivation of feeder cells, plating, enzymatic passaging, and media changes of ESC cultures. Plate addition and removal is continuously tracked and documented.

returned to the incubators, the plate trail is updated with information on the operated process, date, user, media tub barcode, and media tub lot number.

ESC CAN BE RELIABLY CULTURED BY AN AUTOMATED SYSTEM

In a biological validation process, we could show that the Cell^{host} system maintains ESC cultures without affecting growth characteristics and *in vitro* multigermlayer differentiation potential (5). Specifically, both murine and human ESC can be homogeneously plated by the automated cell culture system. Furthermore, repeated automated media changes do not compromise the integrity of the cell layer in fibroblast cultures, murine ESC cultures (mESC), or human ESC (hESC) cultures. Long-term maintenance of mESC and hESC cultures can be performed without appearance of microbial contaminations even when employing antibiotic-free culture media. During four weeks of automated culture, we could show that neither cell morphology and growth

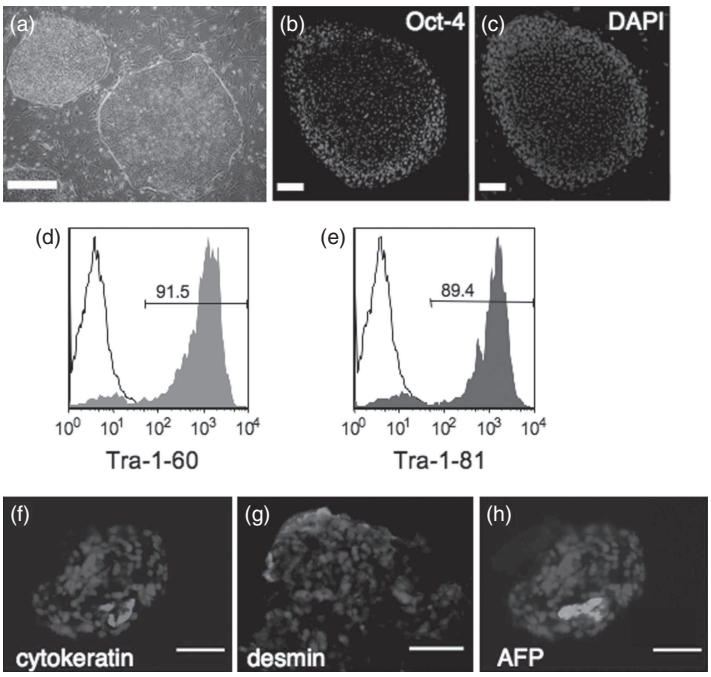

Figure 14.3. hESC cultured by the automated system display typical morphology (a) and maintain expression of pluripotency-associated markers Oct-4, Tra-1-60, and Tra-1-81 (b–e). *In vitro* differentiation potential is conserved as hESC readily form embryoid bodies expressing the germ layer-associated markers cytokeratin (f, ectoderm), desmin (g, mesoderm) and α-fetoprotein (AFP, H, endoderm). Nuclei are counterstained with DAPI (blue). Open histograms correspond to negative controls and filled histograms represent staining of the indicated pluripotency-associated markers. Scale bar = 500 µm (a)/100 µm (b, c)/50 µm (f–h). Adapted from Ref. (5) with permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc. (See color insert.)

characteristics nor pluripotency-associated marker expression and *in vitro* differentiation potential were negatively affected by automated culture (Fig. 14.3). Importantly, no overt karyotype abnormalities were observed during the culture period.

PERSPECTIVES

Embryonic stem cell technology provides attractive perspectives for the generation of unlimited numbers of somatic cells for disease modeling and compound screening. In particular, disease-specific hESC lines available from preimplantation genetic diagnosis (6) represent a powerful tool. However,

automated procedures for stem cell processing are indispensable in order to guarantee a sufficient standard in terms of cell quality and reproducibility. Employing the Cell^{host} system, fundamental processes in ESC culture can be performed in an automated and reproducible manner. Integration of a procedure for the non-enzymatic passaging and the removal of differentiating colonies in hESC cultures would further increase the stand-alone time of the automated culture system. However, mechanical detachment of hESC colonies is all but impossible to automate with a conventional liquid handling system. Attempts to employ a modified tissue chopper for mechanical passaging of hESC cultures (7) might reduce hands-on time, but can hardly even be termed semiautomated. It will be necessary to adopt more unorthodox methods such as laser microdissection and pressure catapulting (8, 9) in order to develop automated processes for nonenzymatic hESC passaging. Eventually, optical read-out systems for cell density, morphology, and fluorescence will be required to bring automated hESC culture to perfection. In this context, the recently described Cell-IQ system holds promise for the automated monitoring and analysis of hESC cultures (10).

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15

QUANTITATIVE 2D IMAGING OF HUMAN EMBRYONIC STEM CELLS

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Human embryonic stem cells (hESCs) are unique in several ways: They grow as tight aggregates of cell clusters *in vitro*, are able to be cultured in an undifferentiated state for an unlimited time period, and demonstrate the ability to form different cell lineages (1). It is the first property of growing in aggregates which makes them both difficult to culture and to monitor accurately *in vitro*. Current means of determining the growth performance of hESC would require daily enzymatic harvest of cells from a culture to manually enumerate them, a process which renders the cells unusable. It is also known that culture quality is highly operator-dependent and that current methods of passaging hESC are highly variable (2, 3). Thus, methods that can quantify key parameters in maintaining a good culture would be invaluable for achieving consistent hESC culture.

Our goal therefore was to develop a stem cell imaging system (SCIS) capable of monitoring and recording the growth performance of live human embryonic stem cell cultures in a fast, accurate, and noninvasive manner. The advantages to the scientist would be better quality control of the hESC culture and the ability to monitor the growth of hESC cultures on a daily basis.

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In this chapter, we demonstrate that the SCIS can perform the following measurements:

1. Size distribution profile of the seeded clusters;
2. Daily monitoring of the same culture of hESC over 7 d;
3. Determination of the growth rate based on increase in area occupied by the hESC clusters;
4. Correlation of cell numbers by comparing cell numbers to area occupied by the hESC clusters;
5. Mapping the position of the seeded cell clusters; and
6. Identification of individual cell clusters and comparing their fold expansion between day 1 and day 2.

SCIS

The SCIS developed for stem cell culture monitoring is shown in Fig. 15.1. The goal of capturing clear images of live cultures presented several challenges due to the constraints of avoiding the compromise of cell health; such as limiting light exposure (phototoxicity) and the need to conduct comparative studies in standard plastic 24-well plates. In particular, the geometry of the wells in the 24-well plates poses problems for an imaging system as the vertical walls and sharp boundaries lead to unwanted reflection and scattering of the incoming illumination required for imaging. The result of this is a loss of uniformity

Figure 15.1. Stem Cell Imaging System (SCIS). On the left is the imaging and lighting system and on the right the software and user interface for data analysis.

in the intensity of the image across the plate. This is not only distracting to the biologist, but more significantly, it also makes automated analysis of the images into occupied/unoccupied areas of cell growth unreliable. The system developed incorporates a customized dark field illumination method that solves these issues and generates images of consistent background intensity. Dark field imaging results in cells being seen as bright objects against a dark background. The images are then archived for user-viewing or analysis by image processing methods into parameters of cell colony growth.

The image analysis facilities currently developed consist of the measurement of confluence (the area of the plate occupied by cells) and the area and centroid position of each of the separate cell colonies. The system software also supports time-lapse logging of images (typically over a week's passage cycle at daily intervals) so that it is possible to keep track of the history of each plate of a comparative experiment. Time-lapse analysis results can then be used to study various aspects of overall and individual colony growth and distribution in a quantified way as illustrated by the results presented in the remainder of this paper.

hESC CELL CULTURE

HES-3 was grown in centre-well organ culture dishes (BD Falcon™) or 24-well tissue culture flat bottom plate (1.9 cm^2/well and working volume of 1.1 mL) coated with Matrigel and conditioned medium prepared from immortal mouse embryonic fibroblast (iMEF) as described by Choo et al. (4). The cells in each well were imaged daily for cell growth study (from day 1 to 7). After imaging, single cell suspensions from triplicate wells were obtained by treatment with 0.25% trypsin-EDTA (Invitrogen). Trypan-blue exclusion method was used to obtain the viable cell counts and viability in triplicate. The viable cell concentration and the area of the cells covered in each well were plotted to determine their correlation. The specific cell growth and the cell cluster area expansion rates were calculated by semi-logarithm plot of viable cell concentration and area covered respectively versus time. At the end of the seventh day of culture, hESC were stained and analyzed by flow cytometry for the canonical pluripotent markers of Oct-4, SSEA-4, and TRA-1-61 (data not shown.)

CLUSTER SIZE DISTRIBUTION

Figure 15.2a shows a typical image, on day 1 after seeding, captured by the SCIS, which is segmented and converted to white images of hESC clusters in a black background. Each cluster occupies a different area and Fig. 15.2b shows some examples of clusters that range from 100 to 1600 pixels. Using a conversion factor of 1 pixel equal to 270 μm^2 the area of the individual colonies

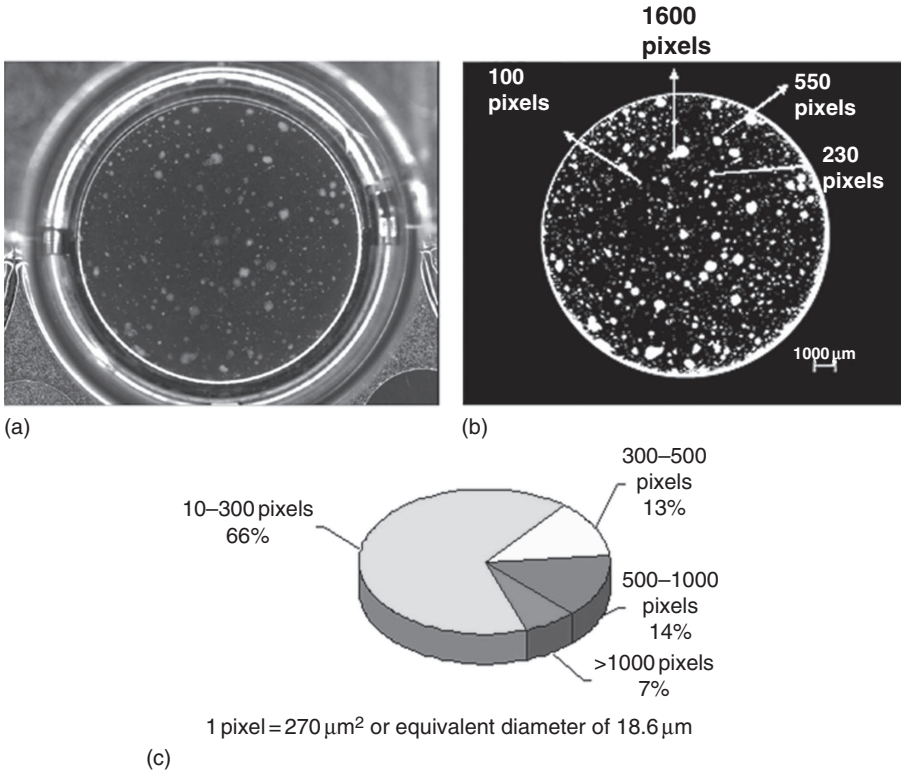

Figure 15.2. Images and size distributions of hESC clusters. (a) A typical example of a captured image in a 24-well plate 1 d after seeding hESC clusters. (b) An image of the hESC clusters is segmented into areas represented by pixels and presented for data analysis. (c) The size/area distribution of clusters are presented in a pie chart showing the percentages of the seeded clusters in different size ranges as represented in pixels. Note that 1 pixel is equivalent to an area of 270 µm^2 or an equivalent diameter of 18.6 µm. (See color insert.)

can be estimated. Figure 15.2c shows a typical example of the distribution of cluster sizes after enzymatic digestion. In this experiment, there is a relatively large percentage (66%) of small clusters in the 10–300 pixel range and a small percentage (14%) of large clusters in the 500–1000 pixel range. It is possible to segment the clusters to different size ranges to determine whether clusters are prepared too small or too large. This allows the operator to determine how consistent seeded clusters are prepared by the same method on different occasions or by different operators.

Growth Rate

Figure 15.3a shows an example of an individual well of hESC clusters tracked over 6 d. Two benefits of live cell imaging are the ability to follow the individual

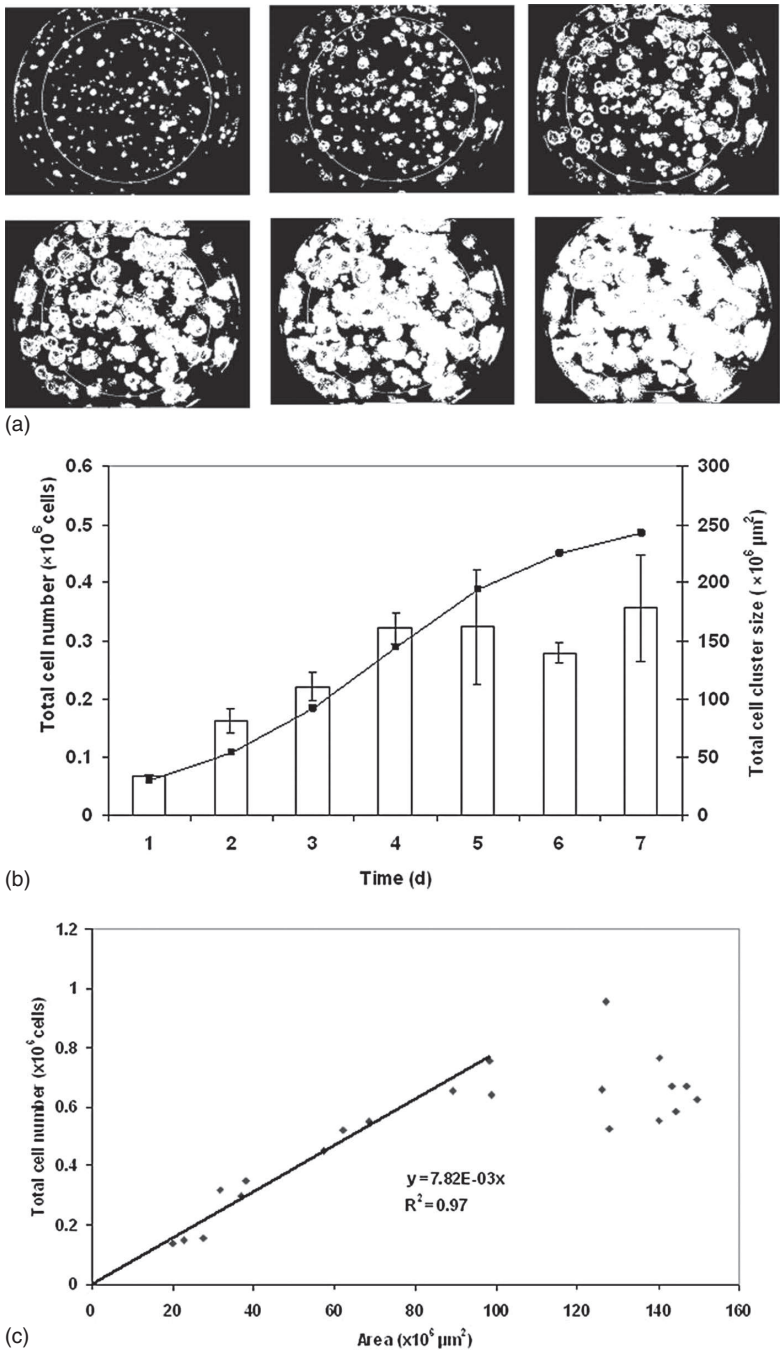

Figure 15.3. Daily monitoring and determination of growth rate of hESC. (a) Day 1 to day 6 images of human embryonic stem cell clusters extracted for data analysis. (b) The area increase (solid line) over 7 d shows a typical sigmoidal growth curve from which growth rate and doubling time can be calculated. The equivalent cell counts are also plotted as a bar chart over the same period. (c) Linear correlation between cell number and area occupied by triplicate wells gives an R^2 value of 0.97 for the first 4 d of exponential growth.

colonies over the growth period and to be able to sum the total area occupied as an indirect measure of cell growth. These data are represented in Fig. 15.3b as an increase in area over time from which the growth rate and hence the doubling time may be calculated. It can be seen that the culture displays a typical sigmoidal curve with an exponential growth period in the first 3–4 d and reaching a plateau at day 5. The doubling time was calculated to be 32 h which is similar to the doubling time of 33 h obtained by triplicate cell counts performed on the same days by harvesting the wells after imaging; cell counts are also plotted in Fig. 15.3b.

After the hESC images are recorded and then cells harvested from the same well and counted by a hemocytometer, it is also possible to establish a correlation between the cell numbers with the recorded image areas. Triplicate cell counts and equivalent images were plotted and a correlation coefficient of 0.97 has been established for the first 4 d of growth as shown in Fig. 15.3c. From the fifth to seventh day, as the cells plateau in growth this correlation seems to weaken as the cells are spreading over a wider area while cell numbers reach the stationary phase with no further increase.

Cluster Mapping and Optimum Cluster Size

Figure 15.4a shows that individual clusters can be mapped on the plate. This shows the position and size of the clusters a day after inoculation, which is a useful feature when seeding culture vessels with a large surface area such as cell factories. Poor distribution of clusters, such as clustering around the central area of a dish; could result in nonoptimal growth or inefficient coverage of the plate. By tracing individual cell clusters, isolated colonies ranging from 10^4 to $3 \times 10^5\,\mu m^2$ can be tracked on day 1 and day 2 to determine their expansion rate. Figure 15.4b shows that there is an average of 2.8-fold expansion (slope) of these clusters between day 1 and day 2. However, on closer examination, it can be seen that the clusters less than $1 \times 10^5\,\mu m^2$ have a more random expansion ranging from one- to about sixfold but averaging about threefold, whereas clusters greater than $1 \times 10^5\,\mu m^2$ have a more consistent expansion about threefold (Fig. 15.4c).

Clusters of 1×10^5–$3 \times 10^5\,\mu m^2$ have an equivalent diameter of 285–625 µm. Based on the last 6 years of experience in hESC culture, we have learnt that breaking seed clusters too small during passaging oftentimes results in the cultures differentiating rather than maintaining their pluripotent state. Especially for new trainees, the time required to achieve this skill is variable. From this initial study, it can be seen that creating seed clusters with a size range greater than about 285 µm should be the goal of hESC culturing. This SCIS provides the means to measure how well one is achieving this goal.

In addition, the effect of varying parameters such as seeding densities, method of passaging on the performance of hESC cultures could be evaluated with this *in vitro* bioimaging instrument. While proof-of-principle has been demonstrated here with feeder-free hESC cultures, hESC culture on feeders

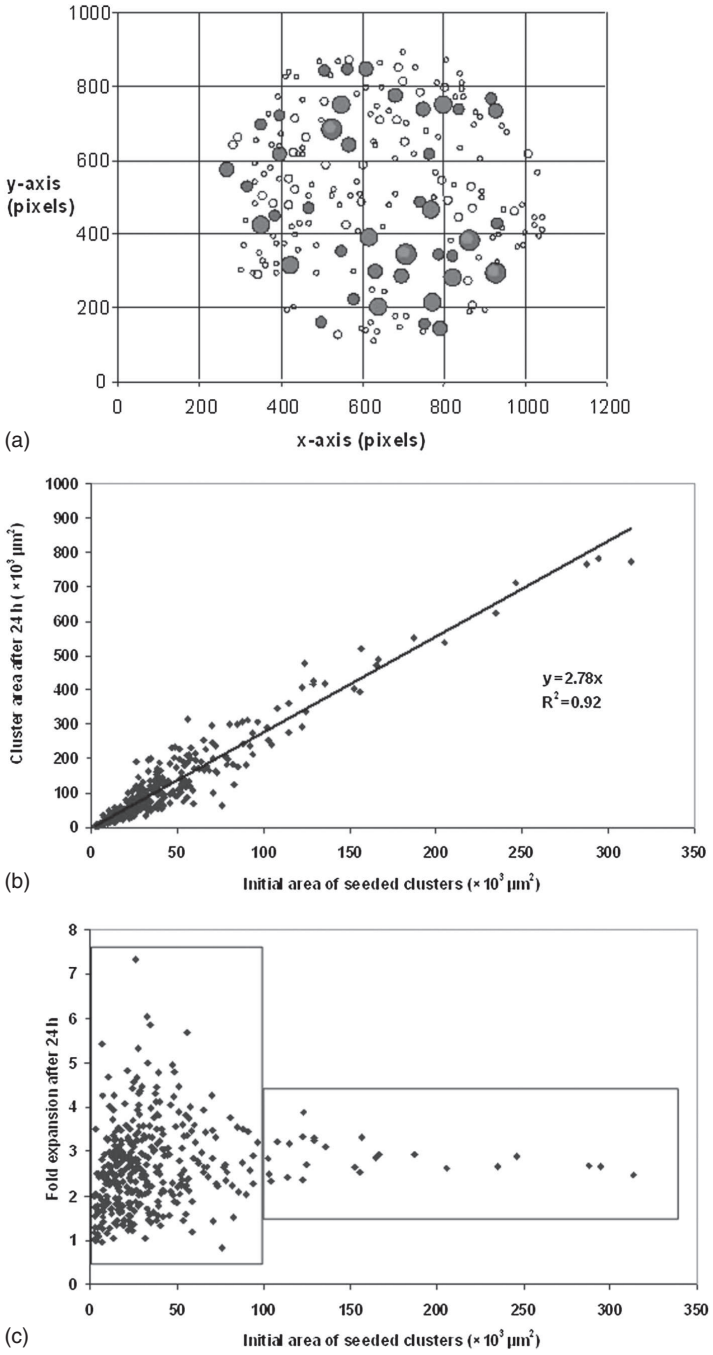

Figure 15.4. Cluster mapping and comparing the expansion of individual colonies. (a) Cluster mapping shows the position and size of clusters distributed within the well. (b) Relatively isolated clumps of hESC were chosen and measured on day 1 and day 2. On average there was a 2.8-fold increase in area between day 1 and day 2 clusters. (c) hESC clusters smaller than $10^5\,\mu m^2$ have a more random expansion profile; whereas clusters from 10^5 to $3 \times 10^5\,\mu m^2$ (equivalent to 285–625 µm in diameter) have a more uniform expansion of about threefold.

can also be monitored in a similar manner as can ESC cultures of other species.

CONCLUSIONS

A SCIS has been developed which is able to monitor hESC culture *in vitro* noninvasively.

We have demonstrated the ability to quantify the following parameters:

1. cluster size distribution profiles;
2. growth rate estimated based on total area increase;
3. mapping of clusters on dish; and
4. individual cluster sizes and identification of optimum cluster sizes (>200 pixels or >285 μm equivalent diameter).

This imaging platform could be a valuable research tool for monitoring hESC cultures under various culture and seeding conditions and may be used for quality control in hESC manufacturing.

ACKNOWLEDGMENTS

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16

NANOBIOTECHNOLOGY FOR STEM CELL CULTURE AND MAINTENANCE

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STEM CELL BIOLOGY MEETS NANOBIOTECHNOLOGY

Stem cell engineering is a promising bioengineering approach to regulate stem cell fates *in vitro* (1). One of its major challenges is to disclose microenvironmental cues that affect the fate of stem cells. It is well known that extracellular factors are important in regulating the fates of adult and embryonic stem cells (ESCs). Differentiation of ESCs is affected by a series of spatiotemporally regulated signals, and thus, by regulating the proper series of signals, it may be possible to differentiate ESCs into any desired cell types efficiently and reproducibly (2, 3). In this sense, nanobiotechnology provides a powerful tool to investigate extracellular signals that regulate stem cell fates as well as to control cell–microenvironment interactions.

Nanobiotechnology is the application of nanotechnology into the life sciences. This research area consists of two closely related sides. One focuses on developing nanoscaled products with biologically related approaches while the other applies nanoscaled tools to biological systems. Nanobiotechnology creates new opportunities in wide areas of science and engineering based on the interplay between nanotechnology and biotechnology.

Emerging Technology Platforms for Stem Cells, Edited by Uma Lakshmipathy, Jonathan D. Chesnut, and Bhaskar Thyagarajan

Recent research has focused on downsizing because analytical performance can be improved for sensitivity and limit of detection at micro/nano scale. These miniaturization efforts derive from the evolution of small-scale devices based on nanobiotechnology. Miniaturization technologies have significant implications for cell biology and cell-based assays. In most conventional culture systems, the cellular microenvironment is significantly different from *in vivo* conditions. *In vivo* cells are regulated by the surrounding cells, soluble factors, and extracellular matrix (ECM) molecules. Recently, miniaturized microfluidic cell culture platforms have been used to supply and transfer nutrients, growth factors, and oxygen, and to drain the waste products through microfluidic channels resembling the human circulatory system (4–10). Automated and high-throughput cell-based assays were achieved using biomimetic cell culture systems based on microfluidics (11). Miniaturized microfluidic technology can be applied to many stem cell research areas such as high-throughput screening and differentiation study.

Microfluidics, one of the major nanobiotechnology fields, has been a key technology for the realization of micro total analysis systems (μTAS) or lab-on-a-chip and the next generation bio-tools for drug discovery (12). Microfluidics covers the design and development of miniaturized devices that manipulate liquid samples at nanoliter volumes, allowing biological assays to be integrated and accomplished on a small scale with minimum time and cost (13, 14). In microfluidic domains, diffusion is the only efficient process for mixing the dissolved contents of the fluids by laminar flows. Most microfluidic devices that have been developed can be used to provide specific functions such as sample pretreatment, separation, dilution, mixing, reaction, and detection.

In this chapter, we introduce two nanobiotechnological approaches for stem cell research. One is an embryonic stem cell divider (ESCD) constructed with microfabrication methods and the other is a microfluidic three-dimensional (3D) cell culture system using hydrogel that can be adopted for stem cells.

ESCD

Human ESCs, characterized by nearly an unlimited self-renewal and differentiation capacity, were first derived in 1998 (15), and since then, they have been emphasized as an influential therapy candidate for incurable diseases. In addition, they have been the center of much attention due to such characteristics as unlimited reproduction and pluripotency in the light of economic and industrial values. The human ESCs have been widely cultured by coculture with feeder cells originated from mouse. However, since the coculture method acts as a bottleneck for transplantation in humans due to the concern on xenogeneic contaminants and contributes to notable variation of experiment, various feeder-free methods have been developed (16). Xu et al., however,

found increased differentiation when ESCs were cultured by a conditioned medium from mouse embryonic fibroblast (MEF) in feeder-free conditions, thereby becoming more important for adequate method of undifferentiated maintenance (17).

The present techniques do not provide a single cell-based subculture method for human ESCs growing in the form of a colony. Recently, Watanabe et al. found that Rho-associated kinase (ROCK) inhibitor, Y-27632, diminishes dissociation-induced apoptosis to human ESCs, increases cloning efficiency, and protects apoptosis even in serum-free suspension culture (18). Although this result shows drastic enhancement of subcloning (up to 27%) and the possibility of single cell dissociation for human ESCs, an adequate protocol for single cell handling has not been established.

Subculture at an optimal time is one of the most important processes and size regularity of the initial ESC clump is a critical point to maintain its own specificity. Currently, ESC dissociation is mainly used by enzymatic and non-enzymatic (chemical and mechanical) methods for detaching ESC colonies from the culture dish and producing ESC clumps without size control. In enzymatic methods, human ESC colonies could be detached by incubation with enzymes such as collagenase and dispase. Before passaging them, detached ESC colonies are required to break into smaller ESC clumps by gentle pipetting. Although this enzymatic method contributes to a large quantity of cell expansion in a short time, it has certain drawbacks such as the possibility to passage with differentiated cells and large variation in clump sizes, as well as high tendency of karyotypical abnormalities (19, 20). Therefore, the method is not suitable for long-term culture of ESCs. A mechanical method can be also used to dissociate ESCs by the use of a cell scraper or a sharp equipment (21). This method is able to maintain the quality of ESCs with relatively small variation in clump sizes and karyotypical stability by selection of undifferentiated cells. However, the mechanical method requires tremendous time- and labor-consuming processes largely dependent on personal skills. Recently, an automated mechanical passaging method was developed by modifying Mcllwain tissue chopper (Mickle Engineering Co., Gomshall, Surrey, UK) (22). Although it significantly reduced labor-intensive, time-consuming, and repeated burdens, and variation of cell number per fragment compared to conventional mechanical method, it is hard to cover colonies located on the edge of the culture dish and shows limited clump shape.

Nanobiotechnology enables microscale control that is why a variety of tools have been applied for embryology and biological application. Zeringue et al. performed the complete removal of the zona pellucida by shorter exposure to lysis solution, and cumulus removal at the zygote stage for embryo development and blastocyst formation by novel microfluidic methods (23, 24). In addition, various platforms for surface treatment have been demonstrated for delicate cell patterning. Derda et al. developed self-assembled monolayer surface arrays to identify peptidic surfaces that support ESC growth and self-renewal, and showed a role for specific surface–cell interactions (25).

To satisfy large-scale expansion with high efficiency and minimization of damage to human ESCs, therefore, we developed an ESCD. A divider was fabricated by photolithography, deep reactive ion etching (DRIE), and poly(dimethylsiloxane) (PDMS) replica molding processes. Since the cells were dissociated by an instant mechanical force after cultivating human ESCs with intact culture condition, damages to cells and cellular deformation were minimized. By this simple method, every cell colony in a Petri dish can be dissociated by unit clumps having regular cell number by one stamping, so that it drastically enhances subculture speed and viability of cells, reducing the time out of an incubator. In addition, the dissociation by regular clump size enables more reliable human ESCs experiment, and the various shapes and sizes of clumps can be made by a designed mask. A simple dissociation method for human ESCs and the fabrication of an ESCD are described herein and the results of biological assays are also discussed.

MICROFABRICATION OF ESCD

To facilitate the dissociation of human ESC colonies during subculture, we constructed a practical and useful device named ESCD. Briefly, photoresist (PR) AZ-1512 (Microchem, Newton, MA) was spincoated at 1500 rpm for 5 s and 2000 rpm for 6 s to make 1.5 μm thickness as DRIE masking layer. After PR patterning by PR developer, dry etching for cutting line was performed by inductively coupled plasma DRIE (Unaxis VL-DSE, FL) equipment. Forty micrometer of height was targeted and etched for 340 s by SF_6/C_4F_8/Ar gas flows. The remnant PR was stripped out and the fabricated mold was immersed into sulfuric acid at 95 °C for 3 h. Then, the silicon mold was diced as large as a 35-mm Petri dish and the pieces were sonicated in ethanol for 15 min. After drying the pieces, they were sufficiently sprayed by a mold release agent (Nambang CNA Co., Korea), and the mixture of PDMS prepolymer and curing agent (Sylgard 184; Dow Corning, MI) were poured onto the diced molds where the ratio of mixture was 5:1. The polymer was cured at 70 °C for 3 h on a convection oven to complete cross-linking.

Configurations of ESCD with square and hexagonal patterns are shown in Fig. 16.1a,b (26). One side length of square and hexagonal patterns was 200 μm (a-a') and 124 μm (b-b'), respectively. The height of both patterns was 40 μm and the ESCDs were made to fit inside a 35 × 10-mm tissue culture dish with 1-mm margin against the wall of the dish to provide enough space for easy and free movement. The fabricated divider was stuck beneath a sleeve inside a homemade stamping apparatus and the sleeve was moved up and down by a guide stand. After locating the culture dish beneath the guide stand, the ESCD was moved downward to the culture dish. In this configuration, the cell-pressing pressure of the stamping apparatus could be controlled by adding a scale weight onto the sleeve.

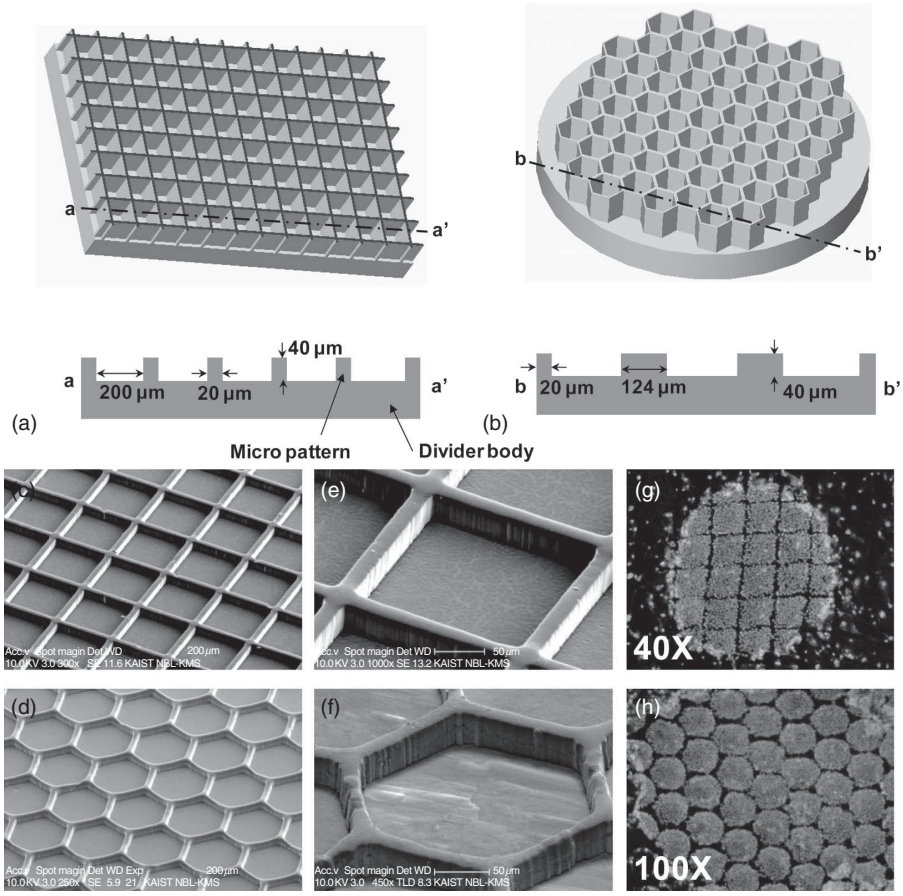

Figure 16.1. The configuration of human embryonic stem cell dividers (ESCD) and dissociation of human embryonic stem cell (ESC) colony. Schematic diagrams of ESCD with (a) square pattern and (b) hexagonal pattern. Scanning electron microscope (SEM) images of PDMS replica. PDMS replicas with (c, e) square pattern and PDMS replicas with (d, f) hexagonal pattern. Images of human ESC colony after pressing with ESCD with (g) square and (h) hexagonal pattern, stained by DAPI. (*Lab Chip* 2007; 7: 513–515) Reproduced with permission from The Royal Society of Chemistry.

CULTIVATION OF HUMAN ESCS

The STO cell line (mouse embryonic fibroblast cell line) was used for feeder cells of human ESCs. STO cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, MD), 2 mM glutamine (Sigma, MO), 0.1 mM ß-mercaptoethanol (Sigma), and 1%

nonessential amino acid (Gibco). Cells were treated with 10 μg/mL of mitomycin C (Sigma) for 1.5 h, washed with phosphate buffered saline (PBS), and then replated into 0.1% gelatin-coated 35-mm culture dishes.

Human ESCs (CHA-hES4 derived at the Pochon Cha University, Korea) were plated at an initial density of 50 colonies per 35-mm dish. Undifferentiated ESCs were maintained by coculture with mitomycin C (Sigma)-treated STO feeder cells in DMEM/F12 medium (Invitrogen, CA) containing 20% knockout serum replacement (Invitrogen), 1% nonessential amino acids (Invitrogen), 0.1 mM mercaptoethanol (Sigma), 100 U/mL penicillin-streptomycin (Invitrogen), and 4 ng/mL basic fibroblast growth factor (Invitrogen) at 37 °C, 5% CO_2 in incubator. Before we developed ESCD, ESCs were mechanically passaged to fresh feeders every 5 d. After carefully isolating ESC colonies from surrounding STO feeder layers, ESC colonies were disaggregated into small ESC clumps by scrapping with homemade tool (mechanical method).

CHARACTERIZATION OF HUMAN ESCD

ESCDs were equilibrated by soaking with ESC culture medium. Air bubbles trapped in ESCD can be easily removed by 70% ethanol washing. Prior to subculture, the ESC culture medium per 35-mm dish was replaced with fresh and prewarmed medium. To dissociate ESCs, ESCD was pressed down onto the surface of the culture dish being sampled. Complete dissociation was confirmed by observing it under a stereomicroscope (Nikon, Japan). Then, cell clumps were detached from the dish by using a rubber cell scraper (Nalge Nunc, NY), harvested using a pipette, and transferred onto an STO feeder layer in a 35-mm dish. ESC culture media were changed at day 2 after subculture and then daily.

By pressing this tool onto human ESCs, it worked perfectly by making regular-sized cutting line onto ESC colonies. The height and width of ESCD are significantly important for clear cell dissociation. First, we experimentally confirmed that 25–30 ESC clumps from one ESC colony can maintain undifferentiated human ESC during subculture. Accordingly, a unit pattern of ESCD was designed to have 40 000 μm^2 areas, and to yield an ESC clump comprising 160 cells. In our observation, using laser scanning confocal microscope (Carl Zeiss Inc, Germany), the thickness of human ESCs was approximately less than 20 μm. Thus, actual height of the patterns should be higher than 20 μm. In terms of height, ESCD should provide enough space to avoid contact with ESCs and compensate bending of the roof of ESCD as vertical pressure. Besides, it was difficult to fabricate a replica from an ESCD mold with high aspect ratio, so that optimal height of ESCD was investigated and set to 40 μm after testing various heights. Furthermore, a sharp cutting line of ESCD is required to minimize the loss of ESCs. However, as it is related to aspect ratio with height, it should also be optimized. After determining the

height as 40 μm, we reduced cutting line as small as 13 μm whose aspect ratio is over 3. However, it was hard to get perfectly uniform cutting lines. In addition, the thin cutting line was easily bended even in slight pressure. Therefore, cutting line to dissociate ESCs was optimized as 20 μm width.

In addition, we constructed a hexagonal-shaped pattern to minimize ESC fragments made after dissociation of ESCD at the periphery region of ESC colony. Figure 16.1c,d exhibit well-detached square and hexagonal PDMS replica. While the replica having square pattern had clear cutting line, that of hexagonal pattern sometimes had more or less irregular shapes at the center of some side lengths owing to directional angle of detachment (Fig. 16.1e,f). The height difference between the vertex and the center of a side length in hexagonal patterns of PDMS replica was about 3.5 μm.

It was characterized for silicon mold and PDMS replica ($n = 8$). The PDMS devices were replicated shorter than the silicon mold in side lengths. Hexagonal PDMS replica showed more or less larger variations in cutting line and height. However, the replica showed satisfactory dimension with high aspect ratio, which is comparable to previous works. Few studies have been reported in PDMS replication with high aspect ratio. Folch et al. studied molding of deep PDMS microstructures (27). They discussed that release in the direction perpendicular to the trenches resulted in a qualitatively lower replication yield. This phenomenon corresponded with our observation.

DISSOCIATION OF HUMAN ESCS USING ESCD

We examined whether cutting line of ESCD is sharp enough to make clear dissociation of ESC colony. Figure 16.1g,h show the dissociated images of ESC colony after being pressed by ESCDs with PDMS-based square and hexagonal patterns. ESC colonies plated in 35-mm dish could be dissociated into regular-sized cell clumps at a time. Both square- and hexagonal-shaped patterns worked effectively. Under our experimental condition, one ESC colony cultured for 5 d could be divided into 25–30 pieces (each piece equals to 40 000 μm^2 in area) of cell clumps in a very short time (less than 10 s). Since a customized cell number of an ESC clump can be easily decided by the design of unit pattern as researcher's requirement, it seems that this method has a big advantage to achieve more stable culture and experimental conditions by supporting uniform ESC clumps.

We imagined that ESCD itself would dissociate ESC colony physically, pressing down by high pressure. However, we observed an interesting phenomenon that relatively low pressure onto ESC colony made only cutting lines morphologically, not dividing them. Then, the pressed cells were naturally taken apart by neighboring cells, dissociated clearly in a very short time. Clear dissociation of ESC colony was confirmed by observing under a stereomicroscope, and further confirmed by DAPI staining. The softness of the elastomeric PDMS, whose modulus is less than 0.01 GPa, is advantageous to overcome the

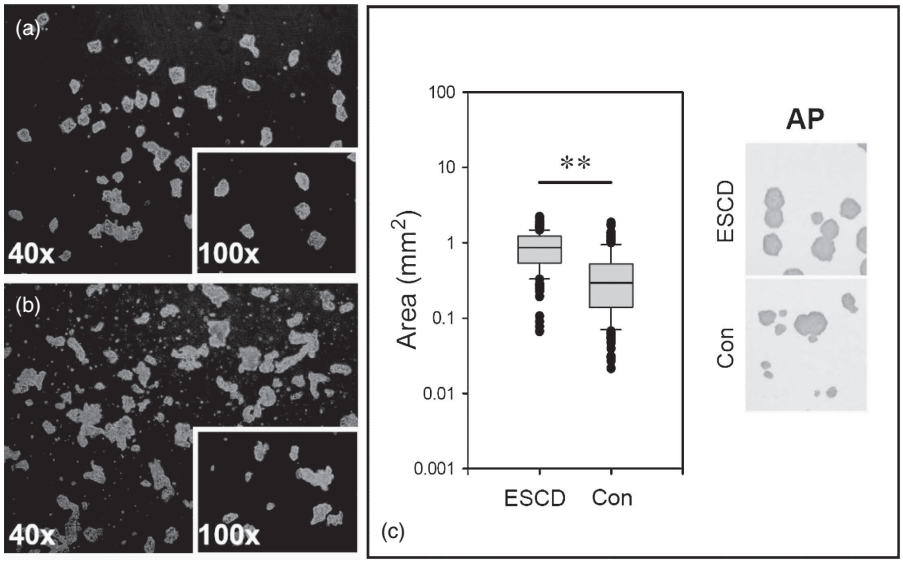

Figure 16.2. Human ESC clumps regularly dissociated by ESCD. Phase-contrast images of suspended ESC clumps produced by using (a) ESCD and (b) conventional mechanical method. (c) Quantitational analysis of recultivated ESC colonies. Graph shows size distribution of individual AP stained-ESC colonies. Efficiency of ESCD method was compared with mechanical method (con). Data represent the mean ± SEM (** $p < 0.001$). To calculate the p value, the values were compared using t-test (paired two samples for means) using Microsoft Excel. (*Lab Chip* 2007; 7:513–515) Reproduced with permission from The Royal Society of Chemistry.

height difference which was observed both in square and hexagonal-shaped patterns.

ESCD-derived cell clumps were easily detached by a rubber cell scrapper and were transferred to a new culture dish for maintenance or further experiments (Fig. 16.2a). Figure 16.2b shows that much more variable-sized ESC clumps and remnant fragments were produced when using conventional (mechanical) method compared to ESCD method. In our observation, it showed a tendency that remnant cell fragments were more easily produced when using square than hexagonal pattern. It seems that the narrower inner angle of square (90°) rather than hexagon (120°) causes more shearing forces during harvest, inducing breakdown of cell clumps.

To confirm attachment and stable proliferation rate of ESCs, 50 cell clumps were randomly selected from suspension and transferred to a new 35-mm dish containing inactivated STO feeder cells. Attached cells were manually counted under an inverted microscope at 2 or 3 d after subculture. Our new tool not

only saved a tremendous amount of handwork and laborious time during subculture process, but it also increased attachment rate up to over 95% while maintaining stable proliferation rate. In a manual clumping method, the attachment rate could not exceed 70% in our hands. Regularity of recultivated ESCs in colony size was calculated by measuring the area of individual ESC colony stained with alkaline phosphatase (AP) in 35-mm culture dish. Using ESCD, it was inevitable to produce irregular-sized ESC clumps at the boundary region of individual ESC colonies, and they could simply be discarded by brief spin down or using cell strainer that was commercially available. Even without such further processes, the ESCD method can provide a higher number of similar-sized ESC colony and less ESC fragments than conventional mechanical method (Fig. 16.2c).

MAINTENANCE OF UNDIFFERENTIATED HUMAN ESCS

After passaging, undifferentiated state of ESCs was confirmed at day 5 by monitoring expressions of pluripotent ESC markers including AP, transcription factors Oct-4, Nanog, and cell surface markers SSEA-3, SSEA-4, Tra-1-60, Tra-1-81 (26). All ESC-specific markers are highly expressed in ESCD-derived ESCs. As a negative control, the expression of SSEA-1 was not detected. Periphery regions of ESC colonies were pressed by cutting line during subculture, but it did not affect the undifferentiated state of ESCs. Cells have been maintained using this tool for up to the 15th passages. During serial passaging, each cell clump of ESC colony produced by ESCD has maintained a similar cell number, proliferation and attachment rate, and undifferentiated state without significant variation.

Manual passaging of human ESCs is the best way to retain normal karyotype, preserving genetic integrity, even after the 100th passages (20). However, this method consumed large amounts of time and required a difficult skill. By saving substantial time for culture and enhancing attachment rate, the developed simple tool will provide an extraordinary advance on large-scale propagation of undifferentiated human ESCs without enzyme treatment.

MICROFLUIDIC 3D CELL CULTURE DEVICE FOR STEM CELLS

Cell circumstance plays a significant role in cell function and behavior, especially cellular signal, cell–cell interaction, and transportation of molecules, etc. (28, 29). Until now, many studies have been tried to make *in vivo*-like environment for animal cells. One of the most principal approaches is constructing a microenvironment (30) and another is cultivating cells by three dimensions. Since microfluidics can provide microscale structures including *in vivo*-like condition and microenvironment, a lot of sophisticated microfluidic tools have

been developed for cell studies, such as chemotaxis (31), cell differentiation by precise concentration gradient (32), and embryo development by minute fluidic control (24).

Some reports have highlighted that 3D culture can be returned to *in vivo*-like original state of cells, such as receptors, gene expression profiles, and other biological activities, more than 2D cell culture (33, 34). Cukierman et al. showed that 3D-matrix interactions exhibit more enhanced cell biological activities and different focal and fibrillar adhesions compared to 2D substrates (29). Therefore, 3D cultivation has been a requirement to acquire reliable and credible cell-based assays. Cell circumstances are also important to ESCs. The differentiation kismet of ESCs is significantly affected by the near microenvironment or niche, which plays a fundamental role not only in cell survival, proliferation, and migration, but also in morphologies of aggregated colony and tissues (35, 36).

Accordingly, we have developed a microfluidic platform to make a biocompatible 3D microenvironment as well as realizing *in situ* concentration dependent cell-based assays. In this section, the principle and some applications of a microfluidic 3D cell culture platform and the availability of cell-based assays by concentration gradient are described. Some potential applications for ESC studies are also discussed.

MICROFABRICATION

The microfluidic device was fabricated by photolithography and PDMS replica molding process. A negative PR, SU-8 (Microlithography Chemical Co., MA), was spincoated on the bare Si wafer by 500 rpm for 10 s and 1600 rpm for 30 s. After flattening and soft baking, the PR was patterned by UV exposure of 350 mJ cm^{-2}. The mixture of PDMS prepolymer and curing agent (Sylgard 184; Dow Corning, MI) was poured onto the fabricated mold and cured at 60 °C for 3 h in a convection oven to complete cross-linking. The holes for inlet and outlet ports were punched and the PDMS replica and a slide glass were sonicated in 70% ethanol for 30 min as a sterilizing and cleaning process. After drying them, air plasma (200 mTorr, 200 W), using an expanded plasma cleaner (Harrick Science, Ossing, NY), was treated for irreversible bonding. Finally, the microfluidic 3D cell culture device was assembled and sterilized with 70% ethanol followed by UV exposure (Fig. 16.3a).

THREE KINDS OF ENCAPSULATION METHODS

The principle of the microfluidic 3D cell immobilization is based on the sol–gel transition of a material. Thus, the sol–gel transition material, materials leading to gelation, and a material controlling sol–gel transition are needed to make stripe-shaped microscaffold. In this study, self-assembling Puramatrix peptide

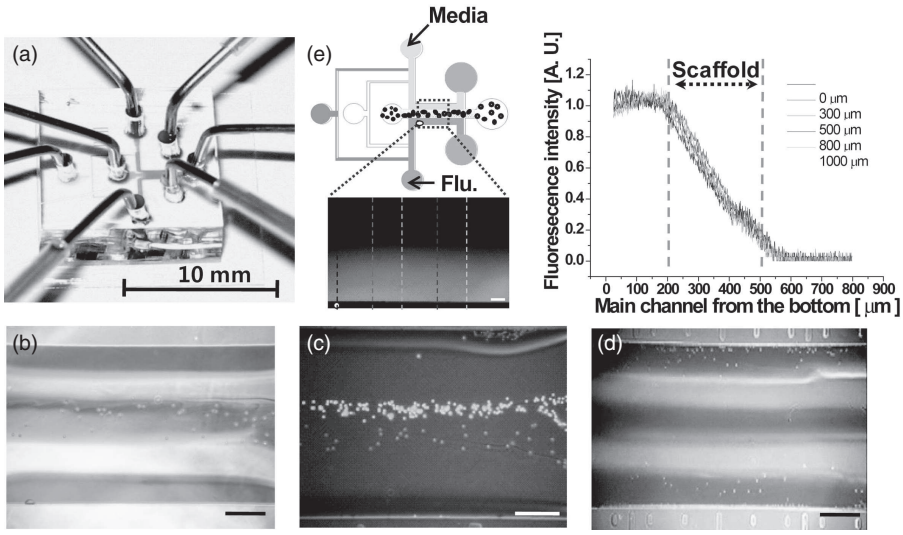

Figure 16.3. A microfluidic 3D cell culture and cell-based assay system by hydrodynamic focusing. (a) Microfabricated device for 3D cell culture and cell-based assays. (b) Fabrication of stripe-shaped micropeptide scaffold. (c) Peptide hydrogel immobilization for 3D coculture. (d) Sidewall immobilization of peptide hydrogel to mimic micro blood vessel. (e) Characterization of concentration profile in a main channel. To measure the concentration profile, fluorophore 488 fluorescence solution and pure water were flowed (scale bar is 100 μm) and the picture shows fluorescence intensity at the main channel of a microfluidic device. The graph means that concentration gradient was linear at the entire area of the scaffold (*see dashed lines*), and it was not so different from the positions of scaffold. Reprinted from *J Assoc Lab Autom*, 2006; 11:352–359, with permission from Elsevier.

hydrogel, media or food dye, and distilled water were used, respectively (37). To create a 3D microenvironment, the distilled water controlling sol–gel transition flows into the main channel of a microfluidic device. Then, the media flow from both sides of the main channel. At this time, the media are focused to both sidewalls of the main channel by distilled water. After stabilization of the stream, the mixture of peptide hydrogel and cells flows into the main channel where the fluid is hydrodynamically focused by the distilled water and media. When the mixture has reached the middle outlet, distilled water is immediately stopped. Then, both sides of media are contacted to the mixture within a few seconds. When the media have reached the mixture, the mixture is also stopped. At this time, as the media are diffused into the mixture, the peptide hydrogel is transitioned from sol to gel phases where the cells existing in the mixture are 3-dimensionally encapsulated by gel-phased peptide scaffold. Finally, as the distilled water is flowed again, we can confirm 3-dimensionally immobilized peptide scaffold where the transitioned scaffold is settled in the middle of the main channel without being washed away.

Under this gelation principle of peptide hydrogel, 3D coculture can be realized by modifying the mixture inlet port. After distilled water has focused the media to the sidewalls of the microchannel, mixtures A and B are flowed and focused to the middle of the main channel by sheath flows. The streams of the two mixtures are contacted to each other and laminar flows are formed. When all streams are stabilized, distilled water is stopped and the media are contacted to mixtures A and B from both exterior sides. Then, two mixtures are transitioned from sol to gel and mixtures A and B are 3-dimensionally encapsulated in the exact vicinity for coculture.

When exchanging mixture and media flows, 3D immobilization mimicking a blood vessel can also be constructed. When the streamlines are formed, the flow of mixture is stopped and the media are diffused to the hydrogel. As distilled water is flowed again, hydrogel patterning for biomimetic micro blood vessel can be fabricated.

As a result, we could simply make a stripe-shaped microscaffold in the middle of the main channel (Fig. 16.3b). A 3D coimmobilization for coculture could also be demonstrated by a 12 μm polystylene bead and a green fluorescent 7 μm streptavidin conjugated microsphere (Polysciences Inc., PA). As shown in Fig. 16.3c,d, immobilized beads were shown in cocultured form. Since the mixtures were of the same material, the two streams formed one straight fluid stream and fused each other. In addition, since the fluids were governed by laminar flow in a microchannel, two beads were not mixed where it could be confirmed by a fluorescence microscope.

In addition, by changing the fluids between the media and the mixture of Puramatrix and cells, we could simply make 3D cell immobilization for mimicking a blood vessel structure, creating a 180 μm thick vessel wall and 440 μm thick vessel (Fig. 16.3d). Faster flow of media contributed to the clear shape of hydrogel pattern, but about 15% variation of hydrogel thickness occurred. There exist various dimensions of blood vessel from a few microns over 6 mm in the human body, and the thickness and geometry of blood vessel walls affect fluid dynamics and response of endothelial cells. Since the thickness of blood vessel walls and the width of blood flow can be controlled by this platform, it shows a possibility of mimic for specific blood vessels as well as vascular diseases, such as coronary sclerosis, by proper geometry designs. Therefore, it will be advantageous to study the relationships between fluid dynamics and a variety of cells, including endothelial cells, marrow stromal osteoblasts, and nerve cells.

3D CELL CULTURE IN A MICROCHANNEL

Hepatocellular carcinoma cell line (HepG2; ATCC HB8065) was chosen for 3D immobilization and cell-based assays (38). The media for HepG2 were made by DMEM containing 10% FBS. Prior to microfluidic cell culture experi-

ment, 1% (w/v) Puramatrix, a sol–gel transition peptide hydrogel, was sonicated for 30 min. The cells were dissociated from the flask at 70%–80% confluence with trypsin (0.25%) ethylene diamine tetra-acetic acid (EDTA; Gibco) after gentle washing with PBS (Gibco). Then, they were resuspended and agitated with media and counted by a hemacytometer (Marienfeld, Germany). After centrifugation, the remnant media were pipetted off, leaving the cell pellet. They were resuspended by adding the calculated sterile 10% sucrose solution. The optimal cell concentration was calculated by the reference that the cell concentration of 1×10^6 cells/mL in three-dimensions was equivalent to a plating density of 1×10^4 cells/cm^2 with respect to maintaining constant cell-to-cell distance, and set to as much as 6×10^6 cells/mL by 10% sucrose solution (39). The sonicated Puramatrix and cells suspended with 10% sucrose were mixed equivalently. The mixture of Puramatrix and cells was injected into the microfluidic channel within 3 min. The 3-dimensionally immobilized cells were cultivated by perfusion method on an inverted microscope plate (Carl Zeiss, Germany) for 4 d. Since Puramatrix exhibits ~3 pH, it needs a fast immobilization process as quickly as possible to minimize the time that cells are immersed within only the hydrogel. Therefore, the mixture of Puramatrix and cells was injected into the microfluidic device within 3 min.

We compared the cell morphology and growth cultured in a 3D microenvironment and a conventional Petri dish (40). In 2D culture, cells reproduced and spread vigorously so that the plate was almost filled with cells after 3 d. However, the morphology was not changed as time went on. On the contrary, cells cultivated in 3D microenvironment were getting bigger, clustered and aggregated with each other as tumor's nature, and their morphologies were significantly different from those in conventional Petri dish culture. Kataoka et al. found that the HepG2 cells exhibited specific functions better in cell clusters than in a monolayer culture (41), and Glicklis et al. confirmed the hepatocyte function in cell aggregation, urea levels, and albumin secretion rate (42).

To examine the cellular function and stability in a microfluidic device, albumin secretion was measured versus time (40). Compared to albumin secretion in bulk-scale scaffolds including collagen composite scaffold, open-cell polylactic acid (OPLA®) scaffold (BD Biosciences, MA), and peptide scaffold, perfusion cultured HepG2 cells in a micropeptide scaffold exhibited over twofold higher albumin secretion rate. Contrary to the tendency of albumin secretion at bulk peptide scaffold, the albumin secretion at micropeptide scaffold increased gradually for the first three days. One of the main reasons of high secretion rate was supposed to be fast recovery from low pH exposure of cells because fresh media were continuously flowed to the main channel of a microfluidic device from the gelation of hydrogel. In addition, media perfusion also contributed to getting high albumin secretion rate. Many reports have supported that perfusion culture is more beneficial than static culture in cellular functions such as protein expression (43).

CONCENTRATION PROFILE IN A PEPTIDE SCAFFOLD

If there was a drug screening system to realize *in situ* cell-based assays as well as 3D culture, it would be a desirable platform in drug discovery. We characterized a concentration profile within fabricated micropeptide scaffold (37). After fabricating 300 μm stripe-shaped peptide scaffold in a main channel, mixture, distilled water, and media were stopped and only fluorophore 488 solution and pure water were flowed at 30 $\mu L\,h^{-1}$. Figure 16.3e represents a fluorescence picture of steady state in the main channel, and 30 s was enough time to become a steady state. In the area formed in peptide scaffold, linear concentration gradients were formed at the entire area. Scaffolds with different thicknesses were fabricated by controlling fluid flow rates, and the profiles showed that the linear region depends on the scaffold thickness. In addition, the concentration gradient did not show distinct difference according to the positions of fabricated scaffold (*see dashed lines*). Compared to other platforms requiring additional components to generate a concentration gradient, it enables 3D cell culture as well as *in situ* linear concentration gradient generation without any complicated and additional microchannels.

By the above-mentioned characteristic, we applied the microfluidic device for cytotoxicity test using a toxicant concentration gradient by menadione. Since stationary phase of the concentration gradient was formed within a few seconds, any effect for initial nonlinear profile was neglected in this cytotoxicity test. Toxicant concentration was set to 200 mM. After cultivation of HepG2 cells, they were stained by CellTracker™ Green CMFDA (Molecular Probes) as shown in Fig. 16.4a. And then, menadione and media were flowed by each side inlet so that we could make toxicant concentration gradient in the scaffold. The gradient was maintained for 1 h, taking a fluorescent picture (Fig. 16.4b). Image analysis of the data was performed with the Java-based image analysis program ImageJ (44). After the obtained images were converted to 16 bits gray scale, they were adjusted by threshold. To calculate cytotoxicity versus concentration, scaffold was divided by a quarter (*see dashed lines*), comparing initial number of viable cells and viable cell number after 1 h at each area.

As shown in Fig. 16.4c, the result showed that the higher the toxicant concentration, the higher the cytotoxicity of cells. Therefore, the platform showed availability for dose–response curves by the scanned image data in a single experimental setup. It has several potential advantages, including simple 3D cell immobilization and cultivation; cytotoxicity test of continuous drug concentration in 3D culture; *in situ* drug–drug interaction by injecting different drugs into side inlets.

APPLICATION FOR MOUSE EMBRYONIC STEM CELL (mESC)

Understanding the mechanisms of ESC differentiation is one of the major issues such that it has been demonstrated by various models, including trans-

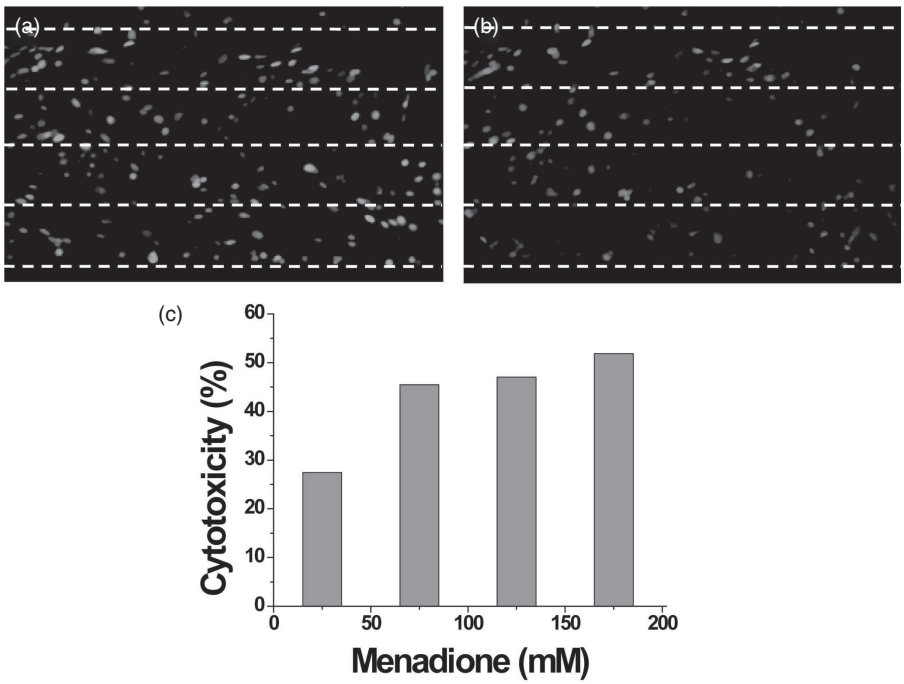

Figure 16.4. Cytotoxicity test by forming linear menadione concentration gradient in a scaffold. (a) Immobilized HepG2 cells were stained by CellTracker™ Green CMFDA (Molecular Probes). (b) After forming menadione concentration gradient in the scaffold for 1 h, cell viability was examined. To calculate cytotoxicity versus drug concentration, the scaffold was divided by a quarter (*see dashed lines*). (c) Cytotoxicity as menadione concentration; the higher the toxicant concentration, the higher the cytotoxicity of cells.

fection with appropriate genes to control differentiation (45, 46). However, these experiments are performed by two-dimension. Therefore, the formation of 3D microenvironment can be another candidate to study ESC differentiation since multicellular structures are more favorable to form normal embryos and severe combined immunodeficient (SCID) mouse teratoma. Chen et al. reported that differentiation of ESC in 3D systems was different from that in monolayer. Topologically different matrixes, soluble factors, and the type of ECM could be critical in directing differentiation of ESCs and formation of tissue-like structures (47).

mESCs (D3 line) were cultured by DMEM including 0.1 mM β-mercaptoethanol (Sigma), 20% FBS, nucleosides, 100 U/mL penicillin-streptomycin (Invitrogen), and 1000 U/mL murine leukocyte inhibitory factor. The cells were adapted by feeder-free cultivation so that 0.1% gelatin coating was performed before cell expansion. Matrigel™ (BD Biosciences) can also be used as a good scaffold, including abundant ECM proteins such as laminin, collagen

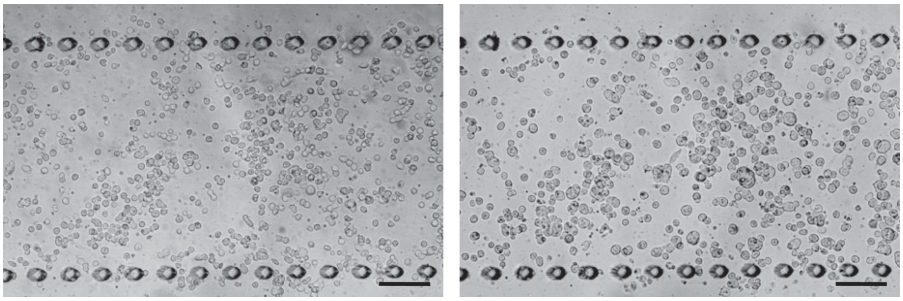

Figure 16.5. 3D immobilized mouse embryonic stem cells (mESCs) in a microchannel by Matrigel. (a) Initial picture after gelation. (b) A picture of mESCs grown for 1 d. The cells were moved and clustered each other, forming spheroid shapes. Scale bars are 100 μm, respectively.

IV, entactin, and a variety of growth factors. After centrifugation of cells, cooled Matrigel was mixed with harvested mESCs and the mixture was transferred to cooled syringe. The mixture in the syringe was injected to a microchannel as quickly as possible, and then the surrounding temperature was raised up to 37 °C, and the media were flowed by the side inlets. Figure 16.5a shows the 3D immobilized mESCs in a microchannel. Since the Matrigel was flowed in the middle of microchannel and gelled by temperature change from 4 °C to 37 °C, the structure was fixed as time went by. We cultivated the cells for 1 d in a microchannel. As shown in Fig. 16.5b, the cells were moved and clustered each other, forming a colony. The forming colony was correspondent to 2D culture, but the morphology was different where 3D culture showed more spheroid shapes.

After cultivating mESCs in microenvironment and 3-dimension, if a drug is injected by one side of the inlet, stem cell-based assays can be realized as continuous drug concentration. Especially, heterogeneity of ESCs is significantly dependent on handling skill and batch of chambers, which can be minimized by using this platform. Therefore, it will be of great potential for ESC studies by performing 3D culture and in situ concentration dependent cell-based assays.

CONCLUSION AND PERSPECTIVES

In this chapter, we described two applications of nanobiotechnology for stem cells: a microfabricated ESCD and a microfluidic 3D cell culture system using hydrogel. Using ESCD, human ESC colonies could be easily and efficiently dissociated into regular-sized ESC clumps without enzymatic treatment. The regularity of ESC clumps was improved compared to that of dissociated by a

conventional mechanical method, and its quality and reliability were confirmed by maintaining undifferentiated ESCs up to the 15th passages. Therefore, ESCD has the potential to contribute to advance quality control of *in vitro* ESC culture and allow large-scale production of qualified ESCs with tremendous time- and work-saving.

To make biocompatible microenvironments as well as realizing *in situ* cell-based assays, a stripe-shaped microscaffold was fabricated in the middle of the main channel. The platform enabled not only 3D cell culture in a microenvironment, but also a linear concentration gradient profile across the peptide scaffold due to molecular diffusion. Based on this characteristic, cytotoxicity test was demonstrated by menadione. In addition, mouse ESC culture was also conducted in a similar microchannel platform. The cells clustered each other by forming a colony so that the morphology showed more spheroid shapes compared to 2D culture. This microfluidic platform can facilitate *in vivo*-like 3D microenvironment, and can have a potential for the applications of reliable cell-based screening and assays, including cytotoxicity test, real-time cell viability monitoring, and continuous dose–response assay.

The promise of stem cells for cell-based therapies in human disorders, tissue engineering, and developmental biology has resulted in a growing interest in applying nanobiotechnology to stem cell research. Understanding the factors that control the proliferation and differentiation process is a crucial step that must be solved prior to potential therapeutic applications. As the knowledge of stem cell development increases, it is apparent that controlled differentiation is a complicated process involving interplay between biochemical and mechanical factors. Microscale control of cellular environments has been used to probe the influence of the spatial and temporal effects of specific cell–cell, cell–ECM, and cell–soluble factor interactions on cell fate. Microfluidic platforms can be very useful in studies of stem cells, providing the controlled cellular microenvironment that is difficult to achieve using traditional culture methods (48). Manipulating the chemical environment of the culture in time and space allows the behavior of stem cells, such as proliferation and differentiation, to be controlled. In the future, microfluidic assays will aid in accelerating drug screening on differentiated stem cells that can be used to model *in vivo* behavior. Since experimental conditions should be identical in order to compare the effects of different drugs, the precise control over the cellular microenvironment that microfluidic systems provide are potentially advantageous. Integration of advanced culturing techniques using heterotypic culture and 3D cues is likely to further increase the value of nanobiotechnology for stem cell research.

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ENGINEERING MICROENVIRONMENTS TO CONTROL STEM CELL FUNCTIONS

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INTRODUCTION

One of the primary goals of modern medicine is to create technologies capable of regenerating the body to its original function following trauma or disease. Until recently, technologies have been limited to the use of grafts, donor organs, and prosthetics. The advances in stem cell technology over the past decade offer a new option and hope for patients. To realize the therapeutic potential of stem cells, it is essential to fully understand the mechanisms by which stem cells self-renew, differentiate, and function. Recent research has developed a plethora of new techniques and devices to control the chemical, mechanical, and/or structural environment for stem cell renewal and differentiation. For each application, it is essential to focus on the most important factors. Flexibility, size, cell capacity, temperature, oxygen tension, nutrition, uniformity, inertness, biocompatibility, biodegradability, and the micro/nanostructure of the substrates are a few examples of characteristics that could be considered in the design. The purpose of this chapter is to define some of the engineering tools being used in stem cell studies.

Stem cells have two defining properties that distinguish them from other cells in the body. The first is their ability to self-renew through cell division. The second is that they can differentiate into specific cell types. Present research has focused on developing technology that will harness these two

Emerging Technology Platforms for Stem Cells, Edited by Uma Lakshmipathy, Jonathan D. Chesnut, and Bhaskar Thyagarajan

properties to expand cells and obtain specific cell types for regenerative medicine applications.

The majority of today's research is focused on two types of stem cells: embryonic and adult. Embryonic stem cells (ESCs) are derived from four- to five-day-old blastocyst-stage embryos that have been fertilized *in vitro* and were donated for research purposes. Blastocysts are thin, hollow structures formed from several hundred cells. The outer layer of cells forms the placenta and other supporting tissues necessary for fetal development. The inner cell mass gives rise to the tissues that form the body. ESCs are pluripotent, meaning they have the potential to differentiate into cell types of all somatic lineages from the three embryonic germ layers. Adult or somatic stem cells are undifferentiated cells that originate from mature tissues and organs. They are multipotent, with the ability to differentiate only into specific cell types of the tissue or organ where they are located. Their main function is to maintain homeostasis in the surrounding tissue. They are found in most tissues of the body, including bone, liver, blood, brain, heart, fat, and skin. The most commonly researched adult stem cells are hematopoietic stem cells (HSCs), endothelial progenitor cells (EPCs), and mesenchymal stem cells (MSCs), all of which can be extracted from bone marrow. HSCs give rise to blood cells such as red blood cells, white blood cells, and platelets. Both these cells and EPCs can also be derived from adult peripheral blood and human umbilical cord blood. MSCs are highly useful in clinical applications as they can be expanded a billion-fold in culture and can be manipulated to produce a wide variety of cell types. They have also been reported to have potential therapeutic uses in coronary artery disease, spinal cord injury, liver regeneration, and Parkinson's disease. In addition to embryonic and adult stem cells, fetal stem cells can be isolated from fetal blood, amniotic fluid, and placenta.

It is widely accepted that stem cell proliferation and differentiation are regulated by physical factors (e.g., mechanical and electrical factors) and the surrounding microenvironment, which is primarily composed of the extracellular matrix (ECM) and soluble factors. The ECM is the biological substrate on which adherent cells attach, migrate, and grow. Besides providing the cells with a structural support, the ECM modulates intercellular communication and regulates signaling events induced by soluble factors. In order to properly harness the full potential of stem cells in regenerative medicine, it is important to understand the mechanisms of how physical and chemical aspects of the ECM regulate stem cell functions. This chapter will describe emerging platform technologies currently being used to engineer the ECM and control stem cell behavior.

ENGINEERING THE SPATIAL DISTRIBUTION AND STRUCTURE OF THE ECM

The spatial distribution of the ECM can be used to control cell shape, spreading, migration, and differentiation. In addition, the latter two can be regulated

by the surface topography of the ECM. Furthermore, the two-dimensional (2D) and three-dimensional (3D) structure of the ECM may exert distinct effects on stem cells. Recent breakthroughs in matrix engineering and nanotechnology have made it possible to have greater control over the distribution, structure, and organization of the ECM. In this section we describe these platforms in detail and highlight both their benefits and shortcomings for experiments.

Matrix Patterning on 2D Surfaces

ECM molecules such as collagen, fibronectin, laminin, and elastin have a great influence on cell signaling and function. Micropatterning of ECM molecules has given researchers insight on their role in stem cell function. For example, ECM gradients can guide cell migration; they have been examined on different micropatterned substrates. The control over the shape and spreading area of MSCs *in vitro* can lead to their differentiation into bone or fat lineages (1). Micropatterned matrices have also been used to control the size of ESC colonies. Microarray of ECM proteins and biomaterials has been used to screen the optimal conditions for cell differentiation (2, 3). Soft lithography techniques such as microcontact printing (μCP), stencil patterning, and microfluidic patterning are commonly used to engineer the distribution of matrix proteins, as discussed below.

μCP is a 2D chemical-based method involving the design of a polymeric stamp, usually made of polydimethylsiloxane (PDMS), with a desired pattern and the subsequent transfer of the pattern onto a surface (see Fig. 17.1a). It generates patterned cell-adhesive and cell-repulsive areas on the surface; complex configurations can be created with a polymer mold designed with "posts" reserved for desired molecules in the micropattern. These molecules can either be matrix molecules (e.g., collagen, laminin, fibronectin), ligands, or linker molecules. Once the mold has been coated with the molecules, the pattern can be transferred to the cell culture substrate through conformal contact. The remaining regions are then blocked with appropriate molecules, such as polyethylene glycol, to prevent cell adhesion. If linker molecules are used for the micropatterning, the cell culture substrate is then incubated with matrix molecules, which attach to the linker molecules, forming the micropatterned adhesive areas. Micropatterning can also be generated using a stencil-based technique. Polymeric membranes with defined holes are laid upon the cell culture substrate and physically block areas characterized as cell repulsion regions. This is generally followed by ligand/protein coating and subsequent blocking, as in the μCP method (4). One disadvantage of the μCP and stencil techniques is the lack of control on the protein/ligand density and activity on the micropattern. However, they are both convenient and relatively straightforward techniques for micropatterning ECM proteins.

Polymeric molds are also used in the development of microfluidic patterning devices. Microchannels created between the mold and contact surface are used to guide the fluid flow of the solution containing molecules to be

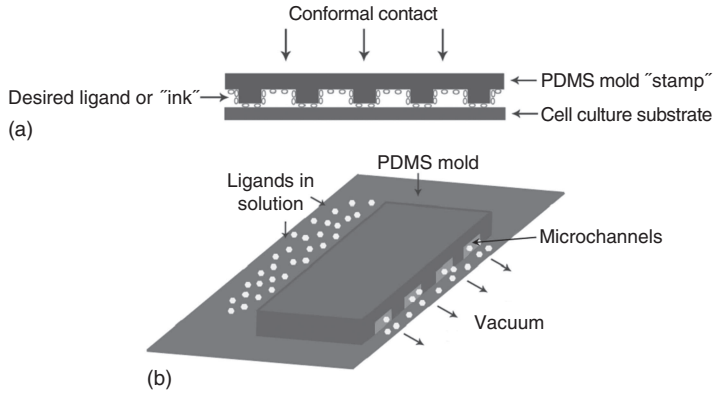


Figure 17.1. Micropatterning techniques. (a) Microstamping patterning. A PDMS mold is created to form a "stamp" and subsequently coated with the desired molecules for the cell culture substrate. The molecules are then transferred to the cell culture substrate through conformal contact. Following removal of the PDMS stamp, the cell culture substrate is often blocked with a molecule such as polyethylene glycol. (b) Microfluidic patterning. The predesigned mold is placed on top of the cell culture substrate. Microchannels formed between the mold and the substrate are flushed with a ligand solution through vacuum pressure, which deposits ligands in a specific micropattern. The mold is then removed and blocking molecules are added as necessary. (See color insert.)

patterned, as shown in Fig. 17.1b. The molecules (e.g., ECM proteins and/or ligands) are then deposited onto the substrate in the chosen micropattern. Step gradients can be formed through patterning strips on which different concentrations of ECM molecules are deposited (5). By using microfluidic networks with multiple inlets, gradients of the ECM molecules can be formed on the substrate (2). Gradients provide a reliable way of directing cell migration through haptotaxis, the migration of cells toward areas of higher ECM density.

In addition to micropatterning, nanolithography can be used to create nanodots composed of clusters of matrix proteins or ligands, which allows for control of molecule distribution at the subcellular level (6).

Engineering Topographic Features at the Micro and Nanoscale

Topographical cues at the micro and nanoscale are also important in the regulation of stem cell organization and behavior. While matrix patterning is usually used in 2D culture, topographic patterning can be extended for 3D culture.

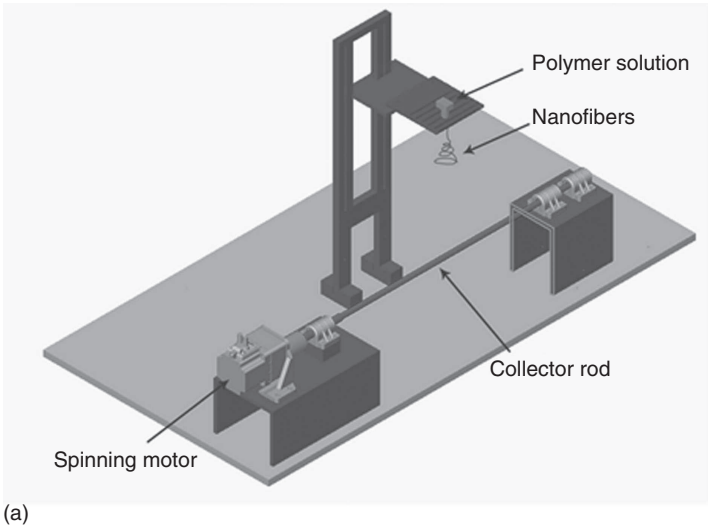
Soft lithography has been used for generating different topography on polymer membranes. Various patterns can be generated on photoresist wafers, and transferred to polymers (e.g., PDMS mold). Many studies have shown that altering the structural shape and size of the cell culture substrate can affect

the morphology, migration, and proliferation of stem cells. For example, stem cells can be cultured on microgrooves or between strategically placed fibrils on the substrate, and the cells will subsequently align in the direction of the microgrooves. The topographically aligned features may promote actin polymerization in the direction of alignment, thus guiding cell migration. It has been shown that cells can sense topographic features as shallow as 11 nm (7, 8).

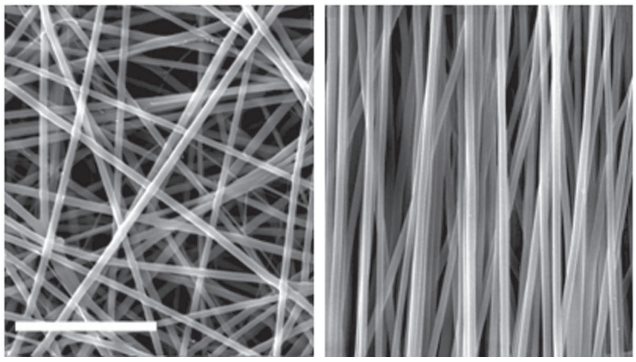
Several technologies have been developed to fabricate micro and nanoscale fibrous matrices, including drawing, self-assembly, template-directed synthesis, phase separation, and electrospinning (9). Drawing is the fabrication of continuous nanofibers through the extraction and solidification of a liquid polymer solution through a sharp probe tip, such as a glass micropipette. Due to the high surface area to volume ratio at reduced length scales, the liquid fiber pulled through the probe tip is quickly solidified by rapid evaporation of the solvent. This technique has been shown to produce nanofibers with diameters ranging from 50 nm to up to a few micrometers. One challenge with the technique is the creation of 3D matrices. The sensitive timing between the drawing and evaporation of the solvent also creates another disadvantage. Self-assembly usually refers to the gradual compilation of peptide-amphiphiles or other molecules that form the building blocks of nanofibers. Intermolecular bonds between the molecules usually strengthen in aqueous solution, creating aggregates of the molecules, and a subsequent basis for the fiber structure (10). Template-directed synthesis involves producing the desired fiber within the pores of a membrane. Many biodegradable, biocompatible polymers, such as poly(DL-lactide-*co*-glycolide) (PLGA) and poly(DL-lactide-*co*-ε-caprolactone), can be used as a template. Patterns are generally etched with a dilute sodium hydroxide solution, and nanofibers are fabricated during a high temperature polymer melt in contact with the pattern. Phase separation is based on the division of two phases due to physical incompatibility. Two polymers are combined in solution and allowed to gel. After an appropriate incubation of the gel, the solvent is then extracted from the solution, leaving a nanoporous structure behind. The main disadvantages of these first four techniques are the difficulty to scale up and their cost to manufacture.

Recently, several laboratories have used electrospinning technology to produce aligned nanoscale fibers for cells to grow on. This technology not only allows the manipulation of 2D surface topography, but also produces scaffolds with an organized 3D structure, which mimics the nanofibrous structure of native matrix fibrils and can be used for tissue regeneration. Electrospinning is the most widely accepted and simplest method of fabricating micro or nanoscale matrices. The technology is based on the theory that the surface tension associated with a single electrically conductive polymer solution droplet can be overcome with an applied electric field. As the voltage from the field increases, the force applied to the molecules in the polymer increases and approaches the magnitude of the original surface tension. When the electric field force surpasses that of the surface tension, the droplet begins to form a cone shape, known as the Taylor cone, and at a certain threshold voltage, ultimately materializes into a nanoscale liquid jet spray. Placement of an

oppositely charged, grounded plate or mandrel underneath the jet spray allows for collection of these "nanofibers" in either an aligned or unaligned configuration. Sheets or tubes are produced through rotation of the collection rod or mandrel, as shown in Fig. 17.2a. Alignment can be established during the elec-



(a)



(b)

Figure 17.2. (a) A schematic drawing of an electrospinner. A polymer solution is pumped through a syringe. As the polymer droplet leaves the syringe, an electric field is applied to it, which overcomes the surface tension of the liquid droplet. The total force results in the materialization of the liquid polymer into a nanoscale, fibrous jet spray. An oppositely charged collector is placed underneath the syringe in order to collect the nanofibers and can be rotated at various speeds to create different alignments. (b) Unaligned and aligned electrospun nanofibers, shown left to right, respectively. Scale bar = 10 μm.

trospinning process by adjusting the rotation speed of the collector or by using a rod/mandrel with a gap of nonconductive region. Alternatively, aligned fibers can be generated after the electrospinning process by applying uniaxial stretch to the matrix. Examples of random and aligned nanofibers are shown in Fig. 17.2b.

Nanofibers can be spun with both natural and synthetic polymers. Natural polymers such as collagen, elastin, chitosan, and alginate have an inherent advantage over synthetic fibers due to their biocompatibility and bioactivity. Synthetic nanofibers, on the other hand, must have their surface chemistry selectively modified so that the stem cells are better accommodated. Fortunately, proteins and other ligands can easily be attached to the substrates following electrospinning. One disadvantage of using natural polymers is the difficulty involved in spinning them into fibers. Chitosan and alginate, two of the most widely used natural polymers for tissue engineering, gel at very low polymer concentrations, which often leads to sprayed beads of polymer or short fibers with embedded droplets. Unfortunately, high concentrations of polymer increase its viscosity, making it difficult to inject into the spray. One approach to overcome these difficulties is to combine the natural fibers with synthetic polymers prior to spinning them. By blending polyethylene oxide, a common polyether, with appropriate ratios of chitosan, the viscosity of higher concentrations of chitosan can be reduced such that the polymer solution can be electrospun into stable fibers (9). Both natural and synthetic nanofibers have been used to create biodegradable scaffolds, which have tremendous potential for stem cell delivery and tissue engineering.

3D Microstructures

Although 2D environments allow researchers to understand the effects of individual parameters on stem cell biology and behavior, they fail to provide the combined effects of forces and signals in 3D that occur naturally in the body. An *in vitro* investigation of topography and micropatterning in 3D culture is vital to gaining a complete understanding of stem cell behavior in the natural 3D environment.

Computer-aided design models can be used to create detailed layered structures for imprinting a particular pattern onto a substrate. This method, commonly known as 3D printing, involves using binder materials to bond polymers together to create each layer. 3D structures are created layer-by-layer by using ink-jet printing. Alternatively, photoresist polymer can be patterned in the same manner using laser lithography. One advantage of this technique is its flexibility to fabricate 3D microstructures. However, it is difficult to print the cells directly onto the 3D structure due to biocompatibility issues, which include the binder materials and laser beam.

Electric fields can also be used to control complicated organizations of cells through the combination of dielectrophoretic (DEP) forces and photopolymerizable gels. Metallic substrates are patterned with photoresist to form a

conductive plate. This plate is then used in conjunction with an unpatterned plate to sandwich unpolymerized gels containing a cell solution. An alternating current is applied to the plates, creating a high electric field where the insulating photoresist layer is not present. The cells then migrate to areas with high DEP forces and are subsequently trapped into the arranged configuration through UV polymerization of the gel. Multiple layers and patterns can be created through repetition of this process. This technique is valuable for generation of complex, multicellular tissue samples.

ENGINEERING THE ECM: EFFECTS OF ECM RIGIDITY AND DYNAMIC MECHANICAL LOADING

Thus far we have discussed the technologies to engineer ECM distribution, surface topography, and 3D structure. However, stem cells *in vivo* are constantly responding to a combination of both static and dynamic forces. In addition, material properties of the ECM affect cell proliferation, migration, and differentiation. The complexity of the *in vivo* environment makes it important for researchers to identify how individual stresses and material properties affect the behavior before determining the combined effects of these factors. In this section we describe current techniques used to control ECM rigidity and apply various mechanical loadings to cells in culture.

Engineering the Rigidity of Extracellular Matrix

Researchers have known for over half a century the importance of material properties in biology. In particular, having an ECM with a modulus comparable to that under typical physiological conditions is essential for normal growth and development of many cell types. For example, Engler et al. discovered that myoblasts grown on substrates with compliances other than that of mature muscle tissue, with a Young's modulus of approximately 12 kPa, will fail to develop properly (11). MSC differentiation also depends on the elasticity of the ECM (12). Studies have also shown that cells tend to migrate toward more rigid surfaces and that cells on soft matrix have a low rate of DNA synthesis and growth (13, 14). Although the exact effects of different matrix rigidities on stem cell behavior is not well understood, a number of researchers hypothesize that stiffness fluctuations alter cell focal adhesions and contractile cytoskeleton structure, and can potentially affect soluble chemical factors (15). Due to the mechanosensitivity of stem cells, it is important to create a reliable method to manipulate the rigidity of the matrix.

In the past few years, natural polymers or hydrogels have been used to create matrices with different stiffness by manipulating the monomer concentrations. Hydrogels such as Matrigel (BD Biosciences) are often used as scaffolds in tissue engineering due to their close resemblance to the cells' native environment. Agarose gels coated with a protein matrix have also been used to generate substrates of varying rigidity. Again, stiffness can be varied using

different concentrations of agarose in the gel. By monitoring the peptide chains present on the substrate, researchers are able to regulate the interactions between the seeded cells and the ligands located on the substrate. However, there are some drawbacks to using natural polymers in hydrogels for the study of ECM rigidity. One of the main concerns is the inability to differentiate the mechanical (the rigidity) and chemical (the ligand density) signals being delivered to the cells through the substrate. Furthermore, the structure of the fibers and porosity of the matrix can vary with the changes in stiffness, which can in turn affect cell response (16).

The recent use of synthetic substrates results in better control of matrix rigidity independently of any chemical influences. Pelham and Wang formulated an elastically tunable, well-defined procedure for producing matrices by using polyacrylamide (PA) gels chemically tethered to glass coverslips (17). One advantage of using PA gels over natural polymers is that the rigidity of the substrates can be easily varied by adjusting the percent ratio of acrylamide to bis-acrylamide crosslinker, while keeping the total polymer concentration constant. This also allows both the surface texture and ligand distribution to remain even. In addition, chemical signals are limited to only desired cell–ligand interactions because the bare PA gels are resistant to cell adhesion. To allow cell adhesion, PA gels must be modified with a specific ligand coating, such as Type I collagen, prior to cell seeding using a heterobifunctional linker such as Sulpho-SANPAH (*N*-Sulfosuccinimidyl-6- [4′-azido-2′-nitrophenyl-amino] hexanoate) (Pierce, Biotechnology). This allows greater control over parameters affecting the cells. PA gels have been found to produce well-characterized and stable substrates for cell seeding.

Effects of Dynamic Loading on ECM and Cell Surface

Stem cells have great potential as a source for tissue engineering such as construction of tissue-engineered grafts. Cells in many tissues and organs (e.g., heart, blood vessels, bone, cartilage) are constantly experiencing stresses. For example, cells in the vascular wall experience anisotropic strain and shear stress whereas cells in cartilage are subjected to compressive forces. Different types of dynamic loading also induce various differential responses from cells. Park et al. found that cyclic uniaxial strain increased the expression of smooth muscle contractile markers in MSCs whereas equiaxial strain decreased the expression (18). These studies demonstrate that MSCs are highly susceptible to slight changes in external loading. Yet the specific mechanisms through which they are affected are still poorly understood. In order to properly study these processes, it is essential that researchers have appropriate tools and devices to simulate the mechanical factors *in vivo*. In this section, we will describe current techniques used to apply dynamic loads to stem cells.

Equiaxial Strain. Equiaxial strain, in which the cells are loaded uniformly in all directions, is one of the most straightforward types of mechanical strain applied to cells. Flexcell International Corporation has developed the

commonly used Flexercell® system for applying equiaxial strain to cell cultures. Cells are cultured on a flexible membrane that moves under vacuum pressure; up to 30% substrate elongation can be achieved through the system under either static or cyclic deformation (Fig. 17.3a.i). *In vivo* physiological cell conditions can be mimicked easily with adjustments to the frequency and magnitude of applied cyclic strain. However, the deformation in the membranes of this system is heterogeneous. Heterogeneity arises when the strain tensor changes with location. Newer versions of the Flexercell system use a design that includes modifications such as using different post shapes to control membrane deformation and improve strain homogeneity.

Some groups have overcome the nonhomogeneity problems with systems similar to Flexercell that employ fluid (positive pressure), platens, glass domes, and pin-shaped posts as the source of strain in lieu of the vacuum, and a platen as a replacement for the flexible-bottomed plate (Fig. 17.3a.ii–iv) (19). Some of the drawbacks to these devices include the inability to operate in an incubator or, in some cases, the incapacity to induce cyclic strain. The friction created by the movement of the membrane over the loading post or platen may also transport undesired heat to the cultured cells in some of the designs. Schaffer et al. and Cheng et al. designed an alternative system that is nearly isotropic and homogenous (20, 21). Their design is composed of six circular, pulsating cultured wells with flexible bottoms overlaying platen with low-friction outer rims. The platens are driven by a motor that peripherally stretches the central portion of each culture membrane; their study showed that up to 33% strain could be achieved with the device.

Uniaxial Strain. Uniaxial strain is anisotropic, a condition under which the cells are strained in only one direction. This type of strain mimics that experienced by skeletal muscle, tendon, ligament, and vascular smooth muscle cells. One method of mimicking this environment in a 2D condition is with a setup similar to the Flexercell® uniaxial system. Well-defined uniaxial strain can be obtained in the middle part of the well by replacing the circular post with a rectangular straight post (Fig. 17.3b.i). Park et al. custom-designed a mechanical device for application of cyclic uniaxial strain (18). Silicone membranes were fastened into stretching frames (Fig. 17.3b.ii). A Teflon bar located at one end of each chamber was attached to a cam/rotator system programmed to move sinusoidally to induce cyclic strain. Uniaxial strain was measured to be uniform throughout the system, with the exception of the orthogonal edges, which experienced slight compression. In order to better simulate the cells' native environment and promote cell adhesion, the silicone membranes were coated with collagen prior to cell seeding. One of the advantages of this system is the uniformity of strain applied to each of the chambers.

3D uniaxial strain models are also used to better simulate the native stem cell environment. In reality, tissues experience a highly complex combination of signals in all three dimensions as the body moves, thus it is important to investigate these signals *in vitro*. Certain types of cells also behave differently

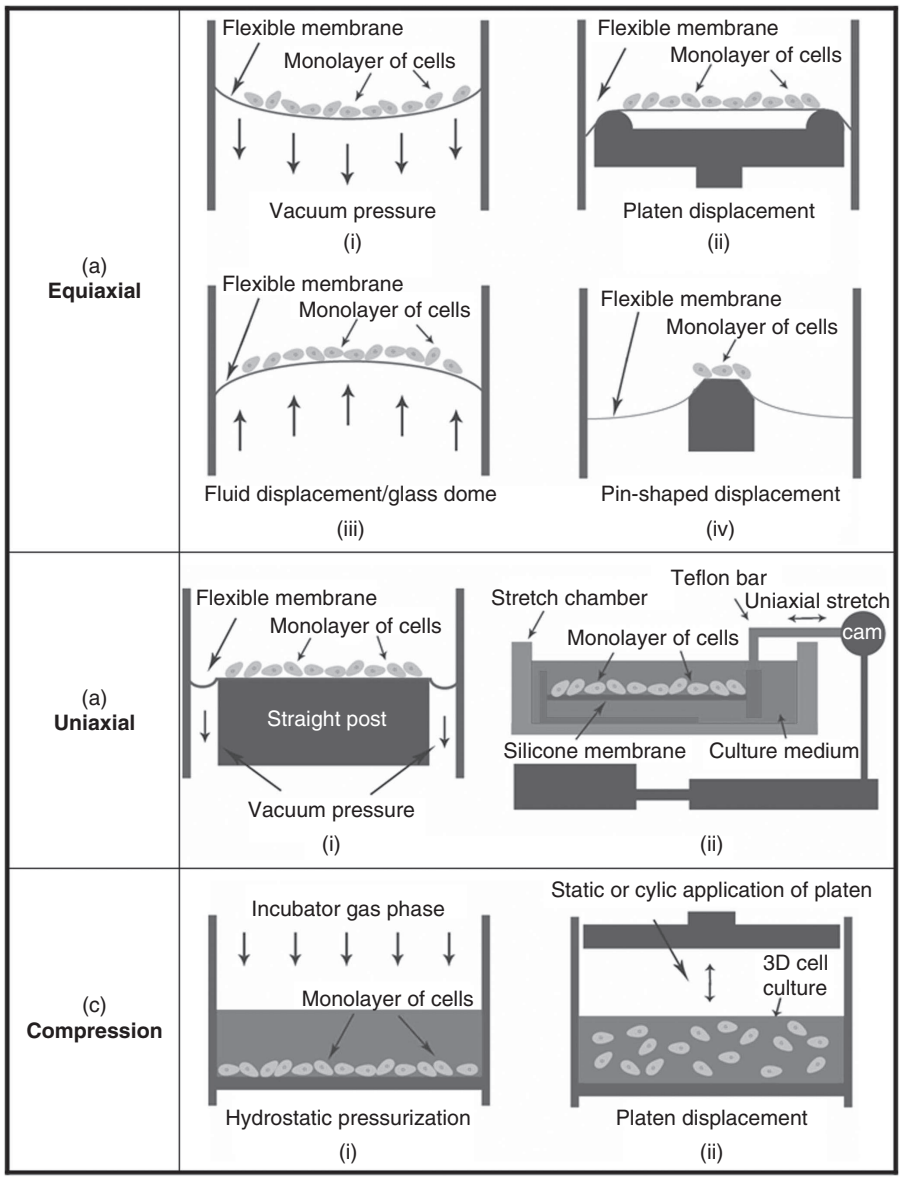


Figure 17.3. Various techniques used to exert dynamic loading. (a) Equiaxial strain. (i) Flexercell® system. Cells are cultured on a flexible-bottomed plate that deforms under vacuum pressure, creating either static or cyclic strain. (ii) Equiaxial strain system where cells are cultured on a flexible membrane and strain is applied through a platen. (iii) Equiaxial strain system where strain is applied through either a glass dome or fluid displacement. (iv) Equiaxial strain system where strain is applied through a pin-shaped displacement. (b) Uniaxial strain. (i) Generating uniaxial strain using vacuum and a rectangular straight post. (ii) A custom-made uniaxial stretch machine. A Teflon bar is attached to a silicone cell culture membrane and controlled by a motor and cam to apply cyclic stretch. (c) Compression. (i) System where compression is applied through hydrostatic pressurization to a monolayer of cells. (ii) System where compressive forces are applied through direct contact with matrix embedded with cells. (See color insert.)

in 2D culture versus 3D culture. For example, fibroblasts prefer a monolayer 2D culture where they can spread evenly and proliferate quickly, whereas chondrocytes grow better clumped in clusters, and are therefore better suited in 3D cultures. One simple method of applying 3D uniaxial strain involves extracting native vessel samples from a host and then applying *ex vivo* stretch to it. Another method involves seeding stem cells into a tubular matrix, followed by the application of stress on the luminal side. Flexcell International Corporation has recently developed the Tissue Train® culture system which is composed of a rubber membrane placed on top of a trough. A gel matrix seeded with cells is poured onto the membrane and the resulting 3D matrix can then be subjected to uniaxial stress.

Compression. Some cells experience more compressive forces than tensile forces. These forces are most commonly exerted on chondrocytes, which are those cells found in cartilage. Most compressive strain studies have been performed on chondrocytes, although osteocytes and endothelial cells have also been studied.

One way of applying compressive force to cell cultures is through hydrostatic pressurization. The basic design consists of a closed system that contains a monolayer of cells covered in media. Pressure is generally applied through the gas phase above the liquid medium, as shown in Fig. 17.3c.i. There are a number of benefits to using this type of setup. All loading is completely homogenous for each sample, and the equipment and application of the force are relatively simple. However, there is some concern for possible changes in the chemistry of the media, for example, the increase of pO_2 and pCO_2 due to high gas pressure, although these changes in the media are less significant if the pressure changes are high in frequency or low in magnitude (22).

An alternative method for employing compressive stress is through direct contact with platens (see Fig. 17.3c:ii). These devices usually require the samples to be in 3D matrices, either embedded in a polymer matrix or from a tissue explant. The 3D samples are loaded into the compression device and the platen is lowered precisely onto the matrix. Cyclic or uniaxial compression is then applied, most commonly through either a pneumatically driven device or through the manual application of dead weights. This allows studies to be highly flexible in the range of deformations given to the sample, in addition to being easy to study the mechanical response of cell/matrix composites. This method is also a closer approximation of the true 3D tissue environment than hydrostatic pressurization.

Shear Stress. Fluid shear stress, the tangential stresses caused by fluid flow, is experienced by many types of cells (e.g., due to interstitial fluid flow), particularly in the cardiovascular system. When cells and tissues are grown in a bioreactor with mixing or perfusion, they also experience fluid shear stress. It is well known that shear stress influences cells through mechanotransduction by

affecting plasma membrane receptors, ion channels, integrins, and other transmembrane molecules. There are two main devices for *in vitro* modeling of fluid flow: the cone-and-plate flow chamber and the parallel-plate flow chamber.

The cone-and-plate system is based on the rotation around a cone axis orientated perpendicular to the surface of a flat plate. Spatially homogeneous fluid flow can be achieved with careful configuration of the system. The magnitude of stress can be altered by manipulating the conic taper angles and angular velocity. However, microscopic visualization of cultured cells is somewhat difficult.

In vitro modeling by laminar flow using a parallel-plate chamber is one of the most convenient methods for examining shear stresses in 2D cultures. A rectangular chamber is outfitted with two slits at the openings of both ends of the device, which creates a pressure differential and consequently, uniform laminar flow. The shear stress and fluid flow rate can be controlled by a perfusion pump. Advantages of this system include increased homogeneity in the shear stress, simplicity of equipment, and the ability to do microscopy. 3D cultures can also be used to investigate the effects of shear stress induced by interstitial fluids.

BIOREACTORS: A REVOLUTION IN STEM CELL CULTURE

Most of cell characterization is performed in 2D culture *in vitro*. Although convenient for examining basic biological processes such as cell proliferation, migration, and differentiation, 2D analyses may give a skewed perspective of the behavior of the cells *in vivo*, an obvious 3D environment. In addition, 2D culture experiments commonly focus on the effects of one particular parameter on cell behavior, rather than a complex combination of factors. In reality, microenvironmental factors such as cell-to-cell communication, external forces from movement, fluid flow, and nutrient transport strongly influence stem cell behavior. To make the situation even more complicated, not only can different combinations of multiple factors affect cell functions such as differentiation, migration, and proliferation, but changes in the quantity of each factor in the combinations can also impact cell behavior. In order to address the challenges of investigating cell and tissue behavior in complex environments, expanding cells efficiently, and engineering tissues for implantation, researchers have developed bioreactors with well-controlled chemical and mechanical environments. There are two main types of bioreactors: those developed for mammalian cell cultivation and those utilized for tissue constructs.

Bioreactors designed for mammalian cell culture usually have a large surface area and efficient nutrient transport systems. To increase the surface area for cell culture, microcarriers, macroporous carriers, stacking plates, and hollow fibers are used in the design. Microcarriers are miniscule beads or particles that can be designed with special surface chemistry that facilitate cell

attachment and growth. Macroporous carriers have the same basic design concept; however, they are slightly bigger and as the name indicates, have larger pore volumes to allow for fluid and nutrient flow to the cells. The simplest of these types of systems are smaller-scaled well-mixed bioreactors, such as shake flasks, stirred vessels, and spinner flasks. This type of bioreactor is most commonly used for proliferation studies of encapsulated cells and cells in suspension culture (e.g., hematopoietic stem cells, embryo bodies). Macroporous carriers are generally used in fixed bed or fluidized bed bioreactors, which are designed mainly for long-term cultivation of mammalian cells for biopharmaceuticals. The carriers are either fixed in columns (fixed bed) or float freely (fluidized bed) in the system. For fixed bed systems, the column is perfused with media to ensure cells are supplied sufficient nutrients (23). For applications such as monoclonal antibody and virus production, recombinant protein manufacturing, and 3D culture of human cells such as hepatocytes, stacking plates, and hollow fiber bioreactors are often used. In hollow fiber systems, cells are seeded at high, tissue-like densities inside or outside hollow fibers. The fibers are usually porous, allowing oxygen and biomolecule transport, but are small enough to inhibit cell infiltration. This type of bioreactor has the potential to fabricate liver and kidney assistance devices. If the pore size can be precisely controlled, larger molecules such as proteins and antibodies can be trapped and concentrated in desired areas, whereas smaller molecules such as lactate and glucose (i.e., waste and nutrients, respectively) can move easily across the membrane of the hollow fibers (23). Perfusion and mixing are often used to enhance mass transport in bioreactors for cell culture, which is critical for the supply of nutrients and oxygen and the removal of wastes and metabolites (e.g., carbon dioxide and lactic acid).

Bioreactors custom-designed for tissue implant generation are highly complex systems that are commonly tailored to be tissue-specific. Each tissue type requires a different geometry with specialized design parameters for external forces, if needed. For tissue constructs cultured in suspension, the most popular design is the rotating-wall vessel bioreactor, in which the construct supporting the cells and tissue are in a free-falling state within the media (23). This design allows the cells to experience low shear forces and a high mass transfer rate. Other bioreactors apply external forces such as dynamic compression, tension, and hydrodynamic pressure to the tissue constructs at physiological frequencies and loading. For example, pulsatile pressure can be applied to vascular grafts to stimulate the remodeling of the matrix, fluid shear stress can be used to precondition endothelial cells on the lumen of grafts, and mechanical stretch can be applied to muscular tissue.

Bioreactor technology has added another dimension to stem cell research. Not only do scientists have the ability to investigate the effects of microenvironmental factors on stem cell cultures and engineered tissues in a more realistic and/or 3D environment, but they now also have the ability to mass-produce cell cultures and tissue samples for either research or regenerative medicine applications.

CONCLUSIONS

The discovery, isolation, and culture of stem cells are tremendous breakthroughs for regenerative medicine and tissue engineering. We not only have the potential to extend the life of those who have been stricken with diseases such as Parkinson's and diabetes, but hopefully will also soon have the ability to engineer tissues and organ. However, in order to truly harness the full potential of stem cells in tissue engineering and regenerative medicine, it is important to have a complete understanding of the behavior of each stem cell type. It is essential that researchers are continually improving current technology and designing new methods of simulating these cells' native environment. Additionally, we must use appropriate techniques for examining stem cell behavior.

ACKNOWLEDGMENTS

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PART V

18

IMPROVED LENTIVIRAL GENE DELIVERY TOOLS FOR STEM CELLS

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Hematopoietic stem cells (HSCs) represent prime starting material for creating customized tissue to treat genetic disorders. Several obstacles confound their preparation. One roadblock is the difficulty in obtaining cells that are immunologically compatible with the recipient. This may be circumvented by modifying the patient's own cells. However, a more serious problem is how to modify or adapt them to suit specific therapeutic applications. Transferring expression or gene knockdown constructs into HSCs to achieve stable production of therapeutic proteins or molecules at physiologically useful levels is often more easily said than done.

Unfortunately, like many of the cell types of interest to biomedical and gene therapy researchers, HSCs are refractory to traditional gene delivery methods. Stem cells, like other primary and nondividing cells, often do not transfect with sufficient efficiency. Electroporation can be harsh, killing a large percentage of valuable cells (1). Direct injection of DNA sequences is laborious, technically demanding and generally yields only a small number of cells expressing the construct of interest (2). These techniques, while useful for small-scale proof-of-concept applications, simply do not offer the efficiency, economy, and safety required for the task of genetically manipulating HSCs for therapeutic use.

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Oncoretroviral delivery tools, while offering greater efficiency of gene delivery, also suffer from numerous technical limitations. Optimized by nature, viruses circumvent most of the toxicity or inefficiencies of artificial mechanical- or chemical-driven gene delivery methods (3). However, once in the cell, a retroviral genome must be reverse transcribed and integrated into the host genome (2, 4–6). Most retroviral systems cannot accomplish this step unless the nuclear envelope has broken down during mitosis (6). Waiting for mitosis can result in reduced integration as the intracellular stability of some retroviral vectors is approximately 6 h (7–10). This problem can be partially overcome by using synchronized cell populations (11).

Unfortunately, successful integration of a retroviral vector does not guarantee the expected phenotype. Work in mice and other species has shown retroviral constructs are susceptible to silencing by host factors which bind to retroviral long terminal repeats (LTRs), effectively shutting down expression of any transferred therapeutic gene (12).

Lastly, standard retroviral vectors display a penchant for integrating near active endogenous promoters (13). Strong viral promoter and enhancer elements in the retroviral LTRs can cause abberrant changes in the expression of adjacent cellular genes. Integration in the wrong hotspot can result in oncogenic transformation and cancer (14, 15). Data from clinical studies has demonstrated that while standard retroviral vectors can perform the job of gene delivery, the therapeutic advantage is attenuated by the potential for extremely negative complications (14, 15). In landmark studies performed in the early 2000s, HSCs modified by Moloney murine leukemia virus (MMLV) to express interleukin (IL)-2Rgamma or adenosine deaminase were used to treat patients with various forms of severe combined immunodeficiency (SCID) disease in France, the United Kingdom and Italy (16–18). These treatments successfully corrected their immune disorders. However, a significant number of those treated in the French study developed T-cell lymphoblastic leukemia, dampening enthusiasm for the use of oncoretroviral vectors in clinical applications.

THE LENTIVIRAL OPTION

Lentivirus has recently played an increasing role in the genetic modification of HSCs. Several proof of concept studies include using lentivirus to stably transduce human cord blood CD34+ cells without vector-based toxicity (3, 19). Osteoarthritis models have elucidated the value of lentivirus for modifying mesenchymal stem cells to express growth factors in a manner conducive to enhancing repair of connective tissue (20). Long-term correction of congenital erythropoietic porphyria has been accomplished in mice implanted with lentivirus-modified HSCs expressing the human version of the deficient enzyme (21). Clinical trials have even been initiated using lentivirus to modify bone marrow cells for human implantation (22, 23).

Several characteristics make lentivirus safer and more efficient as a gene delivery tool (24). One of its key features is the ability to transduce a wide

variety of cells. These include both dividing and nondividing cells as mitosis is not required for lentivirus to cross the nuclear envelope and integrate into the host genome (6, 25, 26). This feature is of key importance in transducing HSCs as gene delivery may be accomplished without cell division and differentiation. Lentivirus' ability to transduce cells is augmented by replacing the normal HIV envelope protein with that of vesicular stomatitis virus glycoprotein (VSVG). This replacement allows lentivectors to bind and transduce a greater range of cell types than the wild type virus (5). Furthermore, unlike oncoretroviral vectors which show preference for integrating at promoter hot spots, lentivirus integration is essentially random, greatly reducing the chance for inadvertantly activating protooncogenes (14, 26).

Despite its notoriety for causing human pandemic, HIV, in a deconstructed and customized format, offers far greater safety than previous generations of retroviral vectors. Commercial lentiviral systems have largely mitigated risk through extensive modification of the viral backbone. First, most of the genes which comprise the HIV parent have been deleted. These include several genes known to play a role in virulence or to be dispensible: Vpr, Vif, Vpu, Tat, and Nef (27). Second, the remaining core genes required to replicate and package the viral sequence have been removed from the actual virus and are generally provided on plasmids as a "packaging mix." Providing these genes in trans reduces to near zero the possibility of generating intact self-replicating virus (5, 6). Invitrogen's Virapower packaging mix places each of the packaging genes on a separate plasmid to maximize safety (Fig. 18.1). Third, the latest generation of lentivectors includes a self-inactivating feature consisting of deletions in the 3' LTR, eliminating viral promoter and enhancer elements, further reducing aberrant transcriptional activity at the point of insertion (27).

One advantage of lentivirus is the simplicity and safety of generating delivery particles in any ordinary BL-2 tissue culture facility. To make virus, constructs are transfected into a packaging cell line such as 293FT cells along with plasmids encoding the minimum complement of viral packaging factors. Within 48 h, particles are secreted into the culture medium (Fig. 18.1). These particles are noninfectious in the sense that they are gutted of HIV's normal complement of viral packaging and virulence genes and are therefore incapable of replicating and propagating. Supernatants containing these secreted pseudoviral particles may be harvested as-is and stored at –80 °C for over 1 year. Alternatively, pseudoviral particles may be isolated by a variety of methods including precipitation or centrifugation, and stored in concentrated and purified form, suitable for *in vitro* or *in vivo* applications.

RECENT IMPROVEMENTS

A healthy, high-titer viral stock is key to maximize lentiviral transduction efficiency and expression. Improvements in the commercial arena have often focused on increasing apparent titer by boosting the expression level of

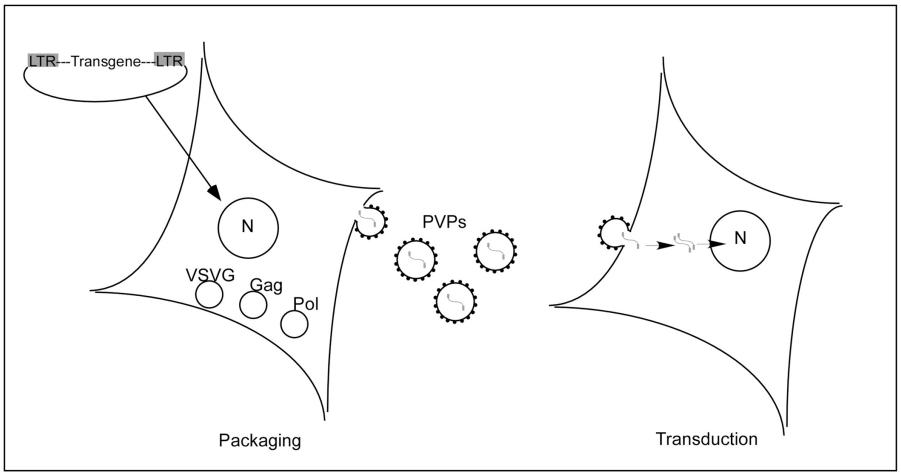


Figure 18.1. Virapower™ Lentiviral packaging and transduction pathway. Virapower lentiviral packaging genes are segregated for increased safety, each on a separate plasmid. The lentiviral construct is embedded in the pLenti vector. Viral genes have been removed and replaced with a transgene expression cassette. Modified self-inactivating LTR sequences (shown as gray boxes) define the ends of the viral construct. Co-transfection of the packaging genes (VSVG, Gag, Pol) and the pLenti transgene construct result in the formation of pseudoviral particles (PVPs) which bud through the packaging cell plasma membrane displaying the VSVG envelope protein on the surface (shown as black knobs). Fresh cells exposed to PVPs become transduced as the particles fuse to the cell surface. Viral RNA enters the cell, is reverse transcribed to create an RNA/DNA double stranded hybrid which is then converted into a DNA/DNA molecule. The LTR ends (shown in gray) of the viral construct are partially cleaved to expose recombinogenic sites. The double stranded viral DNA translocates into the nucleus and integrates randomly into the genome. Deletions in the 3' LTR, which eliminate viral promoter and enhancer sequences, prevent the stably integrated construct from replicating.

lentiviral titering reporters. However, methods which simply boost the signal strength of the on-board reporter do not actually increase the number of functional particles. Rather, they simply make a larger percentage of the existing particles stand out and be counted over background. Unfortunately, expression or detection of the cloned gene of interest in these particles usually does not benefit from the enhancement, making such "improved" vectors of dubious benefit.

Lentiviral vectors have benefited from a number of improvements over the past few years. These include new elements which enhance viral titer and expression in certain cell types as well as a host of convenient selection and detection features. One key element, the Woodchuck Posttranscriptional Regulatory Element (WPRE), has been described extensively in the literature as playing a role in enhancing gene expression (28). Inclusion of this cis-acting

element immediately downstream of an open reading frame results in an increase in nuclear export of the transcript thereby enhancing expression in cell-specific manner. Lentiviral constructs containing the WPRE element exhibited a five- to eightfold increase in luciferase or green fluorescent protein (GFP) expression using lentivirus (29, 30). A central polypurine tract (cPPT) from the HIV-1 integrase gene has been shown to increase the copy number of lentivirus integrating into the host genome especially in nondividing cells. The benefit is cell type specific and results in up to a twofold boost in titer and consequently, gene expression (31).

These elements have been incorporated into a number of commercial and homebrew lentiviral vectors. Recently, Invitrogen introduced an update to their line of lentiviral tools designed to provide increased numbers of functional viral particles and substantially higher protein expression or gene knockdown performance. The so-called HiPerform™ vectors containing the HIV cPPT were created with the WPRE element placed immediately downstream of the cloning site where it directly supports expression of the gene of interest. See below for a discussion of the performance of the new HiPerform™ vectors.

The Invitrogen Virapower™ HiPerform™ line of vectors offer different selection, tittering, and cloning formats to support specific applications. Since lentivectors can be used in either transient or stable expression studies, vectors come with either a drug selection system or a fluorescent protein marker. The blasticidin selection marker embedded in the backbone of pLenti6.3 series vectors, offer stringent selection of cells in which the lentiviral construct stably integrated into the host chromosome. This selection ensures that untransduced cells be killed off and also that the surviving cells do not suppress or silence the integrated lentiviral construct. Only cells that express sufficient levels of the drug resistance gene product survive and propagate.

Not all experiments require the generation of stable cell lines or populations in which 100% of cells have been transduced. In such cases where stringent elimination of untransduced cells need not be eliminated, a fluorescent protein serves as a more useful marker. The plenti7.3 FastTiter vector series contains EmGFP instead of blasticidin in the HiPerform™ vector backbone. This marker enables measuring transduction efficiency far more rapidly than by any other drug selection system as EmGFP-positive cells can be detected and counted by flow cytometry 2 d following transduction. This is in contrast to roughly two weeks required for drug selection where one must wait for killing of untransduced cells and outgrowth of drug resistant foci. With EmGFP, transduction efficiency can also be monitored during the course of an experiment. Furthermore, to enrich the cell population for transduced cells, EmGFP-positive cells can be segregated and collected by flow cytometry. This functionality lends itself to high-throughput applications where one may screen large numbers of constructs plated in high density arrays.

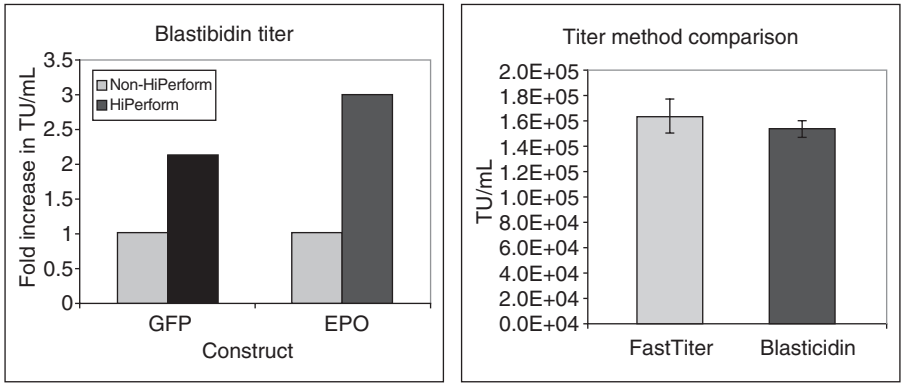
Each HiPerform™ vector is available in either Gateway® or TOPO® cloning format to suit specific workflows. For low-throughput applications,

TOPO® offers speed and ease of cloning of PCR amplified fragments. Gateway® cloning requires the preparation of entry vector clones in pENTR plasmids to allow for recombinational insertion into lentiviral destination vectors. This system is convenient for higher throughput applications as once libraries of entry clones are prepared, the Gateway® system allows rapid *in vitro* transfer of large numbers of clones into pLenti6.3 Gateway® vectors in multiwell arrays either by hand or using automation.

As mentioned above, obtaining a healthy and high-titer viral stock represents a crucial first step in successful use of lentiviral vectors. Expression level is directly correlated with viral dose or multiplicity of infection. Therefore, knowing the titer allows dosing the correct amount of functional viral particles onto cells to ensure that the desired percentage of the population gets successfully transduced. Knowledge of the number of functional particles in the viral preparation is therefore paramount especially when low viral exposure is desired to obtain at most one copy of an expression construct per cell to minimize the chance for insertional mutagenesis.

Titer performance of the HiPerform™ vector, pLenti6.3, was compared to the previous generation vector, pLenti6, which lacks the cPPT and WPRE elements. In this context, titer was measured using the blasticidin reporter present in pLenti6 and 6.3. Expression constructs were made using either EmGFP or erythropoietin (EPO). Lentiviral constructs and packaging components were transfected into 293FT cells in duplicate 10 mL adherent cultures. Pseudoviral particles were harvested at 48 h post transfection, filtered, and used for titering and protein expression evaluations below. In multiple packaging and transduction experiments, vectors containing the HiPerform™ elements produced higher levels of functional viral particles compared to non-HiPerform™ vectors. Both EmGFP and EPO constructs revealed an increase in blasticidin-resistant transductants, effectively yielding at least twofold more functional virus compared to the previous generation vector lacking these elements (Fig. 18.2a).

To determine the feasibility of using GFP as a useful representation of function viral titer, pLenti7.3 constructs containing EPO were used to generate virus. These viral preps were initally used to transduce cell monolayers to determine simply if the EmGFP expression level from the SV40 promoter driving the EmGFP gene in the backbone of the 7.3 series vector was sufficient to visualized the fluorescent signal using a standard inverted Olympus IX70 fluorescence microscope. Transductions were performed in 96-well format, dosing 2–10 μL of virus onto wells seeded with 6000 cells. This was done to keep the multiplicity of infection below 0.3 to avoid saturating the system with virus. EmGFP fluorescence could be visualized within 48 h post transduction. The signal level is not optimized for visual evaluation of transduction efficiency, but is well suited for automated detection of transduction events or sorting of transduced cells by flow cytometer such as the Guava Technologies Personal Cell Analysis-96 (PCA-96) flow cytometer. Since we were able to process multiple 96 well plates per day, one could envision screening hundreds



(a) (b)

Figure 18.2. Titer evaluations. (a) HiPerform™ versus non-HiPerform™ constructs. Hela cells were plated at subconfluent density and transduced at low multiplicity of infection. Non-HiPerform™ constructs in pLenti6 (light bars) were compared to HiPerform™ constructs in pLenti6.3 (dark bars). Constructs expressed either EmGFP or erythropoietin (EPO). Transduced cultures were placed under blasticidin selection for 2 weeks and surviving foci were counted. For each gene, titers were normalized to its corresponding non-HiPerform™ construct. HiPerform™ constructs generated approximately two- to threefold more functional virus per milliliter of viral supernatant. (b) HiPerform™ versus HiPerform™ Fastiter™ comparison. Hela cells were plated at subconfluent density and transduced at low multiplicity of infection with either a pLenti6.3 HiPerform™ construct (light bar) or pLenti7.3 HiPerform™ FastTiter™ (dark bar). pLenti6.3 cultures were placed under blasticidin selection for 2 weeks and surviving foci were counted. EmGFP positive cells in pLenti7.3 cultures were counted 48 h post transduction using a Guava personal flow cytometer. Titer determinations by EmGFP detection was found to be comparable to counts determined using blasticidin selection.

of transductions in semi-high-throughput experiments if desired. The value of the GFP titering approach is that a titer can be obtained within 48 h of transduction in comparison to 2 weeks required with drug (Bsd) selection. Titers obtained from 48-h evaluations were comparable to titers obtained from EPO constructs using blasticidin on the 6-Series vectors (Fig. 18.2b). This is largely expected as both titering mechanisms are reporting events in which virus has successfully transduced a cell and is detectably expressing its reporter.

The key benefit to blasticidin selection is the ability to kill off untransduced and low-expressing cells resulting in the generation of stably expressing cell lines. To demonstrate the stability of the integrated pseudovirus, cells transduced with and expressing the erythropoietin construct in pLenti6.3 were propagated without blasticidin selection for 30 d. Erythropoietin expression was found to be essentially unchanged after 30 d, reflecting the stability of the integrant and its resistance to silencing (Fig. 18.3).

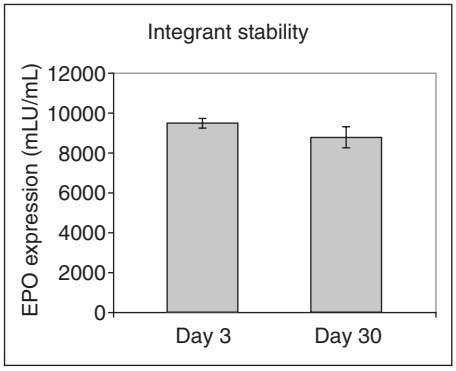


Figure 18.3. Integrant stability. HT1080 cells were transduced with pLenti6.3 containing an erythropoietin construct and maintained without selection. Protein expression was determined by ELISA on day 3 and day 30 post transduction and normalized to cell number. Expression on day 3 and day 30 was equivalent indicating stability of and continued expression from the lentiviral integrant.

Protein expression with HiPerform™ is substantially higher due to the support WPRE provides to the cloned gene (Fig. 18.4). EmGFP clones in pLenti6, 6.3 TOPO® and 6.3 Gateway® lentivectors were transduced into three different cell lines. Cells were dosed with either equal volumes of viral preparation or were treated with defined amounts of virus based on blasticidin titer estimations. Cells transduced with pLenti6.3 constructs showed significantly greater EmGFP fluorescence than plenti6 transductions (Fig. 18.5). Normalization to viral titer shows enhanced expression performance on a per particle basis (blue bars). The even greater increase seen with equal volume dosing reflects the increased viral titer obtained with HiPerform™ constructs (red bars). Similar performance results were seen with EPO and LacZ constructs.

CONCLUSION

Genetically modified HSCs are a powerful resource for research and therapeutic applications. Lentivirus represents one of the best tools to carry out these modifications. The latest generation of lentiviral vectors offers an increase in yield of functional virus higher transduction efficiency and higher gene expression. The ability to generate more virus with increased potency also makes it possible to reach the higher dose levels required for *in vivo* applications (5, 6). Invitrogen's HiPerform vectors offer these improvements for *in vitro* and *in vivo* proof of concept studies. With EmGFP in the backbone of the pLenti7.3 series vectors, high-throughput methods are now feasible. Large

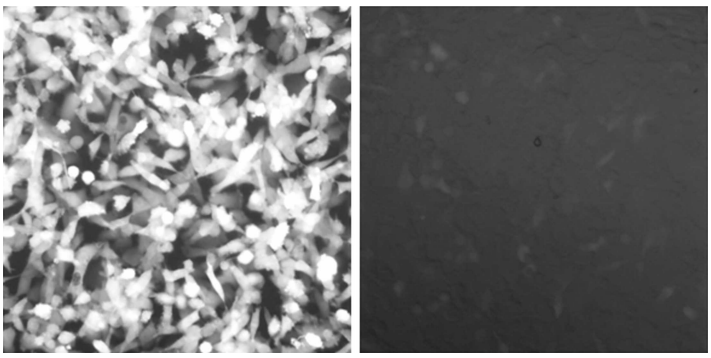


Figure 18.4. HiPerform™ viral constructs generate higher protein expression levels than constructs lacking the new elements. HT1080 cells were transduced with EmGFP constructs in either the non-HiPerform™ vector, pLenti6 (left panel) or pLenti6.3 HiPerform™ (right panel). The HiPerform™ construct displays high efficiency transduction of the cell population and significantly higher EmGFP expression. (See color insert.)

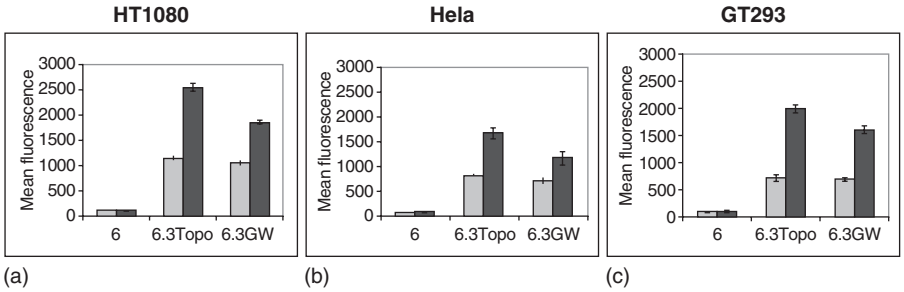


Figure 18.5. Expression as a function of viral dose. EmGFP constructs in pLenti6, 6.3/V5-TOPO®, and 6.3/V5-DEST® were expressed in the cell lines, HT1080 (a), Hela cells (b), and GT293 (c). Virus was dosed onto cells by using equal volumes of viral supernatant (dark bars) or by using equal blasticidin titer units of virus (light bars). GFP expression was quantitated on the GUAVA personal flow cytometer 48 h post transduction. HiPerform™ vectors produce more functional virus and offer higher levels of expression than pLenti6.

numbers of therapeutic constructs can be screened against large numbers of cells using automation.

Making the transition to clinical application requires a degree of safety lacking in retroviral delivery solutions. Positive results from *in vitro*, animal disease models and ongoing clinical trials suggest lentivirus may have circumvented many if not all of the delivery, silencing, and insertional mutagenesis issues of retroviral vectors.

Ongoing optimization efforts promise to further improve the safety and utility of lentivirus for human therapeutic applications (26). Novel lentiviral

envelope proteins are currently being developed using directed evolution techniques to create viral particles with unique cell specificities (32). Lentiviral constructs containing built-in suicide switches are also being devised. Using a variety of strategies, the system aims to automatically eliminate the rare cells in which a lenti insertion results in malignant transformation (33). These anticipated developments coupled with existing lentiviral performance and safety features represent significant advances in gene delivery technology. Together, they offer tremendous potential for both biomedical research and human therapeutic applications.

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19

SLEEPING BEAUTY-MEDIATED TRANSPOSITION IN STEM CELLS

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INTRODUCTION

The last decade has seen a tremendous expansion of research in stem cell biology, providing insights into undifferentiated stem cell characteristics and lineage-specific differentiation as well as the potential for therapeutic application. In basic and translational studies of different stem cell types, genetic engineering will play a key role in bringing this research to fruition. While viral vectors have been used extensively for genetic modification of various stem cell types (1–4), there are several recombinase systems (5, 6) that have recently emerged as nonviral alternatives that can mediate integration and long-term expression that is potentially applicable to primitive and differentiating targets. These nonviral recombinase systems provide alternatives to viral vectors with respect to integrant character (see below), risk of contaminants (replication-competent vector), and simplicity of experimental design.

The most well developed of the nonviral recombinases is the Sleeping Beauty (SB) transposon system (7). SB was molecularly reconstructed from evolutionarily defunct sequences in salmonid fish to generate the first cut-and-paste DNA transposon system known to exhibit significant activity in vertebrate cells. As a tool for cellular engineering, the SB system is comprised of two components (Fig. 19.1); (a) a transposon, consisting of a sequence of inter-

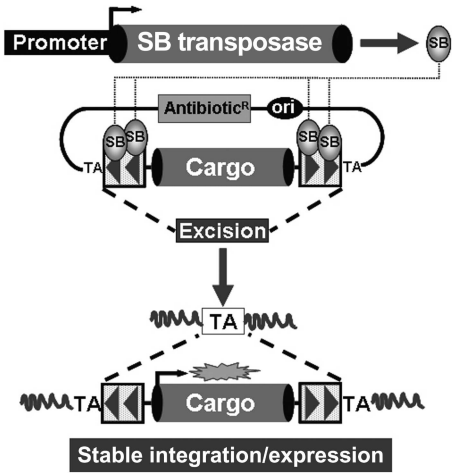

Figure 19.1. The Sleeping Beauty transposon system and transposition. This schematic diagram demonstrates the cut-and-paste mechanism of transposition. For gene transfer applications in somatic cells and tissues, the transposase is frequently supplied *in trans* on a separate plasmid (indicated on top) from the one encoding the transposon, or as *in vitro* transcribed messenger RNA. The transposon ITRs (boxes with triangles) are included on a donor plasmid and flank an expression cassette encoding any exogenous gene sequence ("cargo"). Upon expression, the transposase ("SB") binds to the target sites (shaded triangles) within the ITRs, excises the transposon from the donor vector, and integrates the excised sequence into a TA dinucleotide in the host chromosome.

est flanked by short inverted repeat/direct repeat (IR/DR) sequences, usually delivered on a plasmid by transfection, and (b) SB transposase, usually expressed from a cotransfected plasmid or from codelivered SB transposase-encoding RNA. In the transposition process, the expressed transposase recognizes the IR/DR termini of the transposon, excises the transposon from the substrate plasmid, and inserts the transposon into chromosomal DNA of the host cell. All that is required as a target site for integration is a TA dinucleotide, and thus SB is characterized by a highly random integration pattern, much more so than for retroviral and lentiviral integration patterns (6). SB has been shown to be active in a variety of different cultured cell lines, in somatic tissues *in vivo* and in vertebrate embryos (5). There are numerous modifications of the SB system that have been reported, including modified IR/DR sequences that serve as better substrates for transposition and modified SB transposases that exhibit increased transposition frequency (5). The SB system can thus be used to increase the frequency of nonviral gene transfer in a host cell population, and it can be used to achieve a more predictable nonviral integrant character than otherwise observed for nonhomologous chromosomal nonviral integrants.

The utility of the SB transposon system is limited by its delivery in a particular host cell population and by establishment of conditions for effective transposase expression. When such conditions can be established, transposon-mediated integration has been highly reliable in a variety of cell types. Here we review our recent studies in applying the SB transposon system to achieve nonviral gene transfer in three important stem cell populations: mesenchymal stem cells (MSC), multipotent adult progenitor cells (MAPC), and embryonic stem cells (ES). Each of these populations required particular attention to the gene transfer conditions used, and to the combination of transposon and transposase components provided in achieving effective gene transfer. The nonviral SB transposon system thus provides a special opportunity for genetic engineering of adult and embryonic stem cells for basic inquiry into the regulation of stem cell primitivity and differentiation as well as translational studies into therapeutic applications of genetically engineered stem cells.

TRANSGENESIS OF MSC BY TRANSPOSITION

MSC

MSC, alternatively termed mesenchymal stromal cells or multipotent stromal cells, were initially isolated more than 40 years ago (8). MSC were first defined as osteogenic cells from bone marrow, but investigators later realized that they are capable of differentiating into multiple connective tissue cells; today, MSC are typically defined as cells with a fibroblast-like phenotype that can differentiate into chondrocytes, adipocytes, and osteoblasts *in vitro*. Based on their expression of surface antigens they are characterized as positive for CD73, CD105, CD106, CD166, CD29, CD44, and CD51, while they are negative for CD45, CD11b, and CD14 surface antigens (9–11).

Bone marrow represents the most commonly used tissue source of MSC; however, alternative sources have been used, including umbilical cord blood, placenta, Wharton's jelly, and adipose tissue (12–14). All of these tissues can provide nonhematopoietic stromal cells with the capacity to generate colonies of fibroblastic cells. The colony forming unit-fibroblast (CFU-F) has been used as a measure of clonal MSC proliferation; however, it is likely that not all CFU-F are MSC and vice versa. It is intriguing that despite their apparent homogeneity during *in vitro* culture, MSC appear to be quite heterogeneous *in vivo*. This in turn is relevant for clinical application of these cells since the *in vitro* MSC definition (e.g., expression of surface antigens) and functional readouts (e.g., trilineage differentiation and mixed lymphocyte reactions) may not be reflective of MSC behavior in live organisms.

Clinical Application of MSC

Based on available experimental evidence, MSC in living organisms function as pericytes—adventitial reticular cells with mural (subendothelial) location in the vascular wall. In addition, MSC appear to be poised to answer calls from injured tissues and aid in tissue healing. Remarkably, MSC not only home to the site of injury, but also appear to modulate the inflammatory response in favor of productive regeneration (scarless healing) rather than generation of a fibrous scar. In contrast to what has been a prevailing model over the last several years, recent studies have pointed to a paracrine model of donor MSC in tissue repair of the host (10, 11, 15), whereby donor MSC recruit host stromal cells as well as other tissue repairing cells rather than becoming incorporated themselves into host tissues.

MSC are anti-inflammatory, and while not immune-privileged, they are immunomodulatory (16–19). This capacity has already been harnessed in clinical trials for treatment and prevention of graft versus host disease, a serious immune complication of hematopoietic cell transplantation. In recent elegant studies, LeBlanc et al. have shown that even steroid-resistant graft versus host disease can respond to infusions of MSC (20, 21). In addition, the close approximation of MSC with blood-forming progenitor elements (i.e., hematopoietic stem cells) gave rise to the hypothesis that infusion of MSC can aid in engraftment of hematopoietic elements (21, 22). This appears to be the case based both upon animal studies and on initial clinical data from recipients receiving haploidentical cell transplantation (23, 24).

For any clinical application of MSC, it will be important to define procedures for processing and engineering of MSC *in vitro* and to characterize individual MSC isolates in terms of identity, purity, viability, sterility, potency, dosage, stability, and (retrospectively) efficacy *in vivo*. The potential side effects of MSC therapy include tumor formation and infectious complications. Andreeff and others have shown that donor MSCs home to tumors (25, 26). This capacity can be used for tumor killing, when toxic signals are delivered by *ex vivo* engineered MSC. In addition, some solid cancers likely originate

from a cancer stem cell of mesenchymal origin. Consistent with this, we and others were able to show that osteosarcomas can originate from cytogenetically unstable MSC in mice (27–29). While human MSC appear to be different in many aspects from murine MSC (including their lower propensity for cytogenetic aberrations) (30), malignant cellular transformation still remains an important safety issue as MSC become more widely used in the clinic. Additionally, the immunomodulatory function of MSC can lead to immunosuppression in the recipient, especially in the setting of hematopoietic cellular therapy preceded by a conditioning regimen of chemotherapy or chemoradiation. This may result in activation of latent DNA viral infections (e.g., Epstein–Barr virus or cytomegalovirus) or bacterial and fungal infections, or favor tumor growth (31, 32).

SB-Mediated Gene Transfer in MSC

For the application of MSC in preclinical and clinical studies, it is critical that we have a means of genetically engineering MSC and of manipulating expression of specific genes in MSC. Effective application of such MSC has been elegantly shown in the tumor-killing capacity of MSC (25, 26), as well as in homing of MSC into infarcted myocardium using MSC transgenic for stromal cell-derived factor (SDF) (which home to ischemic heart by recognizing CXCR4 receptor) (33). Using these SDF1-expressing MSC, the mechanical scar in the infarcted myocardium was similar to controls, but the electrical scar was much smaller. Both of these applications (and many others) required the stable introduction of new genes into MSC, thus combining cellular therapy with gene therapy in a synergistic approach: stem cell gene therapy. For that purpose, viral vector mediated gene transfer into MSC has typically been used. As an alternative approach and in order to minimize the side effects of viral transgenesis (34, 35), we and others have investigated a nonviral approach to MSC transgenesis using transposons.

Naked DNA and plasmids without any special capacity for chromosomal integration can be readily introduced into MSC without changing the functional and phenotypic properties of these cells (36). Using transposons with a defined ability to integrate into the host MSC genome, we have routinely been able to achieve >50% transient reporter gene expression and up to 10% stable long-term gene expression (J. Tolar et al., unpublished data). These numbers can be improved when a selection method—either fluorophore-based cell sorting or antibiotic resistance—is used. For any transposon-based transgenesis approach, the fidelity of transposition needs to be verified by insertional mapping of the transposon/genome junctions. These are known and easily recognized for the three species of transposons that are currently available for mammalian transgenesis: SB transposons (7), described herein; TOL2 transposons (37), isolated from the genome of Japanese Medaka fish; and PiggyBac transposons (38), isolated from the cabbage looper moth. In addition, the stability of expression needs to be monitored over time as silencing

due to methylation or histone modification can occur. Constraints of the current transposon vectors are the cargo size and transposition efficiency (39). The latter can be alleviated at least in part by using hyperactive variants of transposases which are becoming increasingly available (5); the former appears to be improved by using the TOL2 or PiggyBac transposon vectors that can carry gene cargo in excess of 10 kb (37, 38).

In summary, MSC are a promising tool in cellular therapy, in particular due to their potential for tissue healing and immune modulation. A genuine benefit can be gained from consistent and high fidelity transgenesis (for cell marking and alteration of MSC function by changing expression of task-specific genes) that transposons have been shown to provide.

SB-MEDIATED TRANSPOSITION IN MAPC

MAPC

MAPC represent a novel class of stem cells derived from bone marrow, muscle, and brain of adult mammals that can be expanded in culture without obvious signs of senescence (40–42). Some MAPC lines can contribute to most somatic tissues when injected into the mouse blastocyst, and mouse MAPC contribute to the epithelium of liver, lung, and gut when infused into sublethally irradiated immunodeficient mice (43–46). MAPC expanded *in vitro* are also capable of reconstituting both myeloid and lymphoid lineages of the hematopoietic system when injected into immune deficient mice (47). Bone marrow-derived stem cells with the capacity for self-renewal and proliferation in culture are important biological tools for studying cell fate and differentiation. These cells also show particular promise for cell-based therapy mainly due to the potential to form almost all somatic cell types (48).

Investigations into the efficacy of MAPC for tissue healing suggest that uncommitted MAPC have as their greatest potential the ability to favorably contribute to blood vessel and muscle regeneration (15, 49, 50). This contribution can occur either directly or indirectly (through cell fusion or by secretion of growth-stimulating factors) as observed in mouse models of limb ischemia (49). Further supporting the positive effect of MAPC-secreted growth-promoting factors are studies demonstrating reduced scaring and improved ventricle function in mice with surgically-induced infarction even when MAPC were detected in the heart for only the first week after local administration (50). These studies are further compounded by experiments demonstrating that increased vascular density and improved cardiovascular performance are dependent upon the immune status of the recipient animal (15). The capability of widespread *in vivo* differentiation combined with secretion of growth-stimulating factors in the repair of damaged tissue makes MAPC an ideal cell population for development of therapies for numerous congenital and acquired disorders.

The capacity of MAPC to participate in the development of vascular tissue also has significant implications for the development of new cancer therapies. Undifferentiated rat MAPC engineered to express green fluorescent protein (GFP) by lentiviral transduction were recruited to and differentiated into endothelial cells in the region of hepatocellular carcinoma cells grown orthotopically in rats (51). This suggests an approach for the use of genetically modified adult stem cells in the delivery of therapeutic agents to established tumors. To realize the full scientific and therapeutic potential of human MAPC, methods are needed for achieving stable expression of gene sequences newly introduced into these progenitor cells with the goal of maintaining expression in terminally differentiated cell types. Our approach has focused on the SB transposable element as a nonviral means of achieving this goal.

SB-Mediated Gene Transfer and Stable Expression in MAPC

The capacity of SB transposase to mediate stable gene expression in MAPC isolated from rodents has relied on Amaxa nucleofection technology, demonstrated to provide efficient gene transfer combined with high viability in these cells (36, 52). SB-mediated transgenesis was first demonstrated by Tolar and colleagues using mouse MAPC (mMAPC) (52). To determine the efficiency of SB-mediated gene transfer, we cotransfected mMAPC with an SB transposase expression plasmid along with two independent transposons, one encoding the fluorescent reporter DsRed and a second encoding firefly luciferase (Luc). Eight percent of mMAPC isolated by single cell sorting maintained expression of both DsRed and Luc, compared to <1% for cells nucleofected with transposon but no source of transposase (Fig. 19.2). The requirement for transposase in achieving stable transgene expression is interpreted as evidence for SB-mediated transposition in these mMAPC cultures.

We have also determined the integration efficiency of SB transposase in MAPC derived from the bone marrow of newborn rat (post-natal days 2–5) (53). For these studies, undifferentiated rat MAPC (rMAPC), were nucleofected with a transposon vector conferring expression of both neomycin phosphotransferase (NEO) and GFP, along with a 1:1 mass ratio of cytomegalovirus regulated SB transposase expression plasmid. Using these conditions, the transfection efficiency ranged from 15%–20% by flow cytometric analysis of GFP positive cells 1 d after transfection. G418-resistant colony formation was increased over control cells transfected with transposon alone by 30-fold when SB cotransfected MAPC were allowed to expand from distinct colonies or when pooled drug resistant clones were subcultured to maintain cell density between 100–500 cells/cm^2.

We have also used SB to introduce a spliceosome-mediated RNA transplicing (SMaRT) expression cassette designed for homologous repair of a nonsense mutation in endogenous DNA-PKcs transcripts of mMAPC from severe combined immunodeficient (SCID) mice (54). In these studies, SCID mMAPC were nucleofected with a SMaRT-encoding transposon along with a second

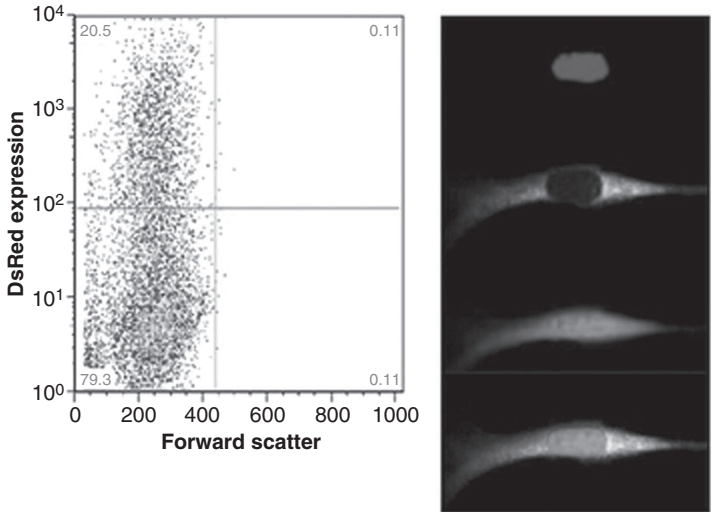

Figure 19.2. Generation of mouse MAPC exhibiting stable expression of DsRed and luciferase. Mouse MAPC were mixed with two independent transposons encoding DsRed or Luciferase and nucleofected along with pCMV-HSB2 as a source of transposase. Gene transfer was demonstrated by flow cytometry of DsRed expressing cells (dot plot). Individual cells exhibiting the highest levels of DsRed expression were sorted into new cultures and expanded. Immunohistochemical analysis of clonal DsRed positive cells was performed using chamber slides to demonstrate coexpression of luciferase (panel). From top to bottom; DAPI (blue), FITC conjugated antibodies specific for luciferase (green), DsRed (red). Cells coexpressing both DsRed and luciferase appear yellow (merge). Adapted from reference 52, with permission. (See color insert.)

transposon encoding DsRed both with and without a CMV-regulated SB transposase expression vector. Two or three days later, the samples were bulk sorted based on expression of DsRed and allowed to expand in culture. In this genetic background, radiation resistance and expression of the full length DNA-PKcs protein was observed only when the SMaRT-encoding transposon and transposase were codelivered to cells. These results demonstrate the necessity of transposase coexpression in order to achieve stable integration and persistent transgene expression in MAPC.

Transgenic MAPC Maintain Stem Cell Character and Ability to Differentiate

Maintenance of cytogenetic stability and developmental primitivity are essential in the genetic engineering of MAPC. In the studies described above, the majority of evaluated mitotic chromosome spreads were diploid with a normal karyotype for both unmanipulated MAPC and cells that had undergone stable

SB transposon integration. Transcript levels for two common markers of multipotency (Oct4 and Rex1) were similar for the genetically engineered cells when compared to unmanipulated MAPC. These results indicate that SB-mediated transgenesis did not affect genomic stability or stem cell character of MAPC. Transgenic mMAPC demonstrated persistent expression upon infusion into immunodeficient mice as demonstrated by whole body bioluminescence imaging and fluorescence microscopic analysis of tissue sections for expression of luciferase or DsRed2 respectively (55).

We also evaluated SB-transposed rat MAPC functionally by the capacity for multilineage differentiation (52, 53). Undifferentiated MAPC engineered for stable expression of DsRed2/Luc or G418-resistance using SB were maintained in culture for differentiation into endothelium, astrocytes, or hepatocytes as previously described (2% or 5% serum and vascular endothelial growth factor to induce endothelial differentiation; basic fibroblast growth factor for astrocyte formation; 2% serum, hepatocyte growth factor, and fibroblast growth factor 4 for hepatocyte differentiation) (40, 41). Following cytokine-mediated differentiation, lineage specific mMAPC stained positive for expression of endothelial markers (von Willebrand factor and CD31), the astocyte marker glial fibrillar acidic protein, or hepatocyte markers (albumin and hepatocyte nuclear factor 1; HNF-1) (52). Rat MAPC induced to differentiate under similar growth conditions were positive for expression of endothelial (Flk-1, Flt-1) or hepatocyte (alpha-fetoprotein, TTR) specific messages (53). These results indicate that SB-mediated transposition did not negatively affect the ability of MAPC to differentiate into multiple cellular lineages in culture or engraft in immunodeficient recipient animals.

Molecular Analysis of SB Transposon Integrants in Isolated MAPC clones

To confirm that stable gene expression was the result of SB transposase-mediated integration into the host cell chromosome, several independent integration events were characterized at the sequence level using splinkerette (56) or inverse (57) polymerase chain reaction techniques to recover transposon-chromosome junction sequences. Recovered sequences demonstrated the hallmark of transposition characterized by precise integration of the transposon-encoded sequence and duplication of a target TA dinucleotide (52, 53). Southern blot hybridization indicated an average of six SB transposon integrants per rMAPC cell genome. This value was threefold higher than previously described for SB10-mediated insertions in cultured human fibroblasts (1–2 transposon integrants per clone) (7, 58, 59) and may be the result of the increased gene loading efficiency of nucleofection-based delivery or an increased frequency of TA-dinucleotide target sites accessible for transposon-mediated insertion in undifferentiated cell types.

In conclusion, we found the SB transposon system capable of delivering exogenous sequences conferring stable expression of reporter genes in MAPC. SB mediated the precise integration of exogenous sequences with varying copy numbers. SB-modified MAPC were karyotypically normal and

maintained their stem cell character, demonstrated by the ability to differentiate into endothelium, astrocytes, or hepatocytes. SB-mediated transposition thus provides an effective nonviral approach for the genetic engineering of MAPC with potential utility in the study of MAPC biology and therapeutic application.

SB-MEDIATED TRANSPOSITION IN EMBRYONIC STEM (ES) CELLS

ES Cells

Embryonic stem (ES) cells can be maintained in the undifferentiated state during extended *in vitro* culture while maintaining a normal karyotype and the capacity to generate all cells and tissues of the adult body. These characteristics provide a unique opportunity for genetic and developmental studies. Culture methods have been developed to support differentiation of human ES cells into blood (60), endothelium (61), cardiomyocytes (62), neural-glial cells (63), hepatocytes (64), pancreas (65), and others (66, 67). Lineage-specific differentiation of ES cells is mediated by exposure to combinations of exogenous cytokines, growth factors, matrix proteins, or stromal cells. The ability to introduce and regulate the expression of specific genes in ES cells has permitted the identification and isolation of specific cell lineages and provides an approach for defining the role of transcription factors, signaling molecules, and cellular proteins during lineage-specific commitment and differentiation.

Testing of SB-mediated transposition in ES cells was previously limited to studies in mouse ES cells. SB activity was demonstrated by excision (68), the first step of the transposition process, and by excision coupled with reintegration (69) of a transposon positioned at a specific site in the genome when SB transposase was provided in *trans.* A gene-trap transposon vector lacking an internal expression cassette was employed to demonstrate that transposition could be enhanced by *in vitro* methylation of the transposon-encoded sequence prior to codelivery with transposase (68). Evidence also suggested that the chromatin status of the transposon can significantly affect the efficiency of excision from a chromosomal locus. However these studies were not designed to address the efficiency of SB-mediated stable gene transfer upon delivery of a transposon vector and a transposase expression vector into ES cells in *trans*. As described below, we tested SB-mediated generation of transgenic human ES cells and evaluated the maintenance of expression following *in vitro* and *in vivo* differentiation conditions.

SB-Mediated, Stable Gene Expression in Human ES Cells

To evaluate the efficiency SB-mediated gene transfer in human ES cells we used nucleofection to deliver a transposon containing a fusion of the GFP and

zeocin (Zeo) antibiotic resistance genes into undifferentiated human H9 cells, with or without a separate plasmid encoding SB transposase (70). One week after nucleofection, we observed approximately 5% GFP positive cells for those samples coadministered transposase-encoding plasmid. After 1 month of selection for zeocin resistance, nearly 80% of human ES cells cotransfected with transposase remained undifferentiated and expressed GFP while cells that were treated with the transposon alone did not yield any zeocin resistant colonies, indicating that transposase was required for stable transgene expression.

The stability of expression from integrated SB cargo sequences was also demonstrated by bioluminescence imaging of firefly luciferase (luc) expression following differentiation of engineered human ES cells under *in vitro* and *in vivo* conditions (70). Luc-encoding transposons were delivered with or without a source of transposase consisting of either an SB-encoding plasmid or *in vitro* transcribed transposase-encoding messenger RNA. Upon continuous culture following nucleofection, human ES cells exhibited luc expression only if coadministered transposon and transposase encoding sequences. Luciferase expression was stable for 5 months during the process of manually isolating and expanding luc+ colonies, and was not affected by the use of a CpG containing promoter (Fig. 19.3a). Both luciferase and GFP expressing ES cells maintained their undifferentiated phenotype as demonstrated by immunohistochemical staining for coexpression of SSEA-4, Oct-4, and Tra-1-60 with luc and by flow cytometric analysis for coexpression of SSEA-4 and GFP, demonstrating that SB-mediated transposition did not perturb the primitive state of undifferentiated human ES cells (Fig. 19.3b).

Stable Expression after *In vivo* and *In vitro* Differentiation of Human ES Cells

Human ES cells are able to differentiate into all three germ layers (endoderm, mesoderm, and ectoderm). To determine the stability of gene expression after differentiation *in vivo*, human ES cells engineered to express luc were injected into the hind leg muscles of immunodeficient mice. An increase in the level of luciferase expression as determined by *in vivo* bioluminescence imaging was observed during the course of teratoma formation. Histological analysis of teratomas harvested at least 3 months after injection revealed tissues representing all three germ layers including neuron (ectoderm), cartilage (mesoderm), and glandular epithelium (endoderm) (70). Immunofluorescence staining confirmed the presence of luc protein within all three germ layers of the differentiated tumor, demonstrating that the SB-engineered human ES cells retained their pluripotent differentiation capacity and sustained gene expression during *in vivo* differentiation.

We also tested the stability of gene expression following *in vitro* differentiation of SB-engineered human ES cells into hematopoietic cells. Luciferase-expressing ES cells were either cultured in suspension for embryoid body (EB)

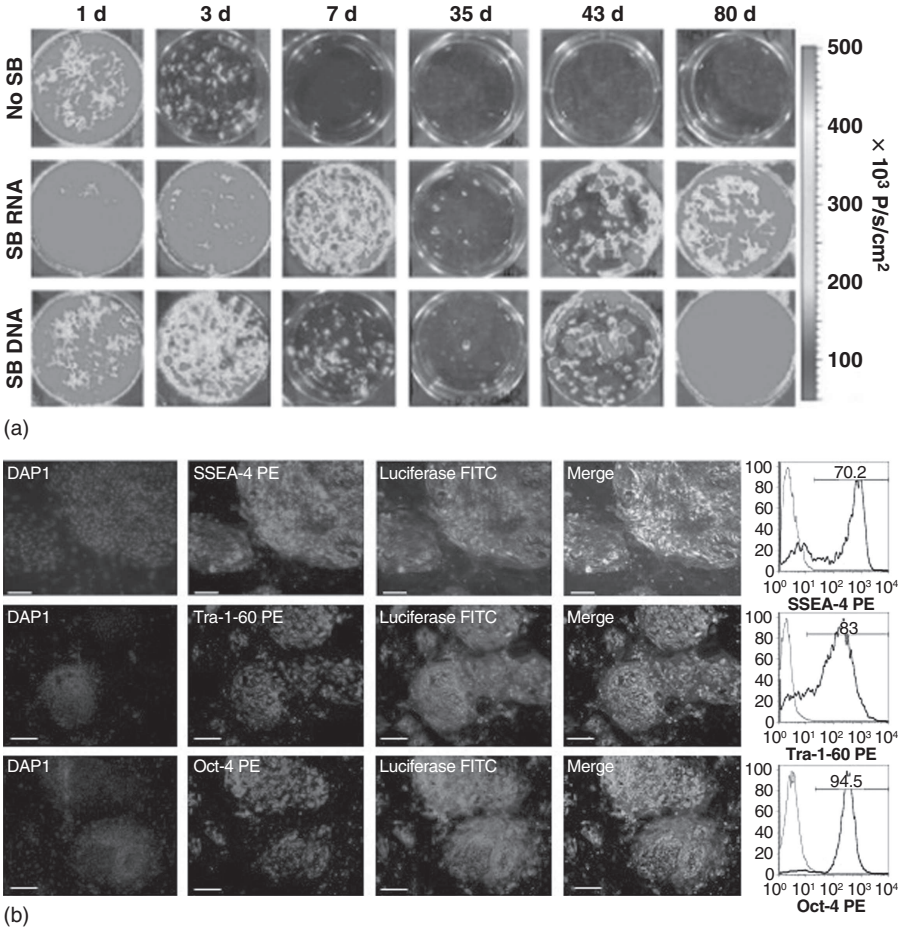

Figure 19.3. Generation of human ES cells exhibiting stable expression of luciferase. (a) Stable expression of luciferase. Human ES cells were nucleofected with SB transposons encoding luciferase along with either no SB transposase (top row, negative control), rU-SB11-U (+SB RNA, middle row), or pPGK-SB11 (+SB DNA, bottom row) as a source of transposase. Luciferase expression was monitored as evidence for transfected cells by bioluminescence imaging. After 1 month of continuous culture, colonies demonstrating the highest levels of luciferase activity (spots pseudo-colored red) were manually transferred to new cultures and expanded. Examples of bioluminescence images obtained from human ES cells maintained in culture for 2–3 months (8–12 passages) under these conditions are depicted. (b) Luciferase positive colonies remain undifferentiated. Immunohistochemical analysis of luciferase positive colonies cultured with MEFs using chamber slides and stained with DAPI (blue; panel 1), PE conjugated antibodies specific for SSEA-4, Tra-1, or Oct-4 (red; panel 2) also demonstrated by flow cytometry (histograms; far right), and FITC-conjugated antibodies against luciferase (green; panel 3). Cells coexpressing both SSEA-4 and luciferase appear yellow (merge; panel 4). Scale bars, 100 μm. Adapted from reference 70, with permission. (See color insert.)

formation or cocultured with S17 stromal cells (60), and the resultant CD34+ cell populations were sorted and grown in methylcellulose-based cultures for hematopoietic colony formation. Luciferase activity was observed in colonies of mature hematopoietic cells as well as in whole populations of cells after differentiation by coculture with S17 cells, in CD34+ cells sorted from the S17-differentiated cell population, and in CD34+ cells sorted from EBs confirming the maintenance of luc expression after hematopoietic differentiation. Furthermore, SB-engineered cells terminally differentiated into CD45 + CD56+ natural killer (NK) cells were also positive for luc by bioluminescence imaging, thus demonstrating maintenance of transgene expression after differentiation of SB-engineered human ES into both myeloid and lymphoid lineages.

Molecular Analysis of Integration in Transgenic hES Cells

To distinguish between transposition and random integration in SB-transfected ES cell clones, we used a plasmid rescue technique to recover complete transposon integrants along with flanking sequences. We found that for 98% of the recovered transposon integrants there was no evidence of flanking plasmid sequence, suggesting a low frequency (2%) of random integration. Direct sequencing of several recombinants revealed integration of transposon sequences into human chromosomes and duplication of a target TA dinucleotide, thus confirming that stable gene expression observed in human ES cells was predominantly due to transposition.

We conclude that SB-mediated transposition is an effective means of integrating new gene sequences into human ES cells, resulting in reporter gene expression that is robust and maintained in the undifferentiated state as well as after differentiation into tissues of all three germ layers *in vivo*, and in particular after differentiation into hematopoietic cells *in vitro*. SB thus provides a nonviral approach for genetic engineering of human ES cells that can be applied to the study of ES cell biology and in the development of ES cells for therapeutic applications.

CONCLUSION

As described in the sections above, the SB transposon system provides a nonviral means of integrating new DNA sequences into several different stem cell types (summarized in Table 19.1). These newly integrated sequences can be used for marking and tracking stem cells during growth and differentiation, to evaluate the role of expressed gene products in the maintenance of stem cell primitivity or in differentiation, or for expression of gene products contributing to therapeutic potential of the stem cell population or of differentiated progeny. While retroviral and lentiviral vectors provide high levels of stable gene transfer in stem cell populations (1–4), the SB transposon system provides a nonviral alternative to integrating defined sequences into stem cell

TABLE 19.1. Sleeping Beauty-Mediated Gene Delivery Applications in Adult and Embryonal Stem Cells

Target (Species)	Transposon/ Transposase	Mass Ratio	Gene(s)	Model	Result	Reference
MSC (Mouse)	T2/CMV-SB	25:1	DsRED/Luc	Wild type	10%	(Tolar et al., unpublished data)
MAPC[a] (Mouse)	T/CMV-SB	50:1	DsRED/Luc	Wild type	8% dsRED/Luc+	(52)
MAPC[b] (Mouse)	T2/CMV-SB	20:1 10:1	Splicesome/DsRed	DNA-PKcs-SCID	Radiation resistant DNAPKcs protein	(54)
MAPC (Rat)	T2/CMV-SB	1:1	NEO/GFP	Wild type	30-fold increase in G418-resistance	(Wilber et al., unpublished data)
ES (Human)	T2/PGK-SB or SB mRNA	1:1 1:2	GFP:Zeo/Luc	Wild type	80% GFP/Zeo-resistant stable 5 months	(70)

Gene transfer was achieved by nucleofection (Amaxa, Gaithersburg, MD) using the following buffers and settings: MSC buffer T, setting T-20; [a]mMAPC buffer T, setting T-20; [b]mMAPC buffer mouse ES, setting T-20; rMAPC butter V, setting U-20; hES buffer mouse ES, setting A-27. Nucleofected cells were immediately resuspended in prewarmed (37 °C) complete growth medium and plated for continuous culture.

genomes that may be attractive for several reasons. Use of the SB system provides integration of defined sequences without the necessity of viral vector packaging, leaving the investigator less constrained with respect to vector packaging requirements and the susceptibility of vector genome RNA to processing and reverse transcription errors (71–74). SB transposon integrants appear to be more randomly distributed than retroviral and lentiviral vectors, for which integration is more closely associated with gene sequences (6, 75–78). Finally, the use of SB transposons rather than retroviral or lentiviral vectors avoids the risk of host cell contamination with replication competent vector that can be generated by recombination between viral vector and packaging plasmids (79).

SB's capability of integrating into the chromosomes of MSC, MAPC, and ES cells provides access through genetic engineering and differentiation to a variety of different tissues including bone, cartilage, muscle, neurons, endothelium, and hematopoietic cells. SB's capability in mediating integration and long-term expression in liver (80, 81), lung (82), and endothelium (83) *in vivo* implies effectiveness of the transposon to integrate into more differentiated cells of these tissues. SB has also been demonstrated to mediate integration and long-term expression in human T lymphocytes (84). Direct nonviral gene transfer into hematopoietic stem cells capable of long-term reconstitution in animals, however, has so far been elusive using SB or any other nonviral recombinase system. Characterization of effective conditions for DNA loading and transposon integration in hematopoietic stem cells will thus constitute a key area of development in this target population, important for basic studies of hematopoietic stem cell engraftment and differentiation as well as clinical applications of genetically engineered hematopoietic stem cells.

Another target cell population for genetic engineering that is of emerging importance is the recent development of induced pluripotent stem cells (iPS), generated from adult fibroblasts by transduction using retroviral vectors encoding a combination of four different gene products (e.g., Oct-4, Sox2, Klf4, and c-Myc [85–88]). iPS cells exhibit many of the characteristics of embryonic stem cells, such as multilineage differentiation potential and thus the capability of generating essentially any cell type. iPS cells can thus be used to generate a primitive cell population from differentiated adult cells, genetically engineered to express a therapeutic gene product, differentiated into the appropriate cellular lineage, and engrafted to correct a genetic deficiency in an animal model of a human disease (e.g., sickle cell anemia [89]). Genetic engineering, including the use of nonviral integrating vectors such as SB transposons, will play an essential role in the biological characterization of iPS cells and their use in the development of new cellular therapies.

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PHIC31 INTEGRASE FOR MODIFICATION OF STEM CELLS

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The integrase from the *Streptomyces* temperate phage ϕC31 possesses several properties that are useful for modification of stem cells. First, ϕC31 integrase carries out unidirectional recombination reactions, thus making the insertion of plasmid DNA efficient and permanent in dividing cells. Second, ϕC31 integrase reactions occur at preferred sites in a sequence-dependent fashion and thus may be safer than random integration for use in combined gene and cell therapy. Third, ϕC31 integrase imposes no size limit on the DNA plasmid to be integrated into the genome, allowing the insertion of large or multiple genes. Fourth, ϕC31 integrase has been shown to produce robust and long-term expression of the integrated gene in diverse mammalian cell types. In this chapter, we examine these properties of ϕC31 integrase, review some of the recent studies that have used ϕC31 integrase in stem cells, and discuss the potential future use of the ϕC31 integrase system and its advantages for modification of stem cells.

PROPERTIES OF ϕC31 INTEGRASE IN MAMMALIAN CELLS

Unidirectional Recombination Reaction Mechanism

ϕC31 integrase, encoded by the *Streptomyces* temperate phage ϕC31 (1, 2), is a member of the serine recombinase family (3, 4). In nature, ϕC31 integrase

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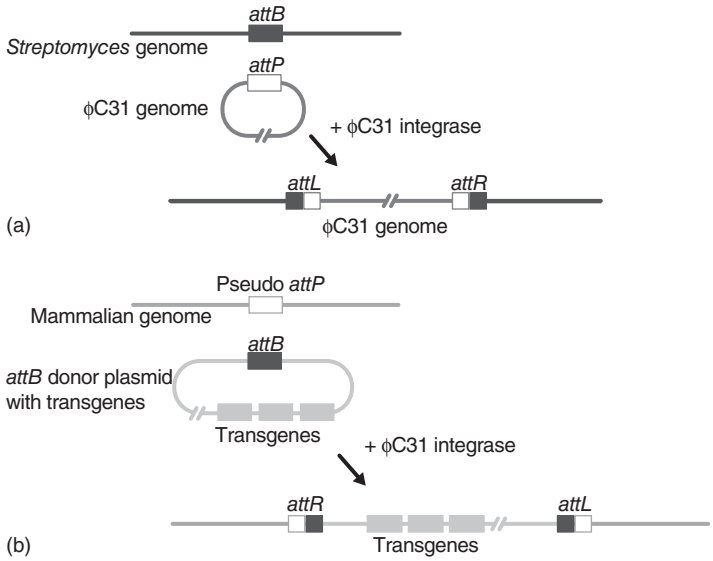

Figure 20.1. ϕC31 integrase-mediated unidirectional recombination reactions. (a) In nature, ϕC31 integrase performs recombination between an *attP* site in the ϕC31 phage genome and an *attB* site in the *Streptomyces* genome, producing hybrid sites, *attL* and *attR* that the enzyme cannot use for the reverse reaction without a cofactor (5). (b) In unmodified mammalian cells, ϕC31 integrase performs recombination between a native pseudo *attP* site in the mammalian genome and an *attB* site on the *attB* donor plasmid carrying the transgenes to be integrated.

carries out a recombination reaction between its attachment site on the phage, *attP*, and the corresponding attachment site on the host bacterial genome, *attB*. This recombination results in the insertion of the phage genome into the host genome (Fig. 20.1a) (1, 2). Unlike Cre and Flp, which are members of a separately evolved tyrosine recombinase family and are resolvases that mediate recombination between identical sites (3), ϕC31 integrase carries out its reaction between two distinct sites, producing hybrid sites, *attL* and *attR*, that the enzyme cannot use for the reverse reaction without a cofactor (5). Also, unlike the λ integrase, a tyrosine recombinase (6), ϕC31 integrase does not require host-specific cofactors to carry out its integration reaction (5). These properties make ϕC31 integrase useful for integrating plasmid DNA into mammalian genomes.

Gel mobility assays using ϕC31 integrase and biochemical studies of the γδ resolvase, a related serine recombinase, have suggested that ϕC31 integrase works as a tetramer (7–9). A dimer first binds to each attachment site. Then two dimers, one that has bound *attB* and another that has bound *attP*, come together to form a stable synapse and carry out the recombination reaction (8, 9). The minimal core sequences for *attP* and *attB* in *E. coli* are 39 bp and

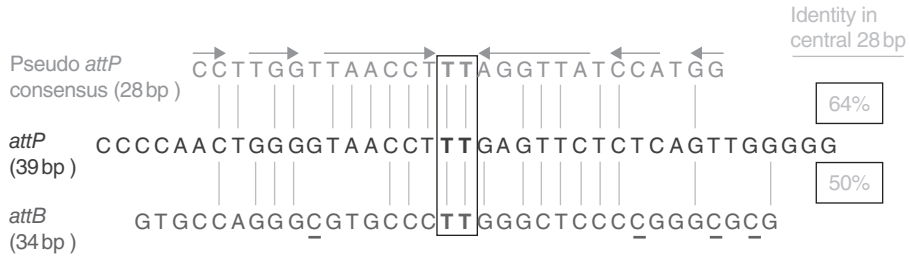

Figure 20.2. ϕC31 *att* site sequences. The 28-bp consensus pseudo *attP* sequence revealed from a detailed analysis of integration sites in human cells (45) is shown at the top. The arrows indicate the palindromic bases. The consensus sequence is 64% identical to the 39-bp minimal ϕC31 *attP* sequence (10) shown in the middle. The 34-bp minimal ϕC31 *attB* sequence (10) is shown at the bottom. Underlined cytosine bases denote the four potential CpG methylation sites in the minimal *attB* sequence. Bases identical between adjacent sequences are noted with line segments between the bases. Percent identities between the adjacent sequences in the core 28-bp regions are noted to the right of the sequences.

34 bp in length, respectively (10). The attachment sites, *attP* and *attB*, share only 50% identity, and each is partially palindromic around its TT core (Fig. 20.2). Although ϕC31 integrase can bind to *attL* or *attR*, the tetrameric complex required for the reverse recombination reaction cannot assemble without a cofactor (8). As a result, the ϕC31 integrase-mediated reaction is unidirectional, and integration events are stable.

Functionality at Pseudo *attP* Sites

ϕC31 integrase was first shown to be functional in mammalian cells by using extrachromosomal assays in human 293 embryonic kidney cells (10). In a subsequent study, ϕC31 integrase was shown to mediate the insertion of *attB*-containing plasmids at previously placed *attP* sites in the genomes of human 293 and mouse 3T3 embryonic fibroblast cell lines (11). Interestingly, the ϕC31 integrase-mediated increase in integration frequency of *attB*-containing plasmids was greater for cell lines with previously inserted *attP* sites than for *attP*-containing plasmids into cell lines with previously inserted *attB* sites (11). In cell lines with previously inserted *attP* sites, there was an approximately 10- to 20-fold increase in integrations above random integration while in cell lines with previously inserted *attB* sites, there was an approximately twofold increase above random integration (11).

Of great interest for gene therapy, ϕC31 integrase was also found to mediate integration at native sequences, called pseudo *att* sites. Again, ϕC31 integrase-mediated integration of *attB*-containing plasmids at native pseudo *attP* sites occurred at a higher frequency, approximately 5- to 10-fold above random

integration, than *attP*-mediated integration at native sites with sequences resembling *attB*, less than twofold above random integration (11). Such preference by ϕC31 integrase for pseudo *attP* sites may stem from the lack of CpG dinucleotide sequences in *attP* while *attB* has four CpG dinucleotide sequences. Such sequences are rare in mammalian genomes (12) and are potential targets for methylation, which may inhibit the integrase reaction (Fig. 20.2).

The ϕC31 integrase system involves cotransfection of an *attB* donor plasmid carrying the transgenes to be integrated and a plasmid expressing the ϕC31 integrase (Fig. 20.1b). The *attB* donor plasmid typically contains ~240 bp of *Streptomyces* sequence surrounding *attB* in addition to the minimal *attB*, since it has been demonstrated that the surrounding sequence positively affects the integration frequency in human cells (10).

Serine recombinases from other phages have been tested to determine whether they can function in mammalian cells in a similar fashion to ϕC31 integrase (13–16). Although these enzymes are functional on their own *att* sites in mammalian cells, ϕC31 integrase has proven to be the most efficient and precise in performing the integration reaction on unmodified mammalian genomes (13–16).

ϕC31 integrase has been shown to efficiently integrate *attB*-containing plasmids at pseudo *attP* sites in many different cell types and in diverse organisms (17). Studies demonstrating the utility of the ϕC31 integrase system encompass gene therapy strategies using *ex vivo* transfection of human primary epidermal keratinocytes (18, 19), human primary fibroblasts (20), human T cell lines (21), and mouse muscle-derived stem cells and human primary muscle precursor cells (22, 23). Furthermore, gene therapy strategies using *in vivo* transfection of mouse liver (24–26), rabbit retina (27, 28), rabbit joints (29), mouse lungs (30), and mouse muscle (31, 32) have been reported. ϕC31 integrase may be beneficial for high-level production of recombinant proteins in Chinese hamster ovary cells (33), and the enzyme has been useful for generation of transgenic organisms, including *Drosophila* (34–38), *Xenopus laevis* (39, 40), the mosquito *Aedes aegyptus* (41), and mice (13, 42, 43). As well, the ϕC31 integrase system has recently been shown to work in mouse neural stem cells and human embryonic stem (ES) cells (44).

Analysis of Integration Sites

A detailed analysis of integration sites used by ϕC31 integrase in three different human cell lines, 293, D407 (retinal pigment epithelium), and HepG2 (hepatocyte) shed light on the sequence specificity of the integration reaction (45). Based on 19 integration sites that were observed multiple times independently and that accounted for 56% of integration events in these cell lines, a consensus motif for pseudo *attP* sites was constructed by bioinformatics analysis (Fig. 20.2) (45). The 28-bp consensus motif is partially palindromic and shares 64% identity with wild-type *attP* (Fig. 20.2) (45). Based on bioinformatics analysis, 48% of the remaining integration sites that were observed only

once in these cell lines also possessed the consensus motif (45). Therefore, at least 80% of integration events appeared to have occurred in a sequence-specific fashion. The finding that all the observed integration events involved the *attB* sequence of the donor plasmid suggested that the integrations were carried out by ϕC31 integrase and were distinct from random integration (45).

The finding that there were more genes near ϕC31 integration sites than near randomly chosen sites (9 genes vs. 4.6 genes per 500 kb) and that genes around frequently used pseudo *attP* sites were more likely to be expressed in at least some cell types (45), was consistent with the possibility that differences in patterns of gene expression may cause differences in patterns of integration sites available for use in different cell types. Consistent with this idea was the observation that a site that was detected only once and was one of among 57 integration sites found in the 3T3 cell line (11) was a predominantly used integration site (called mpsL1) in mouse liver, as detected in *in vivo* gene therapy studies (25, 26).

The analysis of pseudo *attP* sites in the three human cell lines 293, D407, and HepG2 found the distribution of integration sites to be 36.8% within introns, 1.9% within exons and 61.3% in intergenic regions (45). This distribution reflected a modest preference toward transcription units, since approximately 24.4% and 1.1% of the human genome are introns and exons, respectively, while 74.5% is intergenic (46, 47). This distribution was congruent with the finding that ϕC31 integration sites were in regions of relatively high gene density (45). For integration sites observed multiple times, 27.8% were in introns and 72.2% were intergenic, with no such integration site in an exon (45). In contrast, only 28.2% of integration sites were found to be intergenic in a study using 293, Huh7 human hepatoma, HC116 human colon carcinoma, and Hep1A mouse hepatoma cell lines, with 3.5% (3 out of 85) of the sites in exons (48). In a recent analysis of pseudo *attP* sites in human ES cells, 8 out of 23 (34.8%) sites were intergenic, with two sites (8.7%) in exons (44).

For any particular pseudo *attP* site, insertion can occur in either direction (45), presumably due to the palidromic nature of *att* sites. Based on many studies using the ϕC31 integrase, it is thought that a single integration event per cell, resulting in a single copy of the integrated plasmid, accounts for the vast majority of the cells in which integration events occur. Cells in which two integration sites may have occurred have been reported, but it is not clear if they were independent clones (44).

As would be expected if patterns of gene expression underlie pseudo *attP* site availability, differences in integration sites have been found in different cell types. For example, while the integration site at 19q13.31 was used at a high frequency in 293, D407, and HepG2 cells, the integration site at 3q26.31 was found at a high frequency (6 out of 44 colonies) only in HepG2 and not in the other two cell types (0 out of 42 for D407 and 0 out of 31 for 293) (45). Also, a frequently used integration site at 8p22 in human primary epidermal progenitors (19) was not detected in analyses of integration sites used in D407

cells (45), a human T cell line (21), and human ES cells (44). It is also interesting that among the 23 pseudo *attP* sites found in human ES cells, 18, including the two most frequently used sites at 6p11.2 and 13q32.3, had not been detected previously (44).

Although the number of potential pseudo *attP* sites in the human genome has been estimated to be in the hundreds (95% confidence intervals corresponding to 202–764 sites) (45), the actual number of pseudo *attP* sites used in any particular cell type appears to be more restricted. In a study of three human cell lines, only 19 sites accounted for 56% of integration events (45). In human ES cells, fewer than 10 hotspots accounted for 30%–60% of integration events (44). Compared to retroviral vectors that integrate with little sequence-specificity and compared to a transposon system such as *Sleeping Beauty* which requires only a TA dinucleotide sequence (49), the number of potential integration sites for ϕC31 integrase is several orders of magnitude fewer (<1000 for ϕC31 integrase compared to $>10^7$ for *Sleeping Beauty* [50]). The relative ease with which one can characterize and use hotspots that are appropriate for a particular cell therapy may be a critical advantage for the ϕC31 integrase system, since cases of tumorigenesis have been reported for therapeutic approaches using randomly integration retroviruses (51–53).

Lack of Size Limit

An advantage of the ϕC31 integrase system is that there is no limit to the size of the plasmid to be integrated. For example, bacterial artificial chromosomes of sizes up to 133 kb have been inserted with the use of the ϕC31 integrase system to create transgenic *Drosophila* (38). This lack of a size limit is in contrast to retroviral vectors and lentiviral vectors, which allow chromosomal integration of transgenes of sizes up to only about 8 and 10 kb, respectively (54), due to the packaging limit of the virus. Also, an increase in the size of the transposon reduces the efficiency of the *Sleeping Beauty* system (55, 56). One study reported that with a 9.1-kb transposon, the integration efficiency was not any higher with the *Sleeping Beauty* transposase than without (56). For diseases involving large genes such as dystrophin, which is mutated in Duchenne muscular dystrophy, the benefit of having no size limit can be critical. The ϕC31 integrase system has been used to integrate the full-length human dystrophin cDNA (~11 kb) for correction of muscle stem cells, followed by transplantation (23). Another potential benefit of having no size limit is that one can integrate multiple genes in a cell, using just one *attB* donor plasmid.

Safety

Unlike the absolutely precise recombination reaction between wild-type *attB* and *attP*, ϕC31 integrase-mediated insertion of an *attB* donor plasmid at a pseudo *attP* site often resulted in small deletions (25 bps or less) nearby the crossover junction (11, 44, 45). Chromosomal rearrangements and large dele-

tions also occurred in cultured cells, at a frequency of approximately 10% (45). Another study found the frequency of such aberrant events to be as high as 15% (48). Consistent with these observations, long-term expression of ϕC31 integrase may result in abnormal karyotypes in primary human fibroblasts *in vitro* (57). However, to date, there is no evidence for ϕC31-generated aberrant chromosomal events *in vivo*, as opposed to the artificial conditions of cell culture. *In vivo* expression of ϕC31 integrase, even prolonged expression in some studies, has not produced detectable adverse effects in organisms.

The strongest evidence for this tolerability of ϕC31 integrase in mammalian cells is that two different strains of mice genetically engineered to constitutively express ϕC31 integrase in all their cells developed normally and were fertile (42, 43). Also, *Drosophila* and *Xenopus* embryos in which ϕC31 integrase was expressed transiently developed normally (37, 39, 40). Hydrodynamic transfection of hepatocytes in mice deficient in fumarylactoacetate hydrolase ($FAH^{-/-}$) resulted in transient dysplasia of the cells that was more prevalent with ϕC31 integrase, but the dysplasia disappeared within 90 d (25). No tumors or other adverse effects were detected. A similar dysplasia has not been found in gene therapy studies using mice with normal livers (24, 26), so this effect may be specific to the toxic environment in $FAH^{-/-}$ animals. Based on measurement of liver enzymes and histology, ϕC31 integrase showed no toxic effects on normal liver (24).

Increased tumorigenesis has not been found in any studies using the ϕC31 integrase system (24–26). Furthermore, a detailed analysis of integration sites used by ϕC31 integrase in human cells did not reveal any integration sites that would obviously contribute to carcinogenesis (45). The study found three pseudo *attP* sites that were within 1 MB of human genes rarely implicated in cancer (45). However, these pseudo *attP* sites were nearby genes that were linked to carcinogenesis only if translocations with particular partner genes occurred (45), and pseudo *attP* sites near those partner genes were not found (45). Also, these three pseudo *attP* sites were found only once each, indicating low frequencies of integration at these sites (45).

This evidence suggests that ϕC31 integrase is well tolerated by mammalian cells and unlikely to be toxic. In particular, in stem cell studies where ϕC31 integrase is used transiently *in vitro*, it is easy to avoid any rare cells with chromosomal abnormalities by screening the cells and using only clones with a normal karyotype.

Efficient Integration and Robust Long-Term Expression

A potential challenge in using the ϕC31 integrase system is the transfectability of the target cells. However, the system does not require a particular transfection method. Any method of transfection, such as use of lipophilic reagents (11, 18–20, 29, 45), nucleofection (22, 23, 58), or electroporation (21, 27, 31, 32), can be applied. It is recommended that the transfection method be optimized to increase the transfection efficiency.

The efficiency of integration by ϕC31 integrase varies depending on the cell type (11) and other experimental conditions, including the amounts of the *attB* donor plasmid containing the transgene and the plasmid expressing the integrase (25, 29). Integration frequency per transfected cell is often about 0.1%–1.0% *in vitro* (11), but has been observed to be up to 20% in some conditions. For example, approximately 3.6% of transfected $FAH^{-/-}$ hepatocytes in an *in vivo* study underwent integration (25), and this frequency is higher in normal liver (A. Keravala and M. P. Calos, unpublished data). Although a higher efficiency appears to be desirable, the efficiency of the currently used ϕC31 integrase is often adequate. Furthermore, antibiotic selection can be used to obtain either clones or pools of cells that likely have integrated the *attB* donor plasmid. New integrase variants with increased efficiency and specificy are under development (59).

Likely due to the tendency of ϕC31 integrase-mediated integration to occur in regions of gene expression (45), the ϕC31 integrase system has been effective in producing robust, long-term expression of integrated transgenes. Gene therapy studies using *in vivo* transfection of mouse liver (24–26), rabbit retina (27, 28), mouse lungs (30), and mouse muscle (31, 32) in combination with the ϕC31 integrase system, have all resulted in long-term expression of the integrated transgenes. In the cases of mouse liver, when human coagulation factor IX gene was integrated by the ϕC31 integrase after transfection of the liver by high-pressure tail vein injection, expression of human factor IX at a level similar to normal factor IX levels persisted throughout the 250-d experiment, even after partial hepatectomy (26). At the 250-d time point, the serum level of human factor IX introduced with integrase was ~16-fold higher than without integrase (26). After partial hepatotectomy at day 100 to stimulate cell division and loss of unintegrated DNA, the serum level at day 250 was >100-fold higher with integrase than without (26). Similarly, in rabbit retina, the expression of the luciferase transgene persisted for the 4.5-month duration of the experiment and, at the conclusion of the experiment, was at a level ~85-fold above that found without the use of the integrase (27). In mouse lungs, expression of the luciferase transgene fell to background levels after only 2 weeks without the use of ϕC31 integrase, but persisted at twofold above background levels beyond 250 d after cotransfection with integrase (30). In muscle, enhanced transgene expression by the use of the ϕC31 integrase continued throughout the lifetime of the mice for greater than 2 years (31).

Studies using *ex vivo* transfection of human primary epidermal keratinocytes (18, 19), human primary fibroblasts (20), human T cell lines (21), mouse muscle-derived stem cells and human primary muscle precursor cells (22, 23), mouse neural progenitor cells (58), and human ES cells (44) in combination with the ϕC31 integrase system have all shown long-term expression of therapeutic or marker transgenes, either *in vitro* or, in some studies, both *in vitro* and *in vivo* after transplantation (18–20, 23).

For human primary epidermal keratinocytes, the 8.9 kb Type VII collagen (*COL7A1*) cDNA was integrated into the genomes of keratinocytes isolated

from patients with the skin disorder recessive dystrophic epidermolysis bullosa (RDEB), which is caused by mutations in the *COL7A1* gene (19). RDEB cells were transfected with both the integrase plasmid and an *attB* donor plasmid expressing *COL7A1* cDNA. After 10 d of drug selection, >99% of the cells showed normal expression of Type VII collagen by immunocytochemistry (19). After transplantation onto immuno-deficient mice, the corrected RDEB cells regenerated histologically normal skin, and expression of Type VII collagen persisted for the duration of the 14-week experiment (19). Similar positive results were obtained when laminin 5 β3 (*LAMB3*) cDNA was integrated into the genomes of primary keratinocytes isolated from patients with the skin disorder junctional epidermolysis bullosa. When the corrected cells were transplanted onto immuno-deficient mice, histologically corrected skin was produced (18). Fibroblasts from RDEB patients that expressed *COL7A1* cDNA after genomic integration by the ϕC31 integrase have also been shown to correct the disease features of transplanted RDEB skin for 4 months (20).

To examine the utility of the ϕC31 integrase system to treat X-linked severe combined immunodeficiency (SCID-X1) caused by mutations in the common cytokine receptor γ chain (γ*c*) gene, a study was carried out with two human T-cell lines, Jurkat and ED40515(–) (21), which lacks γc expression (60). Pseudo *attP* sites used in the Jurkat cells were analyzed, and a previously unknown pseudo *attP* site at 18p11.2 was identified to be an active integration site in the cell type (21). ED40515(–) cells were transfected with an *attB* donor plasmid encoding the γ*c* gene, along with a plasmid expressing ϕC31 integrase. One of the stably transfected colonies was shown to have undergone integration at 18p11.2 (21). This clone expressed the γc protein robustly after 3 months of culture, as measured by flow cytometric analysis, and also possessed normal IL-2 signaling activity (21).

STUDIES USING ϕC31 INTEGRASE IN STEM CELLS

The possibilities of gene and cell therapies that use the patients' own stem cells, genetically corrected with the ϕC31 integrase system and transplanted back to the patient, are appealing. Studies of mouse muscle-derived stem cells and human primary muscle precursor cells (22, 23), mouse neural progenitor cells (58) and human ES cells (44) have begun to explore the possibilities of such therapeutics.

In the initial study using mouse muscle-derived stem cells (MD1 cell line derived from dystrophin-deficient *mdx* mice) and human primary muscle precursor cells, an *attB* donor plasmid encoding either enhanced green fluorescent protein (eGFP) or an eGFP-full-length-dystrophin fusion protein, in combination with the integrase plasmid, was used to test the ϕC31 integrase system in the cells (22). Either of the *attB* donor plasmids also allowed an antibiotic selection of colonies, and in the case of the eGFP, the use of the ϕC31 integrase system resulted in a 2.2-fold increase in the fraction of antibiotic-resistant MD1

colonies that also expressed eGFP and in a 15-fold such increase in the case of human primary muscle precursor cells (22). Also, the use of the ϕC31 integrase system resulted in human primary muscle precursor cells that were antibiotic-resistant, stably expressed eGFP-full-length-dystrophin, and were able to fuse into myotubes, while no antibiotic-resistant cells were found without the integrase (22). The use of a previously identified pseudo *attP* site at 8p22 in the human genome was verified by polymerase chain reaction (PCR) (22). This study was the first to show integration and stable expresion of the full-length *dystrophin* gene in human myogenic cells (22). In a later study, the use of the ϕC31 integrase system resulted in human muscle precursor cells that were integrated with an *attB* donor plasmid expressing an eGFP-full-length-dystrophin fusion protein and that were capable of fusing with muscle fibers after injection into the tibialis anterior muscle of immuno-deficient mice (23).

The ϕC31 integrase system has been used in mouse neural progenitor cells *in vitro* to provide stable long-term expression of the integrated gene without negative effects on the ability of the cells to grow and to differentiate into either neurons or astrocytes (58). The neural progenitor cells that received an *attB* donor plasmid encoding the luciferase gene and the wild-type ϕC31 integrase plasmid had a 39-fold higher level of luciferase expression at 8 weeks of culture, compared to the cells that received the same *attB* donor plasmid along with a plasmid encoding a nonfunctional variant of the ϕC31 integrase, mutated at the catalytic serine used during the recombination reaction (58). The comparisons were made between two pools of cells that were G418-resistant, presumably due to integration of the *attB* donor plasmid, which also encoded a neomycin resistance gene (58). Integration at the prominent mouse pseudo *attP* site, mpsL1, was verified by PCR in the ϕC31 integrase-modified pool of cells (58). When the numbers of proliferating cells measured by BrdU labeling were compared, there was no difference between the ϕC31 integrase-modified cells and the unmodified control cells (58). The ability of the ϕC31 integrase-modified cells expressing luciferase to grow and differentiate was examined, based on culture for 12 h in media containing BrdU, followed by culture for 10 d in differentiation media containing retinoic acid and a low concentration of growth factors. The ϕC31 integrase-modified cells exhibited no difference from the unmodified cells (58).

Recently, a detailed analysis of the pseudo *attP* sites used in human ES cells was carried out (44), as discussed above. The ϕC31 integrase system worked in human ES cells to produce clones with predictable expression patterns of the integrated transgene (GFP), depending on the promoter elements used to drive it (44). The ES-cell-specific human Oct4 promoter and the constitutive EF1α promoter were used (44). The human ES cell clones with ϕC31 integrase-mediated integration, showed stable expression of GFP with either promoter for at least 4–5 weeks in culture, with the EF1α promoter driving higher levels (44). When the human ES cell clones with Oct4-driven GFP underwent differentiation into the three germ layers of embryoid bodies, GFP expression ceased, as would be expected (44). In contrast, EF1α–driven GFP

expression continued after differentiation (44). This finding suggested that the expression of the integrated transgene could be controlled by the judicious use of promoter elements. It was further encouraging that the ϕC31 integrase system produced no negative effect on the ability of the human ES cells to differentiate, as no difference was found between embryoid bodies formed by ϕC31 integrase-modified human ES cells and unmodified human ES cells (44).

When using the ϕC31 integrase system in stem cells, an initial analysis of pseudo *attP* sites can be valuable. The most frequently used sites, often called "hotspots," can be identified. A hotspot can be then further characterized as to its genomic context and whether it allows for desired levels of transgene expression before and after differentiation to particular lineages. Then a PCR-based method of detecting integration at the site can be developed, which would allow quick identification of clones of stem cells that have integrated the transgene at the particular pseudo *attP* site.

FUTURE USE OF ϕC31 INTEGRASE IN GENERATION AND MODIFICATION OF STEM CELLS

The ϕC31 integrase system may be advantageous for other uses in addition to the insertion of marker genes or therapeutic genes that correct the disease characteristics of either patient or animal model stem cells. For example, in addition to therapeutic genes, one can also place genes that improve the characteristics of the stem cells on the same *attB* donor plasmid. That the ϕC31 integrase system does not have a size limit allows such possibilities. For example, mesoangioblasts are vessel-derived mesodermal stem cells capable of migrating to sites of muscle damage when injected into the bloodstream and differentiating into muscle fibers causing muscle repair (61, 62). The ability to migrate to muscle is enhanced by expression of *α4 integrin* or *L-selectin* (63). Such genes driven by suitable promoters are candidates for addition with the ϕC31 integrase system to improve the properties of these stem cells.

The ϕC31 integrase system may also be used to promote differentiation of ES cells into other stem cell lineages appropriate for therapeutic use. For example, derivation of hematopoietic stem cells from ES cells requires ectopic expression of *HoxB4*, typically introduced on a retroviral vector (64–67). These hematopoietic stem cells can then be transplanted into irradiated mice to reconstitute multilineage hematopoiesis (65). Hematopoietic stem cells derived in such a way have been successfully used in treatment of mouse disease models, using genetically corrected ES cells from immuno-deficient Rag2 knockout mice (66). Genetically corrected induced pluripotent stem cells derived from a mouse model of sickle cell anemia have also been used (64). One study employed inducible expression of another gene, *Cdx4*, in addition to *HoxB4*, to further enhance the specification of ES cells into the hematopoietic lineage (67). These combined gene and cell therapy approaches may be more safely carried out if the ϕC31 integrase system is used to introduce

the genes required for differentiation into hematopoietic progenitors, instead of retroviral vectors.

Our laboratory is continuing studies to improve the specificity and efficiency of ϕC31 integrase. We expect the use of ϕC31 integrase to contribute significantly to the field of stem cell biology and the practical use of stem cells in clinical applications.

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21

CELL ENGINEERING USING INTEGRASE AND RECOMBINASE SYSTEMS

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INTRODUCTION

One of the many challenges in stem cell research is gaining understanding of the complex molecular mechanisms involved in maintenance of pluripotency and lineage development. By using modern molecular biology techniques, it is relatively easy to generate large volumes of data describing the profiles of gene and microRNA expression and methylation in various stem cell populations and cell types which have differentiated from them. By comparing these profiles, lists of differentially regulated genes and miRNAs can be collated giving one a set of genes and pathways to target in cell perturbation experiments. One can then disrupt particular pathways and measure the phenotype of the perturbed cells. Part of the readout of the effect on lineage development has been the use of reporter genes by linking the expression of fluorescent

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proteins (generally green fluorescent protein [GFP]) to a lineage-specific promoter. These types of platforms can provide easy tracking of cells in culture as well as after transplant into animal models.

Attempts at controllable, stoichiometric expression of multiple genes from a single vector in mammalian cells have been reported in last two decades (1). In these reports, several different viral-based strategies were explored to produce two or three different proteins from bicistronic or tricistronic vectors, using various methods such as internal translational initiation by internal ribosome entry sites (IRES), internal promoters, splicing, self-processing polypeptides, proteolytic cleavage of fused polyproteins, and fusion of two genes in frame. What has become clear from these studies is that altering the various elements and their configuration can have significant influence on relative and absolute expression levels of genes in complex constructs. In many cases, expression of specific genes in particular cellular backgrounds requires the testing of multiple elements and configurations in order to achieve the most efficient system for a particular purpose. Assembly of multiple genetic elements can be at least cumbersome and at most impossible using standard restriction and ligase engineering techniques.

Recombinational cloning using bacteriophage integrase (i.e., Multisite Gateway) offers a solution to this problem by providing a powerful technology to assemble multiple DNA fragments simultaneously into a single vector backbone in a predefined order and orientation. In the conventional strategy for Gateway cloning, the assembly of multiplex DNA elements into a cassette of tandem elements in a single plasmid requires the recombination of multiple entry clones with a destination vector in a single LR (recombination between *att*L and *att*R) reaction mixture (2–4). However, use of increasing numbers of entry clones in a single reaction is often inefficient. As an example the power of this technology, we routinely perform and show here tandem assembly of five to ten DNA fragments into a single vector to construct. The resulting multi-cDNA expression clones contain two or three tandemly arrayed cDNA expression elements with multicolored fluorescent protein tags. These multi-element constructs can then be controllably inserted into the cellular genome to provide stable high content cell-based assay platforms both in tissue culture cells and in human embryonic cells and their progeny.

MULTIGENE EXPRESSION VECTOR CONSTRUCTS FOR INTRODUCING HETEROLOGOUS CDNAS INTO LIVING CELLS AND OPTIMIZATION OF THEIR EXPRESSION

The need to simultaneously and stoichiometrically introduce multiple heterologous genes into a single cells, including stem cells, has been gaining importance not only for basic research of various biological functions and drug screening but also potentially for clinical applications such as regenerative medical treatment and gene therapy. Since standard restriction enzyme and ligase techniques

makes assembly of multiple elements difficult or impossible, we rely on recombinational cloning (Gateway) technology which allows the rapid construction of tandem assemblies containing two to four different cDNA expression cassettes in a single plasmid (3,5). These constructs have been used successfully for simultaneously introducing multiple heterologous genes into cells in stoichiometric amounts without gene dosage variation (6,7). The construction of two or more expression elements in tandem in a single plasmid requires the recombination of multiple entry clones with a destination vector in a single reaction mixture. Use of increasing numbers of entry clones up to five in a single LR reaction decreases in efficiency, probably due to inefficiency of recognizing multiple pairs of matched *att* signals simultaneously. This can be mitigated by stepwise LR and BP (recombination between *att*B and *att*P) reactions or by using "modular destination vectors" and "modular entry clones" (5). By using a "modular destination vector," cDNA inserts are sequentially introduced, resulting in a tandem structure with multiple inserts. Whereas the standard destination vector contains only Cm^R and *ccdB* genes flanked by two *att*R signals, this destination vector contains one or two cDNA expression elements, which can be subsequently used in LR and BP reactions to insert a modular set of fragments. This strategy allows the assembly of more than 10 DNA fragments into a single vector. An alternative method would be to use two modular entry clones, which are subsequently recombined with a conventional destination vector (Fig. 21.1). In the strategy described here, one entry clone contains three DNA fragments and the other contains two DNA fragments. When the DNA fragment in the third position of one entry clone is a transcription termination signal (Poly A) and a promoter, the final product would be a tandemly fused dual cDNA expression construct whose transcription is directed by each respective promoter. In this case, each of expression unit consists of two DNAs, one representing a gene of interest and another fluorescent protein tag. Some typical multi-cDNA expression constructs that we have used are shown in Fig. 21.2. This represents two types of expression clones: (a) two or three fused cDNA-tandem expression clones in which each fused cDNA is joined with respective promoter, and (b) operon-type clones containing three heterologous open reading frames (ORFs) (cDNAs) in tandem downstream of a single promoter.

By varying promoter strength, these constructs can be useful for optimizing respective gene expression levels to predetermined and different levels (6). Relative transcriptional activities of the respective cDNA included in the multi-cDNA tandem clones can be controlled by using various promoters with different activities. In eukaryotic cells for instance, many intrinsic genes are expressed in a cell-cycle dependent fashion. Use of promoters, such as those prepared from *cyclin E, cdc2, cyclin B1*, and *aurora A* genes of human genomic DNA which express at G1-S, S-G2-M, G2-M, and G2-M phases of cell-cycle division, respectively, would create testable transgene phenotypes in transformed cells by more closely mimicking the true physiological state. In HeLa, HEK293, and mouse ES cells, the relative activities for *cyclin E, cdc2, cyclin B1*, and *aurora A* promoters are approximately 3.9, 2.0, 1.0, and 1.3, respectively,

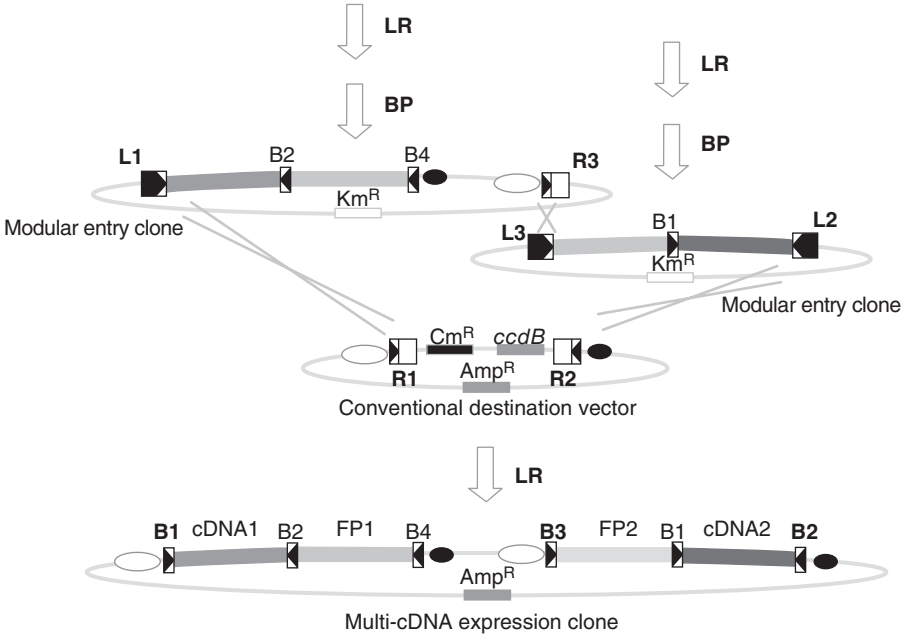

Figure 21.1. Construction of tandem cDNA expression plasmids. Stepwise construction of five DNA fragments on a single vector and an example of usage of this construct are shown. Modular entry clones are constructed from preassembled multi-fragment expression clones by exchanging their vector backbones by BP reaction with donor vectors containing corresponding *att*P signals. Their multiple DNA fragments are then assembled into a conventional destination vector by LR reaction.

compared to 24.1 and 338.0 for *EF-1*α and CMV promoters which express constitutively (6). Taking advantage of the relative difference in transcriptional activity by using these promoters could make it convenient for optimizing transgene expression at near physiological protein levels in living cells.

IRES have been widely used to facilitate translation of a downstream gene in bicistronic vectors (1). In spite of the conventional usage of IRES expressing downstream ORF of polycistronic messages, various IRESs are also useful to express monocistronic messages and the first ORF positioned on polycistronic mRNA. Combination of more than one IRES to express multiple ORFs on a polycistronic message can be used to manipulate expression levels of heterologous genes introduced into the cell. From work done in our lab, we have seen that 11 different IRESs, (Gtx, 2xGtx, 5xGtx, 12xGtx, EMCV, HCV2a, HCV33, HCV45, mHCV2a, mHCV33, and mHCV45) exhibit the relative translational activities of 35, 8, 9, 4, 1, 7, 4, 7, 5, 5. and 5, respectively, when directing the production of enhanced green fluorescent protein (EGFP) protein from the reporter ORF on the monocistronic mRNA, comparing to 39 of Kozak signal (7). Western blot analyses have been reported to parallel the EGFP levels detected by flow cytometry (FCM) and emission intensity determination in

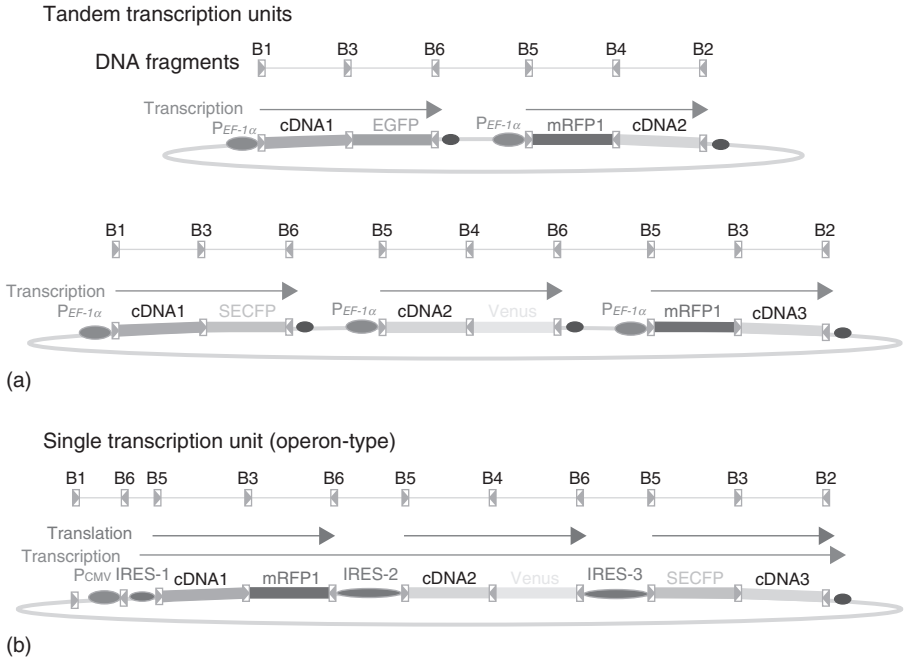

Figure 21.2. Multiple cDNA expression clones with multicolored fluorescent protein tags. (See color insert.)

the above. The encephalomyocarditis virus (EMCV) positioned upstream of the monocistronic mRNA strongly inhibits translation of the ORF, consistent with a previous report (8).

For example, in an operon-type multi-cDNA expression construct containing three cDNAs, (*H2B, EB1*, and α-*tubulin*), each of which fused to different fluorescent protein, (mRFP1, Venus, and SECFP), and directed by the CMV promoter, we placed a 5xGtx IRES upstream of the first cDNA instead of a Kozak signal (shown in Fig. 21.2b), and EMCV at sites between the downstream cDNAs. When this construct is expressed as a single copy (i.e., integrated at a *FRT* site in the HeLa chromosome), expression of differentially labeled multicolor images can be observed in respective single cells and relative expression levels of three cDNAs in the operon-type expression clone can be controlled within a relatively small variation (Fig. 21.3).

CHROMOSOMAL INTEGRATION OF MULTIPLE HETEROLOGOUS cDNAs AT A DEFINITE SITE AND STABLE EXPRESSION IN CELLS

The best way to achieve the long-term stable expression of an introduced transgene is by integrating it into the cell's chromosomal DNA. The conventional

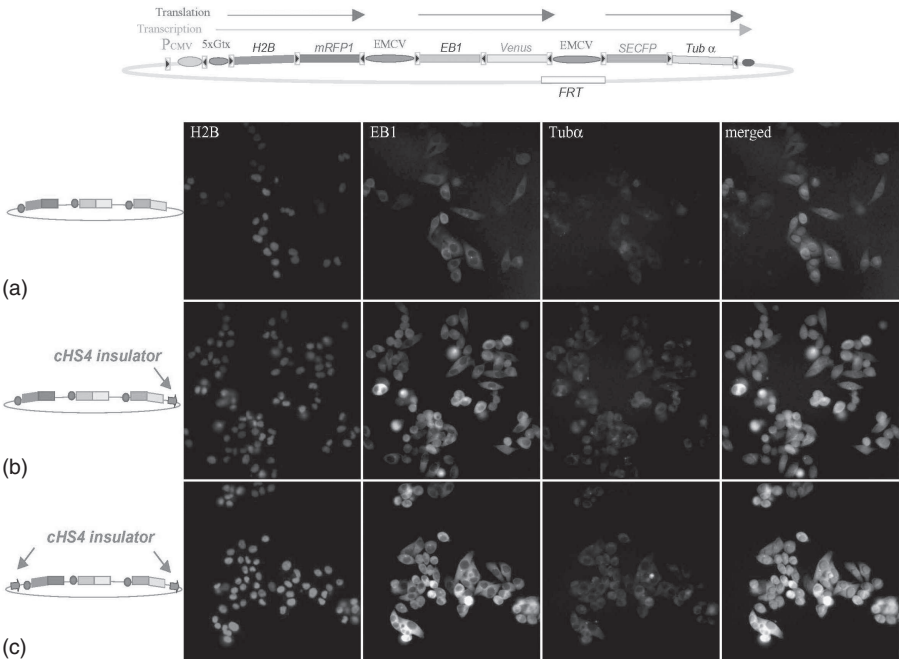

Figure 21.3. Expression levels of an operon type 3-cDNA clone with and without a cHS4 insulator element. Operon-type three fused cDNAs expression constructs were integrated into three *FRT* sites on the chromosomes of HeLa cells. The other interpretations are in the text. (See color insert.)

approaches used to deliver the gene into cells for subsequent integration into chromosomal DNA by spontaneous homologous recombination have generally resulted in very inefficient transduction and random integration. These limitations can be overcome by using site-specific recombination methods to efficiently insert the transgene at a known location in the genome.

SITE-SPECIFIC INTEGRATION USING THE Flp RECOMBINASE SYSTEM

In past two decades, two recombinase systems, Cre/*lox* from P1 phage (9, 10) and Flp/*FRT* from yeast (11, 12), have been developed into the powerful tools for site-specific insertion into the mammalian genome. Both enzymes, Cre and Flp, catalyze recombination between well-characterized short DNA sequences, *lox* and *FRT*, respectively, resulting in integration of a circular DNA into the genome.

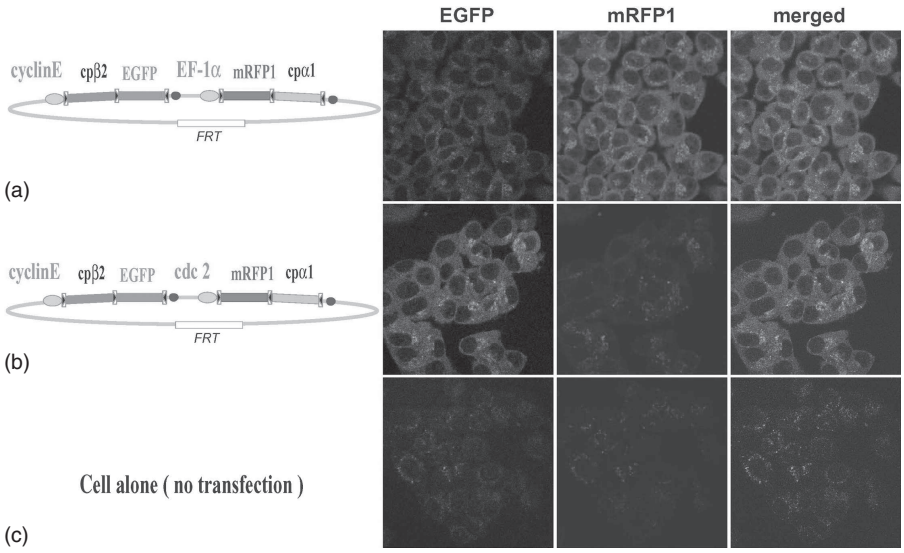

Figure 21.4. Differential expression levels of two tandem cDNA constructs with weak promoters. Differential expression levels were observed by confocal laser microscopy with two fluorescent tagged transgenes of *cpβ2* and *cpα1* introduced into three *FRT* sites on the chromosomes of HeLa cells. (See color insert.)

In Fig. 21.4, we demonstrate that a tandem cDNA-fluorescent protein fusion can be successfully introduced into known genomic locations bearing *FRT* sites in the HeLa chromosomal DNA, and are expressed at predicted levels. In this study, two types of expression plasmids containing protein fusions, CPβ2-EGFP, and mRFP1-CPα1, as well as single *FRT* sites, were constructed (Fig. 21.2a, upper side). These plasmids were then integrated into an *FRT* site in chromosomal DNA by site-specific recombination mediated by Flp recombinase, generating a stably transfected cell. From these plasmids, expression of two cDNAs, *cpβ2* and *cpα1*, were controlled by the *cyclin E* and *EF-1α* promoter respectively, or the *cyclin E* and *cdc2* promoter respectively. The expression plasmid was introduced into HeLa cells harboring at least three *FRT* sites on the chromosomes (determined by Southern-blot analysis of the genomic DNA). After positive selection of the transformed cells using hygromycin, stable transfectant clones were obtained. Figure 21.4 shows coexpression of CPβ2-EGFP and mRFP1-CPα1 in the stably transformed cells, observed by using confocal laser microscopy. The transfected cells bearing a plasmid carrying the *cyclin E/cpβ2* and the *EF-1α/cpα1* in tandem, expressed CPβ2-EGFP at a very low level (whose fluorescent emission was barely visible), compared to mRFP1-CPα1 produced from *EF-1α* promoter (Fig. 21.4a). The cells harboring a plasmid carrying the *cyclin E/cpβ2* and the *cdc2/cpα1* in tandem, expressed CPβ2-EGFP and mRFP1-CPα1, both at very low levels (Fig. 21.4b).

The fluorescent emission intensity of these cells was clearly more distinctive compared with the auto-fluorescence seen from cytoplasm (no transfection of Fig. 21.4c). In transfected cells, fluorescent protein products were only localized in the cytoplasm but not in the nucleus. This feature was similarly observed with stable clones harboring a cassette containing *cyclin E/cp*β*2* and *EF-1*α*/cp*α*1* as well as another cassette containing *cyclin E/cp*β*2* and *cdc2/cp*α*1*. These observations indicate that CPβ2 and CPα1 form heterodimers and localize to the cytoplasm, possibly being associated with actin fibers (13). In fact, the dimer complex formation of CPβ2-EGFP and mRFP1-CPα1 proteins in the stably transfected cells was directly demonstrated by immuno-coprecipitation analysis using antibodies for EGFP, mRFP1 (6).

SITE-SPECIFIC INTEGRATION BY ΦC31 RECOMBINASE SYSTEM

Since both Cre and Flp recombinases create two *FRT* or *lox* sites in close proximity to each other after a recombination reaction, these two *cis*-positioned homologous recognition signals have the ability to participate in an immediate reverse reaction (excision). More recently, a novel integrase from *Streptomyces* phage ΦC31 has been reported to function in mammalian cells (14, 15). This ΦC31 integrase acts on two recombination sites that differ in sequence, *att*B and *att*P, to yield product sites, *att*L and *att*R, which are not substrates for the integrase. Therefore, unlike Cre and Flp, ΦC31 is a unidirectional integrase that only supports integration, which makes it an attractive tool for inserting transgenes stably into the mammalian genome. An attractive strategy using ΦC31 integrase system has been tested in human HEK293 and mouse 3T3 cells (15) as well as in human embryonic stem cells (16) where the cells are cotransfected with a plasmid containing a reporter gene-selection marker cassette and a ΦC31 *att*B site and a ΦC31 integrase-expressing plasmid. Positive selection is used to identify clones that contain a site-specifically integrated plasmid at genomic *att*P (pseudo *att*P) sites in the chromosome. The pseudo *att*P sites have partial sequence identity to *att*P and form naturally occurring targets for site-specific integration of a circular DNA.

The most frequent and common integration sites in the human cell lines HEK293, HepG2 and D407 have recently been identified (17). We have identified nine pseudo *att*P sites in the native chromosomes of a HeLa cell line, and have found that among a set of these potential ΦC31 integrase-mediated integration sites, the most frequently observed integration site has been a pseudo *att*P site at 19q13.31 (Fig. 21.5). To exemplify the utility of this approach, we placed three fluorescent protein-tagged cDNAs, *H2B, EB1*, and α-*tubulin*, on an incoming *att*B plasmid construct and cotransfected with a plasmid expressing the ΦC31 integrase. In the absence of integrase, no colonies bearing integrants were obtained. In the presence of integrase however, many positive colonies were observed consistent with integrase-mediated integration of the tandem cDNA construct into the chromosome. In addition to the hygromycin-

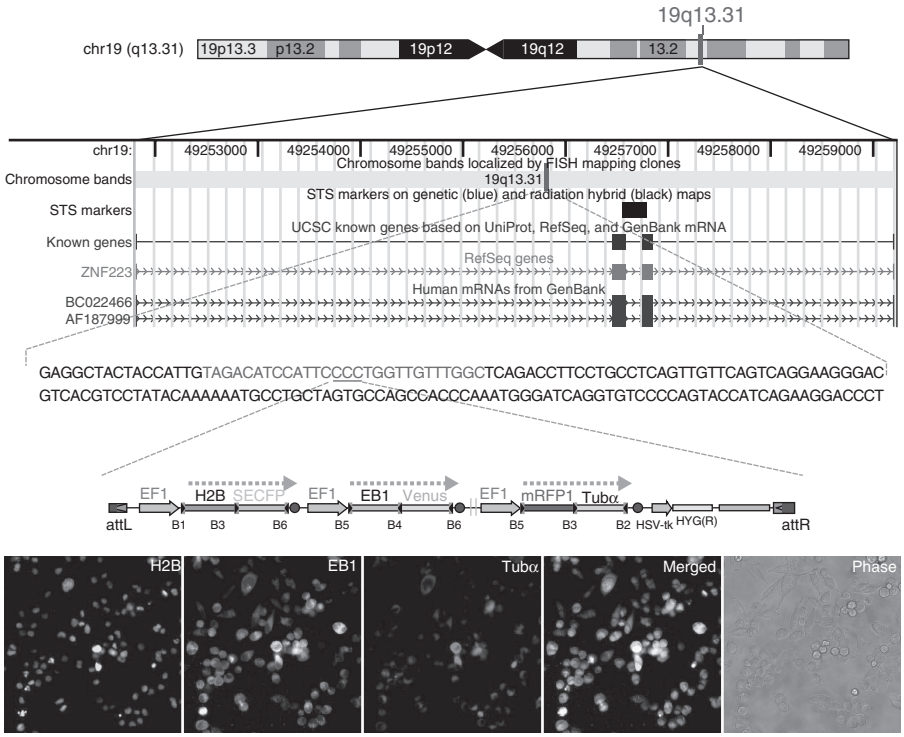

Figure 21.5. Transduction of msGW clone onto chromosome by ΦC31 recombination system. Tandem three fused cDNA expression constructs were introduced by ΦC31 integrase into 19q13.31 pseudo *att*P sites in the HeLa cell genome. The other interpretations are in the text. (See color insert.)

resistant phenotype assay, site-specific integration was also assayed biochemically by the plasmid rescue method described previously (15). The three transgenes at this chromosomal site were observed to be stably maintained and expressed during culture for at least 90 d.

ALLEVIATION OF TRANSCRIPTIONAL INTERFERENCE BETWEEN CLOSELY APPOSED CDNA TRANSCRIPTION UNITS

In stably transfected cells harboring a multi-cDNA expression cassette at a specific site on the chromosome, the expression of tandem transgenes is often subject to mutual transcriptional interference by each of the regulatory elements situated in close proximity (18, 19). Two transcriptionally active cDNAs are often repressed when they are closely positioned on the chromosome. For example, repression of a transgene arising from the upstream active gene(s)

has been observed and is termed "transcriptional silencing" (18–20). Furthermore, repression of upstream gene activity by a downstream gene has also been observed and is termed "promoter suppression" (21, 22).

The diminished expression by transcriptional interference described above can be restored by inserting an insulator such as the chicken β-globin HS4 (cHS4) (23) element between the neighboring expressing two transgenes on a single plasmid (24). The cHS4 element is one of the well-characterized vertebrate insulators that function to shield genes in the genome from neighboring environmental effects like activation or silencing (25). The insulation effect can be observed in either transiently transfected cells or chromosomally integrated stably transformed cells, though the stably transfected cells showed a more marked trend toward restoring the downstream cDNA expression (24).

The multi-cDNA expression construct containing three different cDNAs, *H2B, EB1*, and α-*tubulin*, each of which is fused to respective fluorescent protein tags on a single plasmid (Fig. 21.6a), exhibits the silencing effect on expression of the downstream two cDNAs when this is introduced into *FRT* sites on HeLa chromosome (Fig. 21.6a). Although we have successfully introduced multiple heterologous cDNAs into cells in stoichiometric amounts

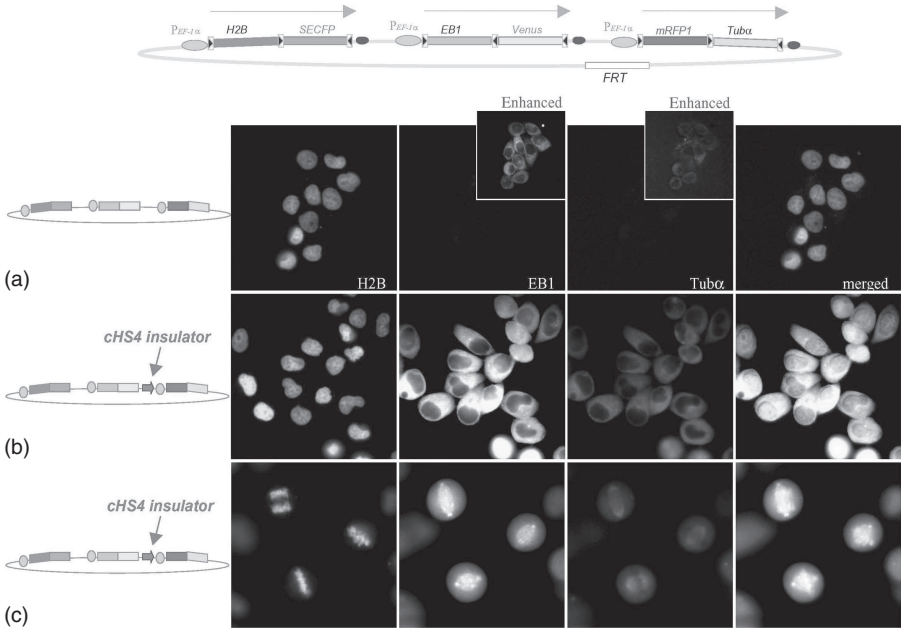

Figure 21.6. Alleviation of transcriptional interference by cHS4 on 3 cDNAs in tandem. Tandem three fused cDNA expression constructs were integrated into three *FRT* sites on the chromosomes of HeLa cells. The other interpretations are in the text. (See color insert.)

without gene dosage variation, the expression levels of these tandem transgenes are not equivalent due to the effect of mutual transcriptional interference. To overcome the problem of diminished expression, we introduced an insulator cassette of two repetitive cHS4 elements between the second and third cDNAs, *EB1*, and α-*tubulin*, respectively. The effect of cHS4 insulator cassette on coexpression of these two downstream transgenes is remarkable, significantly enhancing the visibility of all the fluorescent markers (Fig. 21.6b,c). This effect is specifically mediated by the cHS4 insulator, since it is not seen when a *LacZ* fragment of similar size is used instead (24).

We have shown previously that the silencing effect on the downstream cDNA is relatively weak when a weaker promoter is used for the upstream cDNA and a stronger promoter used for the downstream cDNA (6) (see Fig. 21.4a,b). The molecular basis of mechanisms of either negative effect on tandemly situated cDNAs is not well understood (26). In addition to the transcriptional interference occurring on transgenes, it is well known that the majority of integrated transgenes become subjected to epigenetic silencing by adjacent condensed chromatin when the constructs are integrated into the genome for the stable expression (25). Since the cHS4 insulator has been believed to function in organizing active chromatin conformation by preventing propagation of heterochromatin along the genomic DNA (25) to place cHS4 elements at both extremities of an operon-type multi-cDNA expression cassette shown in Fig. 21.3c might be ideal to protect the transgene cassette from such epigenetic silencing when it is integrated into chromosome. In fact, the expression levels of these three cDNAs are apparently increased by stationing the cHS4 elements in this manner (Fig. 21.3b,c).

DUPLICATE TRANSDUCTION OF TWO MULTIGENE CASSETTES INTO DEFINITE CHROMOSOMAL SITES

The number of cDNAs of interest that can be introduced at one time into genomic DNA of a cell depends on the size of the constructed plasmid molecule (i.e., the transfection efficiency of the cell becomes generally lower as plasmid size increases). The maximum vector size would be estimated to be approximately 15 kb. Mitigation of this problem could be achieved by targeting smaller vectors to separate loci in successive transfections. As an example, we used vectors containing three cDNA- fluorescent protein fusions (Fig. 21.2). Introduction of two different multi-cDNA constructs into different sites on the chromosome would make it possible to integrate up to six fused cDNAs in a cell.

As mentioned above, ΦC31 is a unidirectional integrase that catalyzes only the integration reaction. When a multi-cDNA construct is inserted into a pseudo *att*P site by ΦC31 integrase, this site cannot accept an additional *att*B-construct. The second multi-cDNA construct would be introduced into other pseudo *att*P site in the HeLa cell genome. By successive introduction of two

different tandem multi-cDNA constructs, one a tandem three fused cDNA construct and the other a two fused cDNA construct, we have succeeded in integrating 5 cDNAs fused with fluorescent protein tags into the cellular geneome. The HeLa transfectant harboring the construct containing three fused cDNAs, *H2B-SECFP, EB1-Venus (SEYFP-F46L)*, and *mRFP1-α-tubulin*, at the chromosomal locus 19q13.31 was subsequently transfected with another tandem two fused cDNA construct, *mit-mKeima* and *EGFP-B23*, by the ΦC31 integrase method. The latter construct was integrated at 11q13.1 (Fig. 21.7). Three proteins synthesized from the former cDNA cassette exhibit multicolor image of their intrinsic intracellular localization. The image of two proteins from the latter cDNA cassette is also as predicted (i.e., mKeima with mit tag [the 29 N-terminal amino acids of cytochrome c oxidase subunit VIII] is localized at mitochondria and EGFP with B23 [nucleophosmin] is as expected

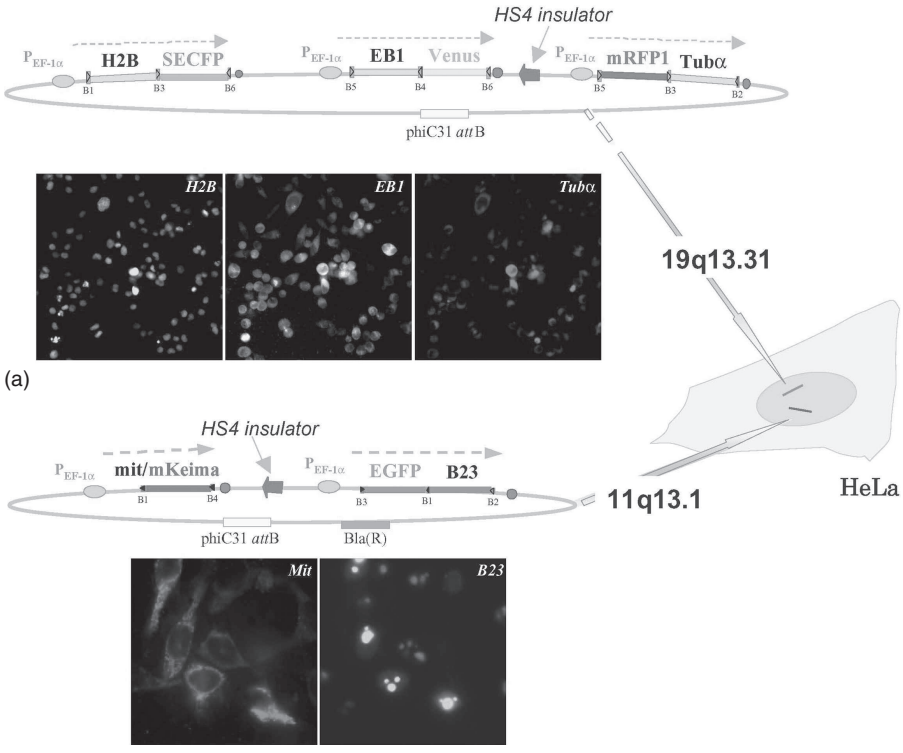

Figure 21.7. Successive introduction of multi-cDNA expression constructs into chromosomes. A tandem three fused cDNA expression constructs and a tandem two fused cDNA expression clones were successively introduced by ΦC31 integrase into pseudo *att*P sites at genomic loci 19q13.31 and 11q13.1, respectively. The other interpretations are in the text. (See color insert.)

at nucleolus). To simultaneously detect these five different fluorescent emission labels by excitation is not easy under our conventional fluorescent microscopy, and this is under investigation by collaborating with the expert scientific group.

APPLICATION OF THESE NOVEL CELL ENGINEERING TECHNOLOGIES TO HESC

Site-specific recombination systems are powerful tools for introducing predetermined modifications into eukaryotic genome by using multigene expression cassettes, thereby facilitating systems biology research by simultaneous multicolor imaging and multigene manipulation in the fields of tissue-regenerative medical treatment and gene therapy. Recent data have shown the applicability of the ΦC31 integration system in both mouse (15) and human embryonic stem cells (16). While little information is available about the presence of pseudo sites in mESC, the pseudo site profile targeted by ΦC31 integrase in hESC is surprisingly unique. The most frequently targeted site is present on Chromosome 13, although there are a few common hotpots between hESC and the previously characterized cell lines. This property of the integrase can be used to generate human ESC lines containing desired expression cassettes.

Regardless of the mode of gene delivery for a particular experiment, expression of the inserted element can be significantly affected by the surrounding activity of its genomic insertion site. Genes placed in an actively transcribed chromosomal location can be expected to be expressed at a higher level than those inserted into inactive heterochromatin. Confounding this "position effect" is the fact that as hESCs differentiate, they go through significant chromatin remodeling leading to silencing of some loci and potentially activation of others (27–29). Expression cassettes randomly inserted into the hESC genome (via viral or other mode) would be subject to locus specific silencing during differentiation, leading to heterogeneous expression patterns in the differentiated cell population. This potential heterogeneity is compounded by the lack of control of copy number and the necessity of performing multiple delivery events for multiple separate expression cassettes. All these issues taken together can lead to unpredictability and cell to cell variability of reporters and other assayable functional elements in populations of cells to be used as assay platforms. Recently, several reports have documented several possible sites in the hESC genome that are potentially useful for this sort of engineering, including the chromosome 13 locus described above (16), the human Rosa locus (30) on chromosome 3, and the "Envy" locus on chromosome 12 (31). Further evaluation of these and other loci may add considerable versatility to future versions of this and similar hESC engineering platforms.

Successful development of systems that can efficiently target constructs to active and non-silencing loci could aid in, and potentially enable, multiple

applications currently anticipated for hESC and lineage positive cells. For instance, by inserting specific read-outs such as fluorescent proteins under the control of specific lineage and signaling pathway promoters, one can envision the creation of stable, sensitive, single or multiple lineage beacons for *ex vivo* differentiation studies and transplant tracking *in vivo*. In addition, this system will also be very useful in developing platforms for cell-based drug screening assays in stem and progenitor cells as well as terminally differentiated human tissue. By inserting appropriate reporter genes at the hESC stage followed by propagation and differentiation to specific lineage cells (i.e., motoneurons), assays could be developed in human cells that would most likely be more consistent and relevant than those currently used in human and rodent primary and transformed cell models. In addition to specific reporter functions, this system will be useful for controllable, stoichiometric expression of cell perturbation molecules including shRNA, miRNA, dominant negative signaling proteins. Since each cell in a population would carry a single copy of the perturbation construct at identical loci, the dosage of perturbation agent would be expected to be homogeneous leading to a similar resultant phenotype from cell to cell.

Given the capacity to rapidly assemble multielement plasmids and efficiently target large constructs hESC, these types of applications can be contemplated singly or in combination with lineage or signaling reporters. These tools can also be afforded more versatility by combining with them existing useful technologies such as Cre/*lox* or Flp/*FRT* integration and excision for in situ modulation of the assay system or complete excision of the engineering construct to create a null control. Finally, given that cells can be targeted to a specific locus, matched allelic series of related assay lines can be created and compared inter-experiment more reliably since each assay construct would be expressed in the same cellular background (same copy number and locus in all cells).

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PART VI

22

HUMAN EMBRYONIC STEM CELL-DERIVED CARDIOMYOCYTES FOR CELL THERAPY AND DRUG DISCOVERY

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INTRODUCTION

When human embryonic stem cells (HESCs) were first isolated, it was immediately recognized that, with their pluripotent and self-renewing capabilities, they hold great promise for the development of cell therapeutics to treat a variety of degenerative diseases (1, 2). The cardiomyocyte is one cell type that can readily be generated from HESCs and has great potential for clinical applications as well as for drug discovery. However, significant technical bottlenecks exist, especially for the development of cell therapeutics. First, HESC culture and scale up remain a challenge (3). Extra hurdles exist for growing cells in a clinically and current good manufacturing practice (cGMP) compliant manner. Second, the differentiation process is largely uncontrolled, and many published protocols rely on spontaneous differentiation in embryoid bodies (EBs). Third, HESCs differentiation at present leads to the production

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of a heterogeneous cell population, requiring enrichment of cardiomyocytes from the other cell types. Purification is of particular importance to remove persisting, undifferentiated HESCs from differentiated cultures given their potential to form teratomas *in vivo*, thereby possessing a significant safety concern for cell therapy. Finally, HESCs-derived cardiomyocytes have to be tested for their ability to improve heart function in relevant injury models, providing the basis for clinical trials. This chapter describes the status of the field including our own recent efforts to enable efficient HES cell differentiation into functional human cardiomyocytes. We further describe cardiomyocyte application in therapy development and drug discovery, and address some of the formidable challenges along the way.

ENHANCING ESC CARDIOMYOGENESIS—2- VERSUS 3-DIMENSIONAL DIFFERENTIATION APPROACHES

Two general strategies to induce *in vitro* differentiation of ESCs have been employed by various investigators, namely differentiation of attached, more or less homogeneous ES cell monolayers (2-dimensional cultures; 2-D) or differentiation in suspension culture, which leads into the formation of cell aggregates termed EBs.

In the 2-D approach, differentiation into a particular lineage is directed by factors in the culture medium and/or matrices used for cell attachment, but is vastly independent of ES cell–cell interaction. Ideally, this strategy would enable a complete, homogeneous transition of ESC into one desired lineage, an approach that apparently requires defined medium/matrix conditions to drive this transition in a cell-autonomous fashion. A mid-throughput screen aimed at finding "golden bullets," compounds that would turn a mouse ES cell monolayer directly into cardiomyocytes, has been performed (4). Combing through a library of 880 bioactive compounds approved for human use resulted in the identification of a single molecule, ascorbic acid, that markedly increase cardiac differentiation in monolayer culture when added into a medium containing 10% fetal calf serum (FCS). However, the method is not broadly applied in the field, indicating limited robustness. For HESC, a sequential treatment of attached cell colonies with activin A followed by bone morphogenic protein 4 (BMP4) was recently published (5). By this method, Laflamme and coworkers observed the relative high induction of about 30% cardiomyocytes and finally achieved >80% cardiomyocyte purity by Percoll density centrifugation.

A modified 2-D strategy was established by Christine Mummery's group. Previously, mouse ESC and pluripotent embryonic carcinoma (EC) cells were cocultured with the endoderm-like cell line END2 to induce directed cardiomyocyte generation (6). The method has now been successfully applied to HESC cardiomygenic differentiation (7) and was further improved by the complete withdrawal of serum from the differentiation medium (8). These examples prove the general feasibility of monolayer differentiation strategies

although none of the methods enabled the complete transition of pluripotent cells into cardiomyocytes. Moreover, 2-D differentiation, and particularly the cumbersome coculture approach, does not lend itself to scalable methods for mass production.

As for differentiation in suspension culture, early work with mouse ES cells showed that EB formation *in vitro* resulted in spontaneous differentiation of ES cells into beating cardiomyocytes (9). Similarly, EB formation from HESC also yielded some degree of spontaneous cardiogenesis (10, 11). Differentiation in EBs, however, is a largely stochastic process, and the degree of cardiogenic differentiation could vary considerably between different HESC lines. Subsequently, several labs have reported addition of factors that could improve the process, both, from mouse and human ESC. The additions of retinoic acid, dimethyl sulfoxide, 5′-azacytidine (5′-AZT), ascorbic acid, and growth factors of the BMP family have all been reported to have a cardiogenic effect in mouse ES cells (12–17), but only 5′-AZT and, to a lower extent, ascorbic acid appear to improve cardiogenic differentiation of HESC (10, 18).

We have recently shown that the coculture approach employing the seeding of HESC on a layer of END2 cells (8), can be replaced by the formation of EBs in a serum-free, END-2 cell conditioned medium (END2-CM), thereby facilitating process scalability (19). HESC differentiation in END2-CM yielded about 10% cardiomyocytes thereby demonstrating that the cardiomyogenic bioactivity of END2 cells is secreted into the medium and apparently coculture independent. Applying this system a candidate molecule screen was performed. The study identified SB203580, a specific p38 MAP kinase inhibitor, as a potent, dose-dependent promoter of cardiogenesis (19). At an optimized concentration, greater than 20% of cells in EBs formed cardiomyocytes. In parallel, an increase of the overall cell numbers was observed finally yielding approximately 2.5-fold more cardiomyocytes compared to the differentiation in END2-CM alone. Besides 5′-AZT, SB203580 is one of the first molecules to act as an efficient enhancer of HESCs cardiac differentiation.

Next, research was conducted aiming at elucidating the cardiogenic activity residing in the "xenogenic" END2-CM. We found that some activity was resistant to heat and protease treatment. Fractionation experiments also revealed that the molecular weight of an active fraction was less than 10 kD. These results suggested that the cardiogenic activity is partially protein independent and imparted by a small molecule(s) or combinations thereof. In addition, comparative gene expression analysis of END2 and MES1 cells, another cell line that was also derived from mouse EC cells but entirely lacks cardiogenic activity, identified enzymes catalyzing prostaglandin synthesis as being over represented in the END2 cell-derived mRNA (20). Conditioned media analysis confirmed that the prostacyclin PGI2 was secreted at significantly higher levels by END2 cells compared to MES1 cells. Interestingly, when END2-CM was diluted with low amounts of unconditioned, serum free medium, the cardiogenic activity was strongly reduced suggesting the presence of an inhibitor. By systematic removal of components from the basic medium, we discovered

that the inhibition of cardiogenesis was mostly attributed to the presence of insulin at levels typically found in serum free media formulations. Indeed, when insulin was removed, our synthetic medium became permissive for HESCs-cardiac differentiation in EBs (20). Importantly, adding PGI2 into the synthetic medium at an optimized concentration resulted in a cardiomyogenic differentiation efficacy equivalent to END2-CM. The process was further improved by the addition of the compound SB203580, yielding an efficient, cGMP and clinically compliant medium for human cardiomyocyte production.

SCALE UP—MASS PRODUCTION AND REPRODUCIBILITY

Although the sequential addition of activin A and BMP4 to HESC was successfully employed for cardiomyocyte generation from HESCs (5), scalability and economic feasibility of this growth factor-dependent monolayer approach needs to be proven. In contrast, EB-dependent differentiation for cardiomyocyte mass production, at least from mouse ESC, has readily been scaled to a 2-L volume (21). Robustness of this strategy will depend on controlled cell aggregation during the phase of EB formation and growth, since EB size was found to be crucial for cardiomyocyte induction in the mouse and human system (22–25). Culture rotation (26, 27) and impeller-based stirring (28–30) has proven to be advantageous for homogeneous EB formation by vastly avoiding extensive ESC agglomeration. By adapting this technique to a 2-L bioreactor scale we have recently enabled the production of more than 1.2 billion cardiomyocytes in a single run (21). Following antibiotic based lineage enrichment from a genetically engineered mouse ESC line, the production of 6.4 cardiomyocytes per input ESC (CM/ESC) was achieved. Implicating multiple steps of process optimization, including lower medium throughput and perfusion-feeding, this value was recently improved to 23 CM/ESC (31). Another index number which is key to assess the commercial viability of production processes for future cell replacement therapies, is the number of cardiomyocytes that can be generated per liter (of a potentially pricy) culture medium. Under optimized conditions, >500 million cardiomyocytes per liter medium (containing 10% FCS) were generated in an 18-day lasting differentiation and enrichment process with mouse ESC.

However, one major difference between mouse and human ESCs is the inability of the latter to aggregate in suspension after they are dissociated to single cells (32). This phenotype seems to be independent of the HESC line, the dissociation method, the culture medium and the seeding density (32, 33). Consequently, all of the present differentiation studies rely on either enzymatic whole colony lifting (thereby separating hESCs from the feeder layer) (34), or other enzymatic and/or mechanical scoring techniques aimed at providing (hardly controlled) preformed hESCs-aggregates for EB formation in suspension (10, 11, 19).

Facing this challenge, Gerecht-Nir and coworkers have employed small cell clumps to inoculate special bioreactors termed slow turning lateral vessels (STLV) and high aspect rotating vessels (HARV) for successfully EB generation from HESC, but cardiomyocyte induction was not documented in this study (24). Recently, HESCs differentiation in standard, impeller-stirred spinner flasks was reported (35), while another study adapted an agarose encapsulation technique to control EB formation from mouse and human ESCs (23). However, all of these studies depended on the cumbersome, difficult-to-control, and hardly scalable preformation of HESCs clumps for process inoculation. In contrast, direct EB formation from single cell suspensions was achieved by seeding the cells on three-dimensional porous alginate scaffolds (25), or *via* the forced aggregation by centrifugation in round-bottom or V-shaped 96-well dishes (32, 33). These studies indicate that HESC dissociation into single cells does not irrevocably induce cell death but suggest that constraining physical cell–cell or cell–matrix interaction combined with chemical cues (from the substrate surface and/or the medium) results in cell survival. While porous alginate scaffolds and the 96-well technique are not straightforward for large-scale inoculation of stirred reactor tanks, these results indicate that single cell inoculation might eventually be applicable to EB generation from HESC. This will be of foremost importance to establish robust large scale differentiation processes.

In summary, *in vitro* differentiation of ESCs is a highly variable process due to continuous changes in cell density (total increase in cell numbers but accompanied by local apoptosis within EBs) paralleled by the formation of differentiated cell lineages, thus creating proceeding variations in cell physiology, cell–cell signaling, cytokine secretion, etc. Nevertheless, recent findings in controlled bioreactors indicate that the reproducible and efficient cardiomyocyte production from ESCs is achievable. Translating highly controlled single-cell EB formation strategies from mouse to human ESC cultures and utilizing our recently developed, fully defined differentiation media are important steps towards this goal.

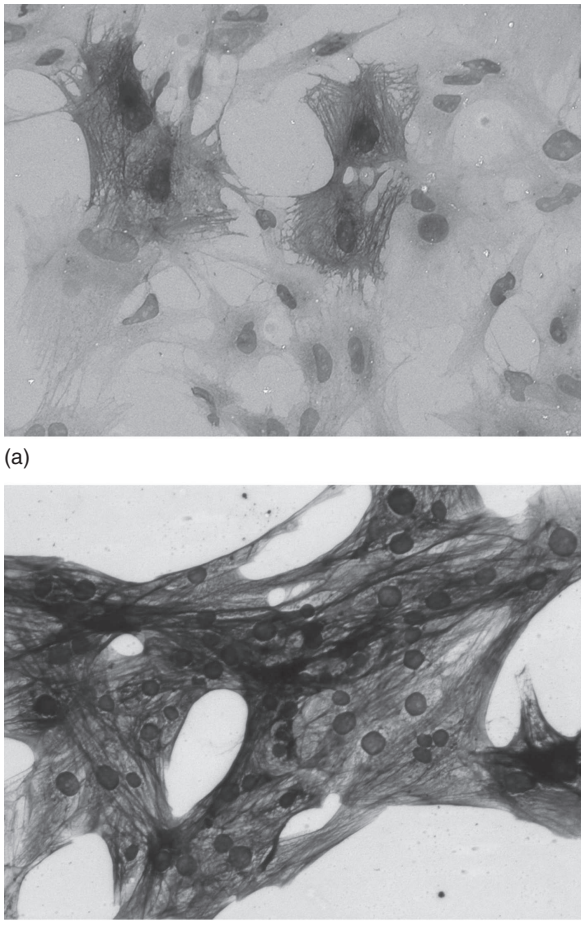
ENRICHMENT STRATEGIES—ENSURING PURITY AND SAFETY

As current differentiation protocols could only generate, at best, 10%–30% cardiomyocytes from HESC, purification strategies are required to enrich this cell type for downstream applications. Purification away from persisting pluripotent stem cells is, especially from a safety standpoint, prerequisite for clinical applications. A relatively pure cardiomyocyte population will also facilitate establishing robust cell-based assays for drug screening. However, until recently, no efficient methods for purifying cardiomyocytes from heterogeneous populations of differentiated HESCs have been described. One crude strategy relies on the visual identification of beating areas and subsequent manual dissection (11, 36). Another reported method relied on the purification

by centrifugation of dissociated EBs through a discontinuous Percoll gradient (10, 37). The enrichment was initially reported to yield up to 70% cardiomyocytes as measured by quantifying the percentage of α-myosin heavy chain (MHC) positive cells (10). Subsequent reports using the same H7 line indicated that this purification protocol routinely yielded only ~15% cardiomyocytes (38), but a modified version of this approach achieved better results if clusters, isolated from the Percoll gradient, were cultured in suspension as "cardiac bodies" (37). The level of enrichment achieved in cardiac bodies was 33%–66% as measured by flow cytometry of α-MHC stained cells. As outlined above, in a more recent report, the level of cardiomyocyte enrichment after an improved differentiation protocol and Percoll gradient centrifugation reached an average value of 82.6 + 6% (ranging from 71%–95%) (5). The authors reported the absence of teratoma formation from this cell population at 4 weeks post-transplantation into nude rat hearts, but notably no assessment beyond this time point was conducted.

An alternative purification strategy is to genetically engineer cell lines to enable lineage selection. The technique takes advantage of the clonal expandability of embryonic stem cells lines after the introduction of a respective selection gene construct. A transgene consisting of the cardiomyocyte specific murine α-MHC promoter driving a neomycin resistance gene was used successfully in mouse ES cells to select for differentiated cardiomyocytes (39). The level of enrichment after G418 selection (determined by α-MHC immunocytology) was greater than 99%. Our laboratory has recently introduced the same construct into the HESC line hES3 (40). Differentiation and subsequent G418 selection of these lines resulted in >99% cardiomyocyte purity assessed by the percentage of α-MHC or α-actinin positive cells (Fig. 22.1). Our results provide evidence that the utilized 5.3 kb mouse α-MHC promoter is cardiomyocyte specific in both species and thus the same level of purity was achievable from HESC *via* this enrichment strategy. The selected human cardiomyocytes expressed cardiac-specific genes as indicated by reverse transcriptase–polymerase chain reaction (RT-PCR) and immunocytochemical analysis, and exhibited electrophysiological and pharmacological properties of embryonic-type human cardiomyocytes as assessed by microelectrode arrays (MEAs) (40). To our knowledge, we have reported the highest level of enrichment of HESCs-derived cardiomyocytes at present. More importantly, by applying a sensitive biosafety model for teratoma formation in SCID mice (41, 42) no teratomas were found for at least 13 weeks after the injection of about 2 million selected cardiomyocytes. In contrast, the same number of differentiated but nonselected cells formed teratomas with high incidence in the same model. These findings strongly underscore the necessity of efficient selection techniques and comprehensive long term safety studies in appropriate animal models for cell therapy purposes.

The mouse α-MHC promoter selected population, however, contains a mixture of cardiomyocyte subtypes, including atrial and ventricular cells, as indicated by the expression of myosin light chain 2a (MLC2a) and MLC2v.



(a)

(b)

Figure 22.1. Lineage selection to generate a pure population of cardiomyocytes. A transgenic construct comprising the murine a-MHC promoter driving the neomycin resistance gene was introduced into the HESC line hES3. The transgenic line was differentiated into cardiomyocytes by EB formation in END-2 conditioned medium and then subjected to G418 antibiotic selection. In the absence of G418 selection (a), about 10% of the cells were positively stained for α-MHC. Upon antibiotic selection with G418 (b), greater than 99% of the cells were positively labeled for the same cardiomyocyte marker. (See color insert.)

The expression of MLC2v appears to be restricted to ventricular cells during human heart development, whereas MLC2a is found in both subtypes (43). For clinical applications, such as replacement of infarcted tissue in a ventricle, it might be desirable to generate ventricular-type cells only. Apparently, one potential method to achieve this goal is to use the MLC2v promoter, which

seems feasible in mouse ES cells (44). Muller and coworkers have used an MLC2v promoter fragment functionally coupled to a CMV enhancer to drive expression of the enhanced green fluorescent protein (EGFP). Purification of cardiomyocytes was accomplished by combining Percoll gradient centrifugation followed by fluorescence activated cell sorting (FACS). Electrophysiological characterization of the purified cardiomyocytes by patch-clamping indicated that about 82% of the cells were ventricular—and 12% were atrial-like, whereas 6% were fetal-like. However, in contrast to the electrophysiology assessment, only 0.1%, of the cardiomyocyte population stained positive for atrial myosin when analyzed by FACS. A more recent report used the human MLC2v promoter driving EGFP in HESC (45). The level of enrichment after FACS sorting was reported to be about 93%. However, molecular and electrophysiological characterization of the EGFP positive cells pointed to an embryonic phenotype and it could not determine whether they were atrial or ventricular cardiomyocytes. Therefore, it is yet inconclusive whether an enrichment strategy based on the MLC2v-EGFP transgene could eventually generate a pure population of ventricular cardiomyocytes from HESC.

HESC-DERIVED CARDIOMYOCYTES FOR CELL THERAPY—HIGH EXPECTATIONS BUT SMALL GRAFTS

Rather than focusing on therapies to curb ischemia-induces heart damage, the initial transplantation of HESCs-derived cardiomyocytes into animal models aimed at biological pacemaker development. Formation of cardiomyocytes with pacemaker activity from HESCs has been extensively demonstrated (7, 11, 34). In conditions such as brachycardia, where the human heart has been electrophysiologically silenced or slowed down, these cells might lead to therapies for correcting defects in cardiac impulse generation. Kehat and coworkers (36) have transplanted a small number of HESC-derived cardiomyocytes in a pig heart model with complete atrioventricular block as assessed by three-dimensional electrophysiological mapping. Macroscopic pacemaker activity was observed in transplanted hearts but further analysis is mandatory to unravel the cellular events underlying this macroscopic observation. HESC-derived cardiomyocytes with pacemaker activity have also been implanted in the left ventricle of guinea pig hearts *in vivo* and optical mapping confirmed the spread of membrane depolarization from the site of cardiomyocyte injection to the surrounding myocardium (46). Again, the exact contribution of the donor cardiomyocytes to this phenotype remains unclear.

In a first attempt to optimize transplantation efficiency, 0.5–10 million Percoll gradient-enriched human cardiomyocytes from HESC were injected into noninjured hearts of athymic rats (38). The selective loss of noncardiac elements in the grafted cell mixture was noted in this study and grafts consisted predominantly of cardiomyocytes by 4 weeks. Heat-shock treatment of donor cells before implantation improved graft size. Notably, Ki-67 expression

and BrdU incorporation of transplanted cells *in situ* suggested greater proliferation in human cardiomyocytes than previously seen in their rodent-derived counterparts.

We have used an equivalent athymic rat model (47) to inject a differentiated, green-fluorescent protein labeled HESCs population (2 million cells per heart) comprising about 12% cardiomyocytes. The constitutive GFP expression allowed us to directly and unequivocally identify the grafted donor cells. An increasing maturation of transplanted cardiomyocytes was found *in situ* and the specific connexin 43 expression patterns at the interface of juxtaposed donor and host cardiomyocytes suggested the presence of nascent gap junctions at 4 weeks post engraftment. A high correlation between GFP and sarcomeric actin immune reactivity also indicated that a preponderance of the GFP-expressing donor cells at 4 weeks were cardiomyocytes, in agreement with previous results in the same model (38).

An early study on the engraftment of HESC-derived cardiomyocytes into infracted hearts was published by Kofidis and coworkers (48). A population consisting of about 1 million cells containing 15% cardiomyocytes was injected into the heart of SCID beige mice post MI. The application of GFP-labeled donor cells was combined with the administration of cytoprotective and anti-inflammatory agents and compared to controls. Measurements of heart function via magnetic resonance imaging (MRI) three weeks post transplantation as well as post mortem histology suggested improved human donor cell engraftment and myocardial restoration when protective agents were coapplied with cells. However, results of the study are limited by the small group size ($n = 5$) and the short term follow-up.

More recently, two other studies have reported the injection of hESC-derived cardiomyocytes into ischemia-damaged mouse or rat hearts and followed functional heart performance by MRI and echocardiography (rat study only). Laflamme and coworkers in Chuck Murry's group (5) transplanted a population of 10 million cells containing about 30% cardiomyocytes into healing infarcts in athymic nude rats 4 d after artery ligation/reperfusion. A subtle compound mix was developed that aimed at opposing ischemia-, inflammation-, anoikis-, and apoptosis-induced donor cell death after transplantation. Transplantation of cardiomyocytes pretreated with this cocktail into ischemic hearts blocked the progressive decrease in cardiac function when assessed at 4 weeks post infarction.

In a second study, Linda van Laake and colleagues in Christine Mummery's group (49) injected 2 million differentiated, GFP-tagged HESCs comprising 20%–25% cardiomyocytes (generated by a coculture approach with END2 cells) into nonobese diabetic/severe combined immune deficiency (NOD-SCID) mouse hearts post MI (terminal left anterior descendent artery ligation). Long-term survival of cardiomyocytes for up to 13 weeks, the latest time point tested, was reproducibly observed and selective cardiomyocyte enrichment was also noted in this study. In interesting similarity to the rat study, a cardiomyocyte-dependent attenuation in the progress of cardiac malfunction

was observed at 4 weeks post infarction. However, the formation of rather small donor cell grafts was observed in both studies. Finally, in the mouse study, significant functional improvement of the cardiomyocyte receiving group (observed at 4 weeks post MI) completely ceased upon reassessments at 12 weeks. While the mechanism(s) underlying the transient benefit is presently unclear, it seems likely that for long term improvement larger grafts consisting of functional and physiological integrated cardiomyocytes would be required.

Assuming that differentiated, HESC-derived cardiomyocytes might have a limited proliferation potential that restricts the formation of meaningful donor grafts after transplantation, other groups have tested the organ-specific differentiation of highly proliferative, pluripotent cells. GFP-tagged HESCs without any pretreatment were injected into the heart (0.5 million cells per heart) of nonsuppressed, immune competent Sprague-Dawley rats post MI (50). Surprisingly, evidence for cellular engraftment for up to 2 months was observed in 5 out of 11 hearts and even some cardiomyogenic differentiation of donor cells was suggested by RT-PCR analysis; unfortunately, no cardiomyocyte-specific immunohistology was provided. These data do not tally with our recent findings in a limited number of immune competent rats of the same strain (47). No survival of transplanted HESC-derived cardiomyocytes was observed in this study, even in the short-term. Another group has also implanted undifferentiated HESCs (0.5–1 million), HESC-derived EBs, and pieces of HESC-derived beating myocardial tissue into normal or infarcted myocardium of athymic nude rats (51). Results from this study clearly suggest that undifferentiated HESCs and predifferentiated human cells in EBs are not directed to form new myocardium after transplantation into normal or infarcted rat hearts but might form teratomas instead.

HESC-DERIVED CARDIOMYOCYTES FOR DRUG DISCOVERY—POTENTIAL TO DEVELOP NOVEL ASSAYS

As the development of a cell therapy from HESCs-derived cardiomyocytes faces many formidable challenges, the more immediate use of these cells might be in the area of basic research and drug discovery. The potential of using ES cells for this application has been reviewed recently by McNeish (52). Due to the rapid depletion of conventional druggable targets (53), pharmaceutical companies are increasingly relying on cell-based assays for identifying novel drug targets. Moreover, cell-based assays are increasingly being used at different stages of the drug discovery process, including evaluation of drug metabolism and drug toxicity. Regulatory authorities also encourage replacing animal models by respective cell-based assays, if available (54). The demand for cell-based assays has been so great that it has fueled other industries such as high-content screening. In contrast to developing cell therapies, producing cell products for R&D is much more straightforward and has the following advan-

tages: (1) limited requirement to generate products under cGMP, (b) no safety and immunogenicity concerns, and (c) no limitations regarding the utilization of genetically modified cell lines.

For many steps in compound screening and validation, it is desirable to use primary cells of a particular lineage, since they exhibit the most relevant phenotypes. However, many primary cell types, such as cardiomyocytes, are not readily available from human sources. This would be of high interest for some applications, whereby mimicking human heart cell-specific physiological features is of paramount importance. HESC-derived cardiomyocytes, therefore, might offer an attractive alternative (55). One area of drug development that could particularly benefit from the availability of *bona fide* human cardiomyocytes is safety pharmacology. Drug-induced cardiotoxicity has become a major concern for the pharmaceutical industry. A long list of marketed drugs have been shown to prolong the QT interval, which is associated with the risk of developing Torsade de Pointe, a dangerous form of ventricular tachycardia (56). The significance of the problem has prompted the International Conference on Harmonisation to publish regulatory guidelines on preclinical and clinical studies to assess new drugs for causing delayed ventricular repolarization, or QT prolongation (57). The conventional assay to assess QT liability relies on testing for inhibition of the human hERG potassium channel, since most drugs prolong QT by blocking this channel (58). However, the hERG channel assay generates a high rate of false positives, a typical example of which is verapamil (59). A cardiotoxicity assay based on HESC-derived cardiomyocytes should have in principle a higher predictive power than the hERG channel assay (60). Recent reports have suggested that mouse ESC-derived cardiomyocytes might be useful for such purpose (61, 62). We have now provided initial data that a purified population of HESC-derived cardiomyocytes could be used in a cell-based assay to predict drug-induced QT prolongation (40). Using the MEA system to measure field potential changes from spontaneously contracting cardiomyocytes, we demonstrated that E-4031, a prototypical hERG channel blocker, induces a concentration dependent prolongation of the action potential duration in our assay (Fig. 22.2). Further research and development is required to generate a homogeneous population of cardiomyocytes preferably with characteristics similar to those found in adult human ventricles. Microelectrode arrays in a 96-well format are currently available, which when utilized with a homogenous and defined cardiomyocyte population, would enable a high-throughput screening assay for drug-induced QT prolongation (63). Importantly, our purified cardiomyocytes that were generated in suspension culture can be dissociated and readily seeded in respective multi-well dishes.

Besides cardiotoxicity prediction, HESC-derived cardiomyocytes might be used to develop assays aimed at facilitating the development of new cardiovascular drugs. Currently, many such marketed drugs target extrinsic factors causing cardiomyopathy, for example, by lowering systemic blood pressure or reducing blood cholesterol levels. The availability of human cardiomyocytes

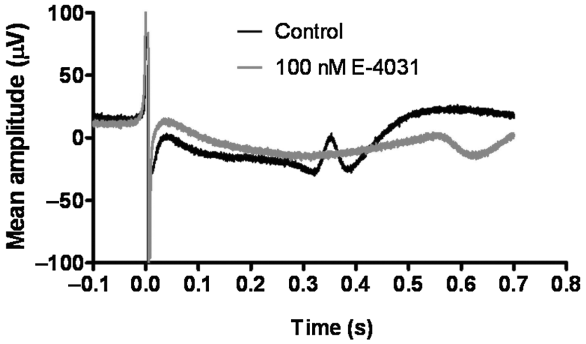


Figure 22.2. A cellular assay for assessing drug-induced QT prolongation. Transgenic cardiomyocytes after G418 selection were dissociated and plated onto a microelectrode array. Extracellular field potentials recorded under control conditions showed spontaneous cardiac action potentials with a duration, or QT interval, of 350 ms. Addition of the hERG channel blocker E-4031 prolonged the QT interval to 580 ms. (See color insert.)

might offer opportunities for the development of novel compounds to improve intrinsic cardiac function by inhibiting cardiomyocyte death in hypertrophy and post myocardial ischemia, increasing heart contractility in dilated cardiomyopathies, or inducing endogenous regeneration of cardiac tissue post myocardial ischemia (55). Since HESC can be genetically modified, it is feasible to generate cardiomyocytes harboring reporter genes to facilitate assay readout. Another interesting option is to develop cellular models cardiac disease by introducing respective loss- or gain-of-function mutations in HESC-lines or to generate novel HESC-lines from patients with inherited disorders by means of somatic cell reprogramming (64), techniques which will greatly facilitate drug discovery of the targeted disorder. HESC-derived cells thus offer considerable advantage over primary cells in that they can be genetically modified, cloned, and expanded as stem cells and then be differentiated to produce the final cellular product on demand. Such a production process will minimize batch-to-batch variations typically seen with primary cells derived from clinical sources.

CONCLUSIONS AND FUTURE DIRECTIONS

The reliable generation of HESC-derived cardiomyocytes with increasing efficiency has been broadly established in numerous labs in recent years. This development has been enabled by the development of robust protocols for the scalable expansion of the "raw material," pluripotent HESC (65). Successful attempts to upscale the mass differentiation of mouse ESC followed by the

sequential enrichment of pure cardiomyocyte populations by us and others (21, 28, 29, 31) is currently being transferred stepwise to human ESCs (23–25, 35, 40, 45, 66), thereby providing sufficient functional material for downstream applications.

Clearly, the therapeutic use of HESC-derived lineages is hampered by numerous hurdles, first of all by safety concerns; this might also explain the delay regarding the initiation of long-announced clinical trials of HESCs-based therapies by stem cell companies (67). In addition, while the successful implantation of HESC-derived cardiomyocytes in animal models has been principally proven, the long-term formation of functionally meaningful donor cell grafts in the heart has yet to be established. The problem of inefficient engraftment of single cells might be overcome by heart tissue engineering approaches (68, 69), that have now also been applied to cardiomyocytes from human ESCs (70, 71). Another stepping stone towards therapeutic applications is the recent derivation of clinical grade HESC-lines that for the first time provide "raw material" that has been fully generated under cGMP conditions (72). Nevertheless, the therapeutic area will clearly need reasonable time to mature, given the fact that providing useful, clinically compliant donor cardiomyocytes is, at best, solving only half of the issues related to developing a therapy. One additional roadblock that was not extensively discussed here pertains to the likelihood of immune rejection of HESC-derived cell products (73). Perhaps cardiomyocytes derived from a HESC line with MHC profile closely matching that of the recipient would allow for a successful allogenic graft. Alternatively, groundbreaking recent advances in somatic cell nuclear transfer (74) and somatic cell reprogramming (64, 75, 76) could one day allow generation of autologous cells and tissues, thereby resolving the immune rejection dilemma.

As outlined above, drug discovery and development should readily benefit from the availability of human cardiomyocytes in the near future. In the area of safety pharmacology, HESC-derived cardiomyocytes might provide a more relevant and predictive assay than hERG channel electrophysiology in assessing drug-induced QT prolongation. Genetically modified or even patient-derived HESC lines would enable the establishment of cellular models of human cardiac disease and facilitate research in pharmacogenomics to study the relationship between genetic variations and differential drug response among individuals (52). Although some of the hurdles facing the development of a cell therapy also apply here (i.e., reliable differentiation, scale up, reproducibility, etc.), the overall goal of generating cells for *in vitro* drug testing should be more readily attainable. Future work should focus on resolving the aforementioned issues, such as the necessity to generate homogeneous cardiomyocyte subtypes (e.g., ventricular cells), as well as the ability to establish more mature cells approximating the adult phenotype in heart muscle. Progress in these areas will help to advance and validate the use of HESC-derived cardiomyocytes for drug discovery applications.

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HUMAN EMBRYONIC STEM CELLS IN DRUG DISCOVERY

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INTRODUCTION

Cell based assays with high human relevance are urgently needed for preclinical drug discovery and safety testing. So far, the industry has mostly been employing animal based systems, both *in vivo* and *in vitro*, but these are not always cost-effective and, besides the issue of clinical relevance of the models, there are ethical and political concerns associated with the use of experimental animals (1). Novel improved *in vitro* models based on readily available, physiologically relevant human cells will result in better precision and more cost-effective assays ultimately leading to lower attrition rates and safer new drugs (2, 3).

It is now 10 years since the first human embryonic stem (human ES) cell lines were derived (4). No doubt, the ability to establish and culture human ES cells marked the birth of a new era in biomedical research. Pluripotent human ES cells have unique properties, that is, the capacity for indefinite self-renewal and for differentiation to all cell types of the human body, that make them exceptionally valuable in a wide range of applications (4, 5). Initial expectations focused mainly on applications in the area of regenerative medicine. Specifically, the ultimate potential of human ES cells lies in the clinical transplantation of differentiated cells for disorders that arise from cellular loss-of-function such as diabetes or Parkinson's disease.

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Less attention has been focused on the possibility of employing human ES cell technologies for the development of new drugs (3, 5–7). Yet the human ES technology has the potential to revolutionize the industrial drug discovery process (5, 6, 8). This chapter will describe the current state of the human ES cell technology platform and discuss some of the hurdles that have to be overcome before the full potential of human ES cell can be realized and the technologies be implemented within the field of drug discovery.

ADULT AND EMBRYONIC STEM CELLS

The expression "stem cell" by itself is not very precise since there are many types of stem cells. The different stem cell populations present varying degrees of developmental potency, however they also share similarities related to molecular mechanisms involved in maintenance of the stem cell state (9). In general, stem cells can be divided into two groups: adult stem cells and embryonic stem cells. The potential of the adult stem cells is widely recognized and these cells were employed clinically for many years in areas such as, for example, bone marrow transplantation.

In this chapter we will, however, focus on the present and future prospects of embryonic stem cells, and highlight some of the possibilities these cells will provide for the development of novel and improved industrial tools for drug discovery. Thus, human ES cells provide a defined and renewable source of normal cells for *in vitro* use, avoiding the necessity for immortalized, abnormal cell lines or repeated preparation of primary material from tissues. Furthermore, their unique properties also make human ES cells extremely valuable for genetic modification such as the stable introduction of reporter gene constructs, that could improve assay throughput or the introduction of specific genes to provide disease models (10).

MOUSE ES CELLS

The first human ES cell lines were originally derived only about a decade ago (4), but for a long time prior to this scientific breakthrough, researchers had been successfully employing mouse ES cells for various purposes. The first mouse ES cell lines were derived in 1981 (11) and already in 1988 it was demonstrated that mouse ES cells could be cultured in the absence of feeders (12, 13). One of the major factors involved, though not the only one, is the leukemia inhibitory factor (LIF) which contributes to the maintenance of pluripotency in mouse ES cells by activating signaling via the STAT3 pathway (14). By combining LIF and BMP4 in N2B27 medium, it is possible to culture mouse ES cells in defined conditions without a feeder layer (15). The mouse ES cell technology has for many years been at an industrially compliant level and provided numerous valuable tools. Most dramatic is the impact of genetically

engineered mice in which gain-of-function and loss-of-function of specific genes can be studied in an *in vivo* setting, applications that rendered the scientists behind these methods the Nobel Prize in medicine 2007. Genetically modified mice allow researchers to validate targets and to screen compounds for efficiency and safety as well as to define pharmacokinetics during drug discovery and development (6, 7, 16).

The *in vitro* use of mouse ES cells has resulted in a considerable wealth of data regarding the molecular mechanisms directing lineage specification and has paved the way for the human ES cell research field. There are, however, many noteworthy differences between humans and mice with respect to patterns of development, expression of differentiation markers, as well as expression profiles of cytokines, cell cycle, and cell death-regulating genes (17). This has left a major gap in our understanding of human embryonic development, tissue specification and organogenesis. The mouse diverged from our common ancestor 75 million years ago, which has led to critical differences in anatomy, biochemistry and physiology starting from the earliest developmental stages (8, 18). In order to increase the human relevance and the predictive value of *in vitro* models, major efforts on human ES cell related research will be required to fully explore and take advantage of these cells' unique potential.

HUMAN ES CELLS—TOWARDS INDUSTRIAL APPLICATION

Current Culture Protocols

In contrast to the mouse ES cells, the culture and maintenance of human ES cells has been, and still is challenging (5, 8). Therefore, not surprisingly, one of the most important issues for human ES cell research today is the proper maintenance and expansion of the undifferentiated cells. Pluripotent human ES cells are generally expanded by mechanical passage in cultures comprising feeder layers of mitotically inactivated fibroblasts. The main advantage of this mechanical transfer method is the possibility to perform a positive selection at every passage by manually separating undifferentiated human ES cells from differentiated cells. In this delicate process, selected areas within individual human ES cell colonies are mechanically cut into small pieces and subsequently transferred to fresh culture dishes. This culture method has been proven reliable and very stable and is used by many groups (4, 19–21). Nevertheless, the method is very labor intensive and time-consuming, making it difficult and expensive to process many cells simultaneously. It is therefore not suitable for scale-up of cell production (22).

Improved Protocols for Human ES Cell Maintenance

Mouse ES cells can easily be expanded by enzymatic dissociation to single cell suspensions, therefore a number of groups have started to develop various

enzymatic passaging methods for human ES cells, which are superior to mechanical passaging with respect to speed and simplicity. However, there are limitations associated with these methods as well. Several enzymatic propagation protocols require dissociation of human ES cells to small clusters of a narrow size range. This causes problems in the standardization and automation of cell expansion and limits the applicability to several important experimental techniques, such as for example, FACS, cell transfection, as well as various high-throughput screening applications. Another important issue with respect to industrial application is the requirement to scale up the production of human ES cells. Facing the increasing demand for human ES cells for different drug discovery and development assays alone, a protocol that allows the use of high split ratios when necessary would be highly beneficial. Recently, Hasegawa et al. reported a method for the selection of human ES cell sublines with high replating efficiency so that selected sub-lines can be propagated at split ratios of 1:50 to 1:100 employing mouse feeders in combination with Trypsin/EDTA (23). In our laboratories, we have been able to demonstrate that by combining the use of human feeder layers and a recombinant trypsin, it is possible to propagate human ES cells by single cell enzymatic dissociation at split ratios in the same range as reported by Hasegawa (23). Importantly, this method can be applied widely to normal human ES cell lines and it does not require selection of sub-lines (22).

A recent study combined enzymatic passaging of human ES cells with a synthetic ROCK-inhibitor resulting in markedly increased cell survival during propagation (24). Taken together, these results further underlines the potential of enzymatic passaging protocols to meet the industry's requirements and to produce human ES cells in large quantities for example, for high throughput screening (HTS) applications.

Mouse ES cells can be cultured in the absence of supportive feeders, both in serum containing media as well as in chemically defined media (15). Human ES cells can be propagated in the absence of feeder cells on a suitable growth substrate such as Matrigel™ using feeder cell conditioned medium (25). The first derivation of human ES cells in a defined medium was reported (26) and several defined media for feeder-independent propagation of human ES cells have been described (27, 28). No doubt, the use of chemically defined media is preferable for any application within the field of drug discovery and development (26, 29). Nevertheless, despite the large number of reports on various culture conditions for human ES cells, no universal protocol has been widely adapted. Attempts to identify, select, and standardize robust and universally applicable protocols for human ES cell culture are currently being undertaken, for example in the large consortium of the International Stem Cell Initiative (30). The European Union's 7th Framework Program is also aiming to address the challenges regarding human ES cell cultivation.

Much progress has been made over the recent years concerning derivation, expansion, and characterization of human ES cells. Therefore, it is now realistic

to believe that human ES cell lines can soon be generated and cultured in a standardized manner for various cell-based applications in drug discovery and development. We consider the consequent automation of processes as well as the use of bioreactor technology as promising paths to bring human ES cell production to an industrial level (31, 32). In a parallel effort though, it is equally important to develop adequate, robust and efficient characterization methods to verify the quality of the manufactured cells.

Quality Control of Human ES Cells

Pluripotent human ES cells have the capacity for extensive, or possibly even indefinite, self-renewal *in vitro*. Several investigators have reported cultures of human ES cells for more than 200 passages and we have similar experiences from our own laboratories and cell lines (33).

The ability to maintain human ES cells in culture for extended periods of time is a great benefit, but also entails that rigorous quality control is an absolute demand to avoid the loss of human ES cell attributes over time. The quality of human ES cells *in vitro* can change rapidly due to for example, spontaneous differentiation or genomic alterations. Principles, known today from the industrial production of other mammalian cell types, such as master cell banks, working cell banks and controlled batches, must therefore also be applied for any human ES cell line.

Human ES cells are characterized by their expression of a number of molecular markers, largely consisting of markers previously used for mouse ES cells and human embryonic carcinoma (EC) cells. For the purpose of continuous quality control of human ES cells in culture, combinations of markers can be measured on the protein level using immunocytochemistry (34), FACS (35, 36) or on the gene expression level using real time quantitative PCR or focused microarrays (37, 38).

Various genetic aberrations have been acquired by cultured human ES cells as reported by several groups (21, 39, 40) and therefore cytogenetic evaluation must be an important element in the routine quality control of the cell lines. Unfortunately, it is not clear in which way culture conditions and the occurrence of chromosomal abnormalities relate to each other. Individual human ES cells in a mosaic-like population of cells may harbor a genetic aberration, which renders an advantage in survival or growth. The likelihood that these cells will rapidly grow in number is increased in an enzyme-based passage system with high split ratios, since the proliferation pressure on each and every cell in such a system is higher. This can be highly problematic in the context of industrial mass culture of human ES cells and it is therefore appropriate to point out the importance of thorough genomic surveillance at regular time intervals.

Finally, the ultimate requirement of any human ES cell line is pluripotency. The potential to give rise to derivatives of all embryonic germ layers

(mesoderm, ectoderm, and endoderm) can be tested *in vivo* by xenografting undifferentiated human ES cells to immunodeficient SCID mice, followed by the histopathological evaluation of the resulting teratoma (4, 41, 42).

Pluripotency can also be assayed *in vitro* by either allowing the human ES cells to differentiate spontaneously (22, 41, 43) or by inducing differentiation via an embryoid body (EB) step (42). Specific cell types derived from different germ layers can subsequently be identified by immunocytochemical analysis.

Human ES Cell Technology Applied in Drug Discovery

The aim for the pharmacological industries is to develop the next generation of safe and effective drugs. This task requires the availability of high quality and relevant human cell models to evaluate novel targets and candidate compounds in a close to physiological environment. Due to their unique attributes, human ES cells have major potential uses within the area of drug discovery and toxicity testing. The use of human ES cells and their differentiated derivates in the drug discovery process spans from early target identification and validation studies via the use in lead generation, cellular screening, metabolism and pharmacokinetic studies, to the use of various stem cell technologies in toxicity testing (8).

Human ES Cells in Screening

The HTS technology of today has widen the field of applications for cell-based methods in drug discovery. Cell-based HTS today may be based on 96, 384 or even 1586 well formats (6, 44) and it has been employed with many different cell types. As discussed earlier, the culture conditions for mouse ES cells are well explored today and mouse ES cells can be cultured in chemically defined medium on a feeder free surface (15), facilitating and simplifying the design and analysis of any HTS. By generating genetically modified mouse ES cell lines, the application range is very wide. For example, by introducing reporter genes such as GFP directly into promoters of special interest, relevant effects can be studied in specific cell types. At present, more specific differentiation protocols are required to advance the application of both human and mouse ES cells in drug discovery. One way forward may be to enrich the cell population via lineage specific expression of antibiotic-resistance proteins (5), a method that has been successfully employed to select and enrich for neural precursors (45, 46) as well as for human ES cell-derived cardiomyocytes (47).

The experience generated from the mouse ES gene target technology needs to be translated and adapted to human ES cells. As discussed earlier, even if the advances within the human ES field have moved forward fast, the validations of the recently reported chemically defined and feeder free culture systems needs to be performed on a wide range of human ES lines. Furthermore, extensive characterization is needed to ensure the robustness of the new

culture systems. Not surprisingly, the human ES cells provide the scientists with challenges also within the transgenic technology, but there are good reasons to be positive and expect that reporter lines based on human ES cells soon can be easily and precisely generated (5, 48, 49). In 2001 Eiges et al. reported the first transfection of human ES cells (50) and in 2007 the first human ES line harboring a reporter line became commercially available (49). No doubt, the ability to target well-expressed loci allows for easy creation of transgenic human ES cell lines expressing markers of interest. Such tools would thereby enable the usage of human ES within HTS, and bring this technology an important step forward. In addition, genetically engineered human ES cells are important for understanding differentiation pathways and for identifying factors needed to manipulate cell lineages, as well as for identifying and validating disease targets using for example siRNA- or compound-library screens (3).

Human ES Cells for Developmental Toxicity Testing

The fields of toxicology and public health share the common need for relevant tests that can determine the potential hazard of a compound exposure. These compound tests could be employed both on new compounds during the development stage, but also to test already existing compounds for which few or no health data exists (51). Within the European Union alone, more than 30,000 chemicals are intended to be tested for toxicological effects including teratogenicity (52) based on European chemical policy. Traditionally, developmental toxicity is performed in animal experiments using rodents. However, by the introduction of the EST (the mouse embryonic stem cell test) (53) the ES cell technology has made it possible to improve the precision of the tests *in vitro* as well as to reduce the number of tests that is based on the use of animals. The EST measures the growth inhibition of mouse ES cells (D3) and 3T3 fibroblasts as well as differentiation of mouse ES cells to indicate the embryotoxic potential of chemicals. Presently much effort is spent in refining the current prediction model (Fig. 23.1) in order to further enhance the predictive power of the EST (54).

It is very important to identify the potential toxic hazard of novel drug candidates at an early stage of development in order to reduce late stage attrition. However, models such as the EST are still restricted by the lack of relevant and validated cell types. The optimal cell types would obviously be human and they should display the appropriate organ phenotype. Specialized cell types can be derived via differentiation of human ES cells. One advantage of human stem cell technologies is that the use of cells derived from a single human ES cell line would minimize the donor and preparation variability. The combination of genetic engineering with the use of human ES cells or their derivatives shows great promises to their future use in the field of toxicology.

The areas where most safety related attrition of new drug candidates occur are the cardiac and hepatic fields. Therefore, the rest of this chapter will focus

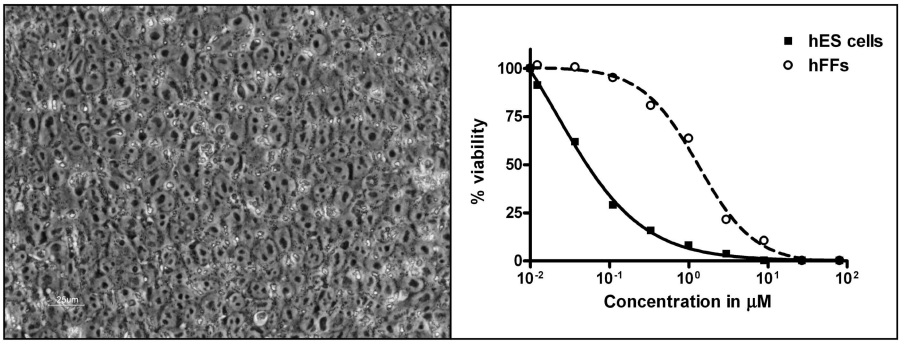

Figure 23.1. (a) Photomicrograph of a pluripotent human ES cell colony, (b) Dose response curve for a strong developmental toxicant tested on human ES cells versus human foreskin fibroblasts (hFFs) in a 96-well plate based cytotoxicity test. The data were obtained by measuring intracellular ATP content per well and were normalised to the untreated control. The pluripotent human ES cells display a considerably higher sensitivity to the embryotoxicant in comparison to the hFFs.

on cardiomyocytes and hepatocytes derived from human ES cells and how *in vitro* assays based on these cells can improve the drug discovery process.

Human ES Cell-Derived Cardiomyocytes

About two decades ago it was first reported that mouse ES cells could differentiate into cardiomyocyte-like cells (55). After the first isolations of human ES cell lines, several groups also reported on the establishment and characterization of spontaneously contracting cells derived from human ES cells (56). The availability of beating human cardiomyocytes would provide drug discoverers with improved model systems for investigating the efficacy of cardio active substances and for assessing potential adverse side effects of new drug molecules. However, at present, human primary cardiomyocytes are not available for pre-clinical drug discovery due to lack of donor material and practical difficulties associated with myocyte isolation from heart tissue. As an attractive alternative, human stem cells can be exploited to derive functional cardiomyocytes for *in vitro* applications in drug development (3, 8).

The most common method for obtaining cardiomyocyte-like cells from human ES cultures is by inducing cell differentiation through EB formation (57). However, this differentiation protocol is rather laborious and the final yield of cardiomyocytes has so far been relatively low, making it necessary to invest time and resources to develop improved protocols for inducing and sustaining cardiomyocyte differentiation from human ES cells. It should be noted that the knowledge about human cardiogenesis is limited and many of the critical factors regulating the signaling cascades involved in this process remain to be determined. Recently, directed differentiation systems based on

the application on specific growth factor combinations (58, 59) or END-2 coculture (60) indicate that up-scaling of cardiomyocyte production is feasible. Importantly, human ES cells are also excellent models to study the molecular mechanisms of regulation involved in cardiogenesis and the results from such studies will be instrumental for further improvements of current differentiation protocols (61, 62) Transgenic enrichment strategies aimed at overcoming the heterogeneity of the differentiated populations have also been pursued (47). Interestingly, the drug-resistant cardiomyocytes responded to chronotropic stimulation and displayed cardiac specific action potentials, indicating that their functionality was retained irrespective of the genetic modification of the stem cell population (47).

The bulk of characterization data obtained from human ES-derived cardiomyocyte are encouraging. For example, the morphology and ultrastructure of human ES cell-derived cardiomyocytes share similarities with adult cardiomyocytes although the myofibrillar and sarcomeric organization indicate an immature phenotype in the stem cell-derived population (57, 63–65). The embryonic/fetal phenotype is not unexpected since the process of *in vitro* differentiation is substantially different from the *in vivo* situation in many aspects. Nevertheless, cardiac myocytes of different phenotypes have been identified (e.g., atrial, nodal, and ventricular) further underscoring the usefulness of human ES cells for generation of multiple sub-types of cells from the same source (66, 67). Human ES cell-derived cardiomyocytes also express cardiac specific markers on the gene and protein level (8). Another advantage of cardiomyocytes derived from human ES cells is that they can be maintained in culture for extended time periods, up to several weeks and sometimes even months, without losing their spontaneous contractile capacity. This allows for repeated noninvasive examination of the same cell preparations and also makes studies of *in vitro* myocyte maturation possible.

The application of cardiomyocytes derived from human ES cells in drug development can generally be divided into cardiac drug discovery and safety assessment of novel compounds in development. The availability of normal human cells is likely to improve the precision of almost any *in vitro* assay since targets and leads can be studied in close to physiological environments. Genetic engineering makes it possible to introduce or delete genes in the human ES cell-derived cardiomyocytes, which in turn opens up a range of new opportunities for the development of *in vitro* cell based assays for target identification and validation.

One of the main causes of drug safety liabilities is cardiotoxicity. The primary reason for ventricular repolarization disturbances is blockage of the hERG channel, which may cause a QT interval prolongation and subsequent ventricular tachycardia. Thus, many of the established *in vitro* test systems focus on the analysis of drug action on hERG channel function. Notably, QT prolonging drugs belong to diverse therapeutic classes including both cardiovascular and noncardiovascular drugs (68). Obviously, there is a substantial need for identifying the risk of drug-induced QT prolongation as early as possible

during drug development. Presently, due to the limited availability of human cardiomyocytes, scientists are restricted to using transformed cell lines and animal-derived cells or tissues in the pre-clinical phase. To evaluate drug induced QT prolongation, the optimal sub-type is the ventricular cardiomyocyte. The electrophysiological properties of these cells can be investigated in single cell preparations using patch-clamp techniques (67). Clusters of human ES cell-derived cardiomyocytes can for example be applied for transmembrane action potential (TAP) recordings or in micro electrode arrays (MEAs) in which rhythm, route and origin of excitation, repolarization, and conduction can be analyzed through the recorded extracellular field potential (69). Cardiac field potential properties have been studied before and their correlation with cardiac action potential data has been described (70). Recently, the effect of D-sotalol on delayed repolarization was demonstrated in human ES cell-derived cardiomyocytes using a MEA system (71). In addition, incubation of human ES cell-derived cardiomyocytes with the hERG-channel blocker E-4031 resulted in action potential prolongation (66, 72), (Fig. 23.2). These results lend further support to the development of improved assays based on human ES cell derived cardiomyocytes for safety pharmacology.

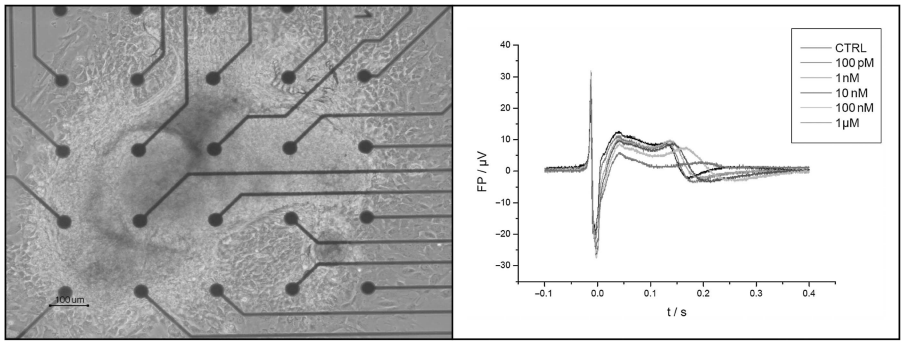

Figure 23.2. Spontaneously contracting cardiomyocyte clusters were derived and isolated from human ES cells, and subsequently plated on microelectrode arrays (MEA) (a). After adhesion of the clusters to the MEA surface, the electrical activity was recorded as the extracellular field potential by the substrate integrated electrodes of the MEA. The addition of the QT-prolonging drug, E-4031, causing hERG channel blockade was measured as a delayed repolarization of the cardiac field potentials (b). Increasing drug concentrations were added to obtain cumulative dose response curves. Notably, E-4031 did not affect the beating frequency over the experiments but caused a sustained prolongation in the low nanomolar concentrations of the cardiac field potential. This is indicative for a hERG channel block known to be caused by this compound. The data were recorded in collaboration with Dr Thomas Meyer (Multichannel Systems, Germany). (See color insert.)

Human ES Cell-Derived Hepatocytes

The human liver controls a number of key metabolic functions, and the most abundant cell type of the liver is the hepatocyte. One immediate application of human ES cells would therefore be to differentiate them into hepatocyte-like cells. The metabolism in the liver and the interplay between the different cell types of the liver and other organs are major drug targets for, for example, metabolic and dyslipidemic diseases. Moreover, unpredicted human metabolism is today one of the major reasons for removal of drug candidates from pharmaceutical projects (8). Finally, hepatotoxicity is causing more than 50% of failures due to toxic effects, and is one of the dominating causes of late-stage attrition among pharmaceutical compounds. Unfortunately the complexity and function of the liver is not reflected by any cell type available for *in vitro* applications in drug discovery today. None of the commercially available hepatocyte-like cell lines produces relevant levels of metabolizing enzymes and they have a distribution of other important proteins that is substantially different from the hepatocyte *in vivo* (3, 73). Primary human liver cells are available, but they rapidly lose functional properties when cultured *in vitro*, and therefore the usefulness of these cells relies on repeated sourcing, which is a major limitation (3, 74). Stem cell differentiation into hepatocytes is therefore of great interest for drug discovery since an easily accessible source of high quality hepatocytes substantially would facilitate the development of new drug discovery strategies and provide possibilities to perform *in vitro* metabolism studies and toxicity assessment with high relevance.

Results from differentiation of mouse ES cells have shown that hepatocyte-like cells can be derived from these cells using a variety of different culture conditions. However, the outcome from these different culture protocols never gave rise to a homogenous, or close to homogenous, hepatocyte-like population but resulted instead in heterogeneous cultures containing several other cell types besides the hepatic lineage (75, 76). Gouon-Evans et al. have showed that the efficiency of mouse ES cell differentiation and maturation can be improved by using a combination of activin A, BMP-4 and bFGF. In this way, an increased proportion of cells positive for α-fetoprotein and albumin was generated (77). In contrast, a recent report from Parashurama and colleagues suggested follistatin to be superior to activin when the amounts of endodermal cell types were compared after differentiation of mouse ES cells. Furthermore, the authors reported on a more homogeneous cell population using this novel approach (78).

The first reports from the human ES field described spontaneous differentiation of such cells without specific efforts to enrich for hepatocyte-like cells (79). These initial approaches were later followed by directed differentiation strategies and modified culture conditions that supported hepatic differentiation of human ES cells (80–84). The cells obtained showed the expected hepatocyte-like morphology and expressed some hepatocyte-associated markers,

for example, albumin, α-1-antitrypsin, and cytokeratin 8 and 18. Also on the RNA level, typical hepatic transcription factors were expressed by the hepatocyte-like cells (80–84). The functional analysis of these cells indicated glycogen accumulation, inducible Cytochrome P450 activity, production of urea and albumin, and uptake of indocyanine green. For the industrial use of human stem cell-derived hepatocytes, the presence of specific biotransforming enzymes in the cells are of great importance. Soto-Gutierrez and coworkers provided the first evidence for actual drug-metabolizing effects in human ES-derived cells (85). However, a first detailed study on more specific metabolism of pharmaceutical compounds by human ES cell-derived hepatocyte-like cells were recently presented (86) when several drug-metabolites were detected simultaneously by using LS-MS. It was for many years not established if the described hepatocyte-like cells originate from a population of cells differentiating from definitive endoderm, which is considered the origin of the liver in mammalian development. Cells with de-toxifying capacity and a phenotype resembling hepatocytes are also part of the extraembryonic endoderm. Due to the limited availability of specific markers capable of discriminating between extra- and definitive endoderm, the results from previous studies have been somewhat inconclusive in this respect (3). Recent studies have begun to shed some light on this issue and the derivation of definitive endoderm (Fig. 23.3) from human ES cells (87), and later the derivation of hepatocyte-like cells from definitive endoderm, was reported (86, 88). For hepatocyte differentiation, human ES cells were induced by activin A, and further treated with FGF-4 and BMP-2. The resulting cells showed expression of hepatic genes and the presence of protein-markers, in addition to exhibiting functions similar to

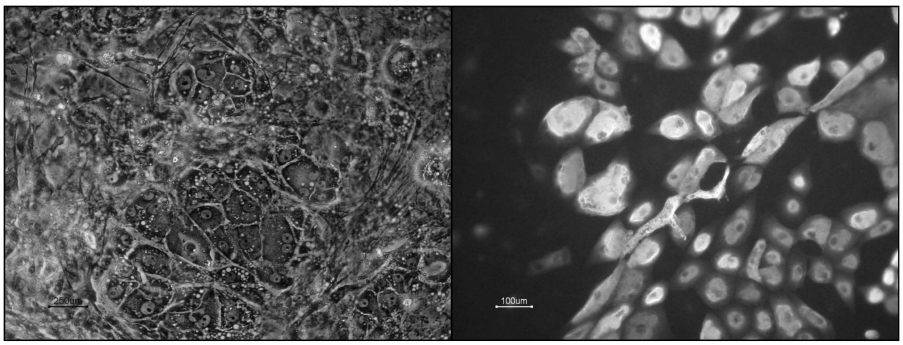

Figure 23.3. Phase contrast image showing hepatocyte-like cells derived from hES cell line SA002 via definitive endoderm (DE-Hep) after 21 d in culture. The cells exhibit a hepatocyte-like morphology; they are big, rhombic, granulated and often binucleated. Immunofluorescent labelling of CYP3A4/7 in DE-Hep derived from SA002 after 38 d in culture. (See color insert.)

adult liver cells. Importantly, these metabolically competent cells were derived using a protocol differentiating the cells specifically via definitive endoderm and liver progenitors (86). In the near future we should be able to control the further differentiation of the definitive endoderm cells towards the hepatic lineage with high yield, and specifically determine if these cells are functional enough to be useful for broad drug discovery and toxicology applications. Obviously important functions such as metabolic competence, biotransformation capacity, and transportation of exogenous compounds need to be further investigated.

Within the drug discovery process the hepatocytes represent key cells and they are used for testing novel targets in, for example, metabolic and dyslipidemic diseases. These cells have also broad uses in studies of liver metabolism and pharmacokinetic properties of novel compounds, as well as in hepatoxicity testing; so no doubt, the need for novel improved models for human hepatocytes is great assessment. It should be noted that unexpected human metabolism and pharmacokinetic problems comprise a major reason for removal of potential new drugs from pharmaceutical projects. Primary hepatocytes from rat and human are currently used as the gold standard in drug metabolism studies, but the tools available today still lack accurate predictive power (89).

Unfortunately, toxicity is often observed only in the late phases of the drug discovery process. Much effort has been spent in order to establish predictive human hepatic cell populations that could be assayed *in vitro*. The models available are based on human cancer cell lines or isolated primary cells, but these have significant drawbacks. The best option today, with respect to functional differentiation, is human primary hepatocytes, but issues related to the acquisition and variability in this material result in practical constraints that limit its usefulness. Therefore, numerous animal experiments are still performed, however without satisfying predictability. Recently it was reported that the mouse ES cells had the potential to differentiate into functional hepatic cell thus providing access to cell material for assessing hepatotoxicity (90). All effort should therefore be taken into applying this knowledge into the human ES cell differentiation protocols. Finally, the human liver is an organ consisting of many cell types besides the hepatocyte. For example Kupffer cells, stellate cells, and cholangiocytes are adding important pieces to the complex architecture of the liver. Therefore, to be able to better understand and predict positive and negative effects of new drugs *in vitro*, more complex models are required. This further underscores the potential for human ES cells as a source for human hepatotoxicity models, since basically any cell type can be generated from the pluripotent stem cells. Although speculative, there is a great hope that human ES cell research will pave the first way to mimic simple liver tissue, thereby dramatically improve the chances to accurately predict human toxicity *in vitro*.

Without any doubt, a reliable source of human hepatocytes would be invaluable for *in vitro* experiments throughout the entire drug discovery process.

iPS Cells as Alternative or Complement to Human ES Cells

Recently, human somatic cells were successfully reprogrammed into pluripotent stem cells (91–93) by introducing different transcription factors into skin cells. These induced pluripotent stem (iPS) cells were proven pluripotent in teratoma studies. No doubt, this method eliminates the ethical issues concerning the use of surplus embryos for the derivation of stem cells, making iPS cells more accessible for the research community around the world. Further, iPS cells could facilitate and improve the generation of relevant patient- and disease specific pluripotent stem cells. However, additional research is needed to learn more about the importance of the differences at the level of transcriptome and epigenetic modifications of DNA that is reported for iPS cells and human ES cells (91–93). Finally, iPS cells will also face similar difficulties as the human ES cells do when it comes to purity of cultures as well as to the challenge of how to direct the cells into a specific cell type.

CONCLUDING REMARKS

Human ES cells provide unparalleled possibilities for *in vitro* studies of normal human cells for basic research as well as for industrial applications. We dedicated this chapter to the discussion of novel applications of human ES cells in drug discovery. The human origin, the capacity of indefinite self-renewal and the potential to differentiate into highly specialized cell types make human ES cells unique for use in an *in vitro* assay platform. Human ES cell-derived hepatocytes and cardiomyocytes can give rise to improved models to assess adverse effects of new drugs early in the development phase. No doubt, there are challenges to deal with, such as the scale-up requirements and the need to improve the homogeneity and yield of the human ES cell-derived functional cells using enrichment or selection techniques. However, the human ES cell research community is now in an intensive and highly productive phase and it is likely that many of the most important hurdles, such as scale-up of production and directed cell differentiation will be overcome in the coming years. Provided the appropriate resources, in combination with sound stem cell research guidelines and regulations, the human ES cell technology platform has the potential to exponentially improve many aspects of drug discovery and safety pharmacology.

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24

CHARACTERIZATION AND CULTURING OF ADIPOSE-DERIVED PRECURSOR CELLS

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INTRODUCTION

Bone marrow mesenchymal stem cells (BM-MSCs) can be seen as promising candidate stem cells in regenerative medicine. However, the aspiration of the bone marrow is morbid and the actual volume of bone marrow that can be procured from the patient is limited. An alternative potential source of stem cells obtainable in large amounts that has a reasonably high concentration of

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stem cells is adipose tissue. Multipotent stem cells isolated from adipose tissue are capable of differentiating into adipogenic, chondrogenic and osteogenic lineages when subjected to appropriate induction stimulants (1). Donor site morbidity limits the amount of marrow that can be obtained and thereby extends the time in culture required to generate a therapeutic cell dose. Thus, the volume of human marrow taken under local anesthesia is generally limited to no more than 40 mL and yields approximately 2×10^6 nucleated cells (2). Obtaining a larger volume of bone marrow will necessitates the use of general anaesthesia, increases donor site morbidity (3, 4) and further dilutes the stem cell fraction with stem cell-free blood (2). By contrast, a typical harvest of adipose tissue, under local anaesthesia, can easily exceed 200 mL and yield at 2×10^8 nucleated cells per 100 mL of lipoaspirate (5).

DESCRIPTION OF THE ADIPOSE TISSUE

Adipose tissue consists of adipocytes embedded in a vascularized loose connective tissue, which is divided into lobules by stronger fibrous septa carrying the larger blood vessels, in order to provide each lobule with an independent blood supply. Within the lobules, the cells are round or polygonal. Loose connective tissue and septa both contain all other cellular components characteristic of fibrous tissue. Fat deposits serve as energy stores, sources of metabolic lipids, thermal insulation (subcutaneous fat), mechanical shock absorbers (soles of feet, palms of hands, gluteal fat, synovial membranes) (Gray's Anatomy). It occurs in abundance in subcutaneous tissue, around the kidneys, in the mesenteries and omenta, in the female breast, in the orbit behind the eyeball, in the marrow of bones, in the subcutaneous tissue of the foot, and as localized pads in the synovial membrane of many joints.

The adipose tissue has a remarkable ability to undergo considerable changes in volume during the lifespan of an individual. Although relatively small increases in volume can be accommodated by changes in the amount of lipid stored in individual adipocytes (hypertrophy), larger changes are mediated by the generation of new adipocytes (hyperplasia) accompanied by coordinated expansion and remodelling of the adipose vasculature (6, 7). This dynamism may be mediated by resident stem cells, which can be enzymatically isolated adipose tissue (Fig. 24.1) and separated from the buoyant adipocytes by centrifugation. A more homogeneous population then emerges in culture under conditions supportive of BM-MSC growth.

The most important features of adipose tissue as a cell source might be the relative expendability of this tissue and the consequent ease of procurement of large quantities with minimal risk. Liposuction is a common surgical procedure: more than 450 000 elective liposuction surgeries were performed in the United States during 2004 (American Society for Aesthetic Plastic Surgery 2005). It is also safe: an American Society for Dermatologic Surgery study of outpatient cosmetic liposuction performed between 1994 and 2000 showed

Structure-function

Adipose	Bone
Soft tissue	Hard tissue
Energy reserve	Skeletal support
Mechanical damping	Calcium storage
Insulation	Hematopoiesis

Endocrine roles

	Adipose	Bone
Responds to	Insulin (diabetes)	PTH
	Growth hormone	1.25 Vitamin D3
	Thyroid hormone	IGF
Secretes and synthesizes	Leptin	
	Adiponectin	BMP
	Angiotensinogen	TGF beta

Types of adipose tissue

Tissue type	Function
White	Energy storage
Brown	Skeletal support
Mechanical	Weight-bearing Stress protection
Bone marrow	Space occupying (passive), lipid metabolism, hematopoiesis, osteogenesis (acrtive)

Figure 24.1. Comparison of properties and function of bone marrow and adipose tissue.

zero deaths on 66 570 procedures and a serious adverse event rate of 0.68 per 1000 cases (8). Similar statistics can be found for Europe.

PUMP VERSUS SYRINGE PROCEDURE

Presently, the aspiration of adipose tissue for autologous transplantation has been largely restricted to the syringe procedure, as the vacuum pump was considered unsuitable for aspirating fat for autologous lipoinjection. As 90% of the adipocyte's cytoplasm consists of lipids, under the negative pressure of the vacuum pump, adipocytes were lysed with foam observed in the tubing connecting the cannula to the pump. This issue seemed to work against the paradigm of aspirating fat with a vacuum pump. However, this lysis effect is also present in syringe aspiration, thus resulting in low numbers of surviving adipocytes and/or BM-MSC. Compounding the problem is that adipocytes may have limited proliferative potential. These characteristics further contribute to poor graft survivability and reduction in the tissue amount aspirated from either procedure. Rethinking, an alternative strategy is to liberate the

adipocytic precursor cell populations from within the graft tissue and use a more potent cellular mixture. Although these adipocytic precursors are present within the graft, these cells are trapped and surrounded by necrotic tissue. There is also decreased metabolite diffusion within the graft, further aggravating its necrosis. Instead, adipocytic precursors like preadipocytes and mescenchymal precursor cells have high proliferative potential and are capable of adipocytic repopulation. These indigenous characteristics would be unlocked and could contribute to eventual survivability of the implanted graft. Leading from this, the knowledge of cell types in the heterogeneous population that did survive the aspiration processes, both syringe and pump liposuction, therefore becomes a relevant issue for applications in regenerative medicine.

From a study performed by our group, there were comparable metabolic activity and adipogenic responses from cells derived from both vacuum and syringe lipoaspiration methods. Our study initiated the discussion toward the possibility of aspirating fat due for transplantation, with a vacuum pump.

ADIPOSE-DERIVED STEM CELLS (ADSCs) PROPERTIES AND CHARACTERIZATION

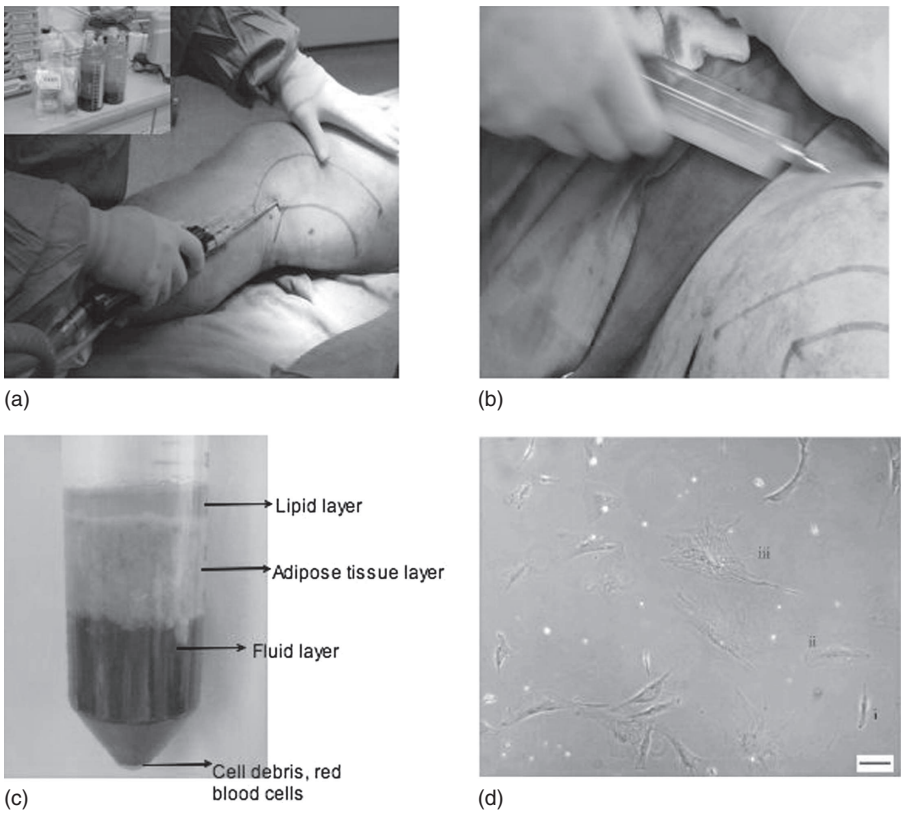
A common misconception of the general public associated with ADSCs is that the donor must be overweight or obese to have sufficient adipose tissue available for harvest. Comparing adipose tissue harvested from obese body mass index (BMI) patients with that from normal BMI patients, did not show any significant increased amounts of stem cells (9), however further studies on larger patient groups need to confirm this preliminary data. In addition, preliminary data also showed that there were no significant differences between ADSCs from patients with and without type II diabetes in terms of their BM-MSC characteristics and osteogenic and adipogenic potential (10). Besides direct differentiation of ADSCs to the desired cell types, assays performed on the factors secreted by ADSCs also revealed the presence of multiple angiogenic and antiapoptotic cytokines (11).

CELL SURFACE CHARACTERIZATION

The cell surface phenotype of human ADSCs is quite similar to BM-MSCs (10). Both stem cell types are positive for CD105, STRO-1, and CD166. CD117 (the stem cell factor receptor) is expressed by embryonic stem cells, hematopoietic stem cells, BM-MSCs, and ADSCs (12–14). In addition to these multipotent markers, ADSCs and BM-MSCs display numerous other molecules including CD29 (beta-1 integrin, which plays a critical role in therapeutic angiogenesis [15]), CD44 (hyaluronate receptor, which is crucial to the development of neoextracellular matrix and plays a role in numerous pathologic

and physiologic events), and CD49e (alpha-5 integrin, important for cell adhesion to fibronectin). ADSCs also express high levels of CD54 (ICAM-1) when compared with BM-MSCs (16). ICAM-1 is a member of the immunoglobulin supergene family and can be upregulated in response to numerous inflammatory mediators and cytokines (17). In addition, ADSC lack the expression of known hematopoietic and endothelial markers such as CD3, CD4, CD11c, CD14, CD15, CD16, CD19, CD31, CD33, CD38, CD56, CD62p, CD104, and CD144. ADSCs do not express the HLA-DR protein and the majority express MHC Class I molecules (5), suggesting their potential for allogeneic transplantation (18). One difference in the surface marker expression appears to be the reciprocal expression of VLA-4 (CD49d/CD29) and its cognate receptor VCAM-1 (CD106). It was observed that there is expression of VLA-4 but not VCAM-1 by ADSCs from the majority of donors (10), but the trend is reversed in BM-MSCs (16). These molecules are involved in hematopoietic stem and progenitor cell homing to and mobilization from the bone marrow (19, 20). In culture, these cells express cell surface markers that are similar to those expressed by BM-MSCs, including CD105, SH3, STRO-1, CD90, and CD44, but they do not express the hematopoietic marker CD45 nor the endothelial marker CD31 (16, 21–24). However, in studies in which both marrow and adipose tissue were obtained from the same individuals, we detected some differences in protein expression: ADSCs express CD49d and not CD106, whereas BM-MSCs express CD106 but not CD49d (25). The significance of this finding is not clear but it is interesting that these molecules represent part of a receptor–ligand pair that has an important role in hematopoietic stem cell homing to, and mobilization from, bone marrow (26, 27). CD34 is a widely used marker of hematopoietic stem and progenitor cells (28, 29) but is also highly expressed in vascular endothelial cells and their precursors (30, 31).

The putative niche of adipose-derived stromal/stem cells (ADSCs) was examined by Zannettino et al. (32) using surface marker proteins, associated with mesenchymal and perivascular cell phenotypes, including STRO-1, CD146, and 3G5. Immunofluorescence staining of human adipose tissue sections revealed colocalization of STRO-1 and 3G5 with CD146 to perivascular regions. To evaluate the capacity of the CD146, 3G5, and STRO-1 specific monoclonal antibodies in order to isolate human clonogenic ADSCs, FACS analysis was used. Clonogenic fibroblastic colonies (CFU-F) were found to be enriched in those cell fractions selected with either STRO-1, CD146, or 3G5. Flow cytometric analysis revealed that cultured ADSCs exhibited similar phenotypic profiles in relation to their expression of cell surface markers associated with stromal cells (CD44, CD90, CD105, CD106, CD146, CD166, STRO-1, alkaline phosphatase), endothelial cells (CD31, CD105, CD106, CD146, CD166), haematopoietic cells (CD14, CD31, CD45), and perivascular cells (3G5, STRO-1, CD146). It was demonstrated that the immunoselected ADSC populations maintained their characteristic multipotential properties as shown by their capacity to form Alizarin Red positive mineralized deposits, Oil Red



O positive lipid droplets, and Alcian Blue positive proteoglycan-rich matrix *in vitro*. Furthermore, ADSCs cultures established from either STRO-1, 3G5, or CD146 selected cell populations, were all capable of forming ectopic bone when transplanted subcutaneously into nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice.

We also performed surface marker analysis on a selected patient population and the results (Fig. 24.2) are comparable to what is reported in the literature.

DIFFERENTIATION POTENTIAL OF ADIPOSE STEM CELLS

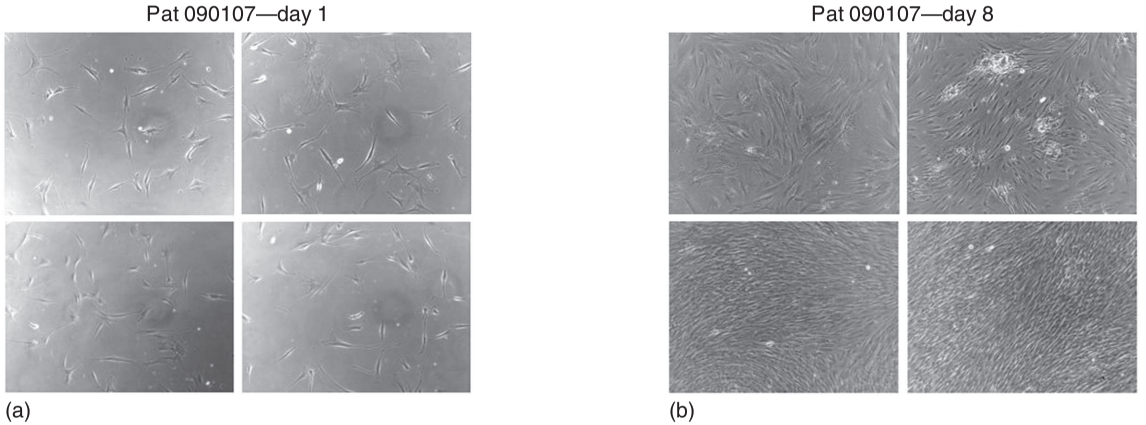
Thus, for many years, researchers studied the adipogenic potential of preadipocytes within the stromal vascular fraction of adipose tissue (33–35). Subsequently, adipose tissue contains a multipotent cell population with properties that are similar, although not identical, to those of BM-MSCs (1, 23–25, 36, 37).

Figure 24.2. In a general liposuction protocol, epinephrine in normal saline solution (1:1 000 000) cooled to 4oC was used (Asken S 1988). This fluid was first infiltrated into the site to be aspirated, via stab incisions. The epinephrine/saline solution infused vasoconstricted the blood vessels to minimize blood loss. After 15 to 20 min, the liposuction cannula was inserted through the previous stab incisions in the abdomen. The pump assisted liposuction procedures were performed with a power-assisted Lipoplasty device, PAL-200® (Microaire, VA, USA) fitted with a size 4 Triport III tip cannula. The maximum negative pressure of –100 kPa (–30 in Hg) was created with a Hercules® liposuction machine (Well Johnson, AZ, USA). A sterile 600 mL drain bottle (Primed®, Halberstadt, Germany) was interposed between the PAL-200 device and the liposuction machine to collect the fat aspirated.
The mixture of adipose tissue and fluid, are then transferred to the tissue culture laboratory for processing. (a) The plunger of the syringe was drawn out and its position maintained by an appropriately sized "Snapper" (Bryon, USA), to generate the negative pressure in its lumen. The fat aspirated was then emptied into a 70 mL sterile container (Sarstedt, Austria). (b) Prior to washing and digestion, a gross view of the various factions observed in the crude adipose tissue obtained from the pump assisted liposuction was observed after spinning down the adipose tissues for 5 min at 100 × g at room temperature. A similar observation was made for the syringe assisted samples. It was observed that the liposuction procedure (both pump and syringe) resulted in a prominent upper lipid layer. There are significant erythrocytes present which were removed with subsequent washing with sterile PBS. Image showing the initial centrifugal separation of the different layers of pump-aspirated adipose tissue in a 50-mL Falcon tube. This step facilitated the efficient separation of red blood cells from adipose tissue. (c) Colonies of PLA cells. Morphologically diverse population of cells showing the heterogeneity of the cell types present within the stroma of the adipose tissue. (i) fibroblastic, (ii) multipolar, (iii) "flat." Scale bar—100 µm. (d) The mixture of adipose tissue and fluid, were then transferred to the tissue culture laboratory for processing (extensive washing—digestion—washing). The entire fraction of the middle layer (arrow) will then be pipeted out in a cell culture flask.
A typical harvest of adipose tissue, under local anesthesia, can easily exceed 200 mL and yield more then 50 million nucleated cells per 100 mL of lipoaspirate. Thus, 200 mL lipoaspirate will typically yield in excess of 100 000 P0 precursor cells; a differential of approximately 40-fold more than that present in 40 mL of marrow. ge 0 ADSCs 3 d after plating liposuction occasionally macrophage-like cells contaminate the mesenchymal pre. (See color insert.)

Several groups have estimated the frequency and yield of bone marrow MSCs by applying clonogenic assays for either fibroblastoid-like colonies (CFU-F) or colonies expressing alkaline phosphatase (CFU-AP) (38–41). Clonogenic assays have typically been used to quantify MSCs in marrow (1, 23, 24, 37). Usually cells are plated at a density of 1000 cells/cm^2 and cultured for 3 weeks. Colonies consisting of more than 50 cells are then counted. Using these assays, the number of MSCs in bone marrow is generally found to be approximately 1 in 25 000 to 1 in 100 000, (39, 40, 42). The frequency of CFU-F

Primary antibody	Conjugate	Becton Dickinson (BD) catalogue number	Leong et al.	Zuk P. A. et al.	Gronthos S. et al.	Lee R. K. et al.
CD90	R-PE	555596	**98.95**	**Low**	**Very high**	**Very high**
CD44	R-PE	550989	**94.95**	**Low**	**Very high**	**Very high**
CD29	R-PE	555443	**89.17**	**-**	**Very high**	**Very high**
CD73	R-PE	550257	**81.26**	**Low**	**-**	**-**
CD31	R-PE	555446	**2.00**	**Absent**	**-**	**-**
CD45	R-PE	555483	**1.40**	**Absent**	**Low**	**-**
CD14	R-PE	340683	**0.66**	**Low**	**-**	**-**
CD71	R-PE	555537	**0.17**	**Low**	**-**	**-**
CD34	PE-Cy5	555823	**0.16**	**Absent**	**-**	**Absent**

Figure 24.3. Summary of surface marker analysis of ADSL's by different research groups. The cell surface phenotype of human ADSCs is quite similar to BM-MSCs (see Table). ADSCs lack the expression of known hematopoietic and endothelial markers such as CD3, CD4, CD11c, CD14, CD15, CD16, CD19, CD31, CD33, CD38, CD56, CD62p, CD104, and CD144. In contrast, CD105, STRO-1 and CD166 are three commonly used markers to identify cells with multilineage differentiation potential and are consistently expressed on ADSCs and MSCs. CD117 has been shown to be expressed on an array of totipotent or pluripotent cells including embryonic stem cells, hematopoietic stem cells, MSCs and ADSCs. In addition to the in the literature defined multipotent markers, ADSCs and MSCs display numerous other molecules including CD29, CD44, and CD49e. Interestingly, ADSCs also express high levels of CD54 (ICAM-1) when compared with BM-MSCs. ICAM-1 is a member of the immunoglobulin supergene family and can be up regulated in response to numerous inflammatory mediators and cytokines. Interestingly, less than 1% of ADSCs express the HLA-DR protein and the majority express MHC Class I molecules, showing their potential for allogenic transplantation. Analyzing the current literature on surface marker expression, it is interesting to notice the reciprocal expression of VLA-4 (CD49d/CD29) and its cognate receptor VCAM-1 (CD106). Thus, we have observed expression of VLA-4 but not VCAM-1 by ADSCs from the majority of donors. This is the reverse of the BM-MSC expression pattern of these molecules. While the nature of this difference needs further investigation, this observation is intriguing since these molecules are involved in hematopoietic stem and progenitor cell homing to and mobilization from the bone marrow.



Patient 250407—Pico Green assay

Population doubling time

Doubling time (days) (1–8 days)		Doubling time (days) (2–8 days)		Doubling time (days) (2–6 days)		Doubling time (days) in log phase (1–4 days)	
DMEM	3.47	DMEM	3.59	DMEM	3.09	DMEM	1.97
DMEM+FGF	1.64	DMEM+FGF	1.86	DMEM+FGF	1.56	DMEM+FGF	1.06
MesenPRO	1.34	MesenPRO	1.44	MesenPRO	1.17	MesenPRO	1.09
MesenPRO+FGF	1.12	MesenPRO+FGF	1.24	MesenPRO+FGF	1.00	MesenPRO+FGF	0.74

Figure 24.4. (a) Light microscopy of passage P2 ADSC 1 d after plating. (b) Light microscopy of passage P2 ADSC 8 d after plating. (c) DNA content in adipose tissue-derived stem cell culture in different media, measured by Pico Green assay. Columns represent means and error bars represent standard errors. Data were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey's post hoc test. Statistically significant differences between samples and control (Dulbecco's modified media [DMEM]) are indicated by asterisks, where: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

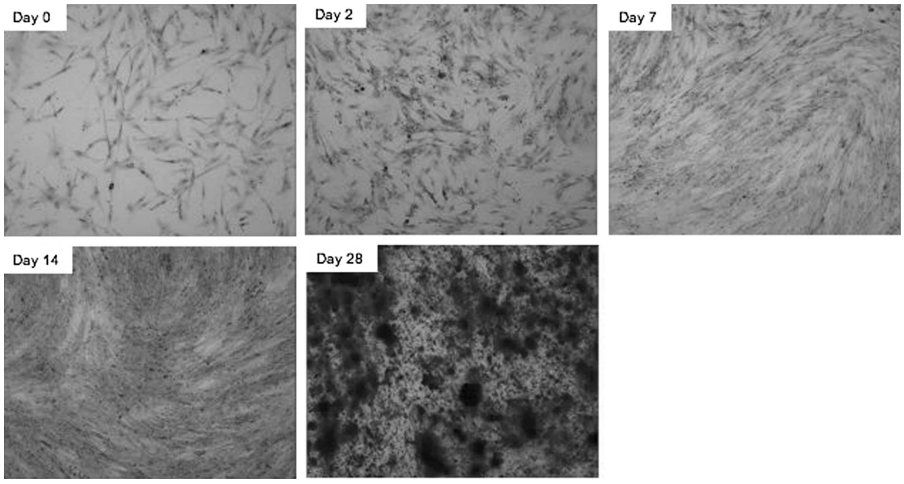


Figure 24.5. The Alizarin Red staining of ADSCs cultured for 4 weeks under osteogenic conditions. (See color insert.)

and CFU-AP is in the order of 1 in 100—some 500-fold more than that found in marrow (43). However, many authors have found this frequency is affected by age, gender, presence of osteoporosis, and prior exposure to high-dose chemotherapy or radiation (39, 41, 44, 45).

ADSCs and BM-MSCs both possess the ability to suppress a mixed lymphocyte reaction in a dose-dependent and time-dependent fashion (46, 47). Furthermore, Rodriguez et al. (36) have demonstrated that clonally derived, multipotent cells from adipose tissue are immunoprivileged, both *in vitro* and *in vivo*. This suggests that, similar to BM-MSCs, ADSCs might have potential as immunoprivileged universal donor cells with the capacity to be used in the allogeneic setting and to reduce graft-versus-host disease (47).

SPECIFIC PROLIFERATION AND DIFFERENTIATION CAPACITY OF ADSCs

Adipose-derived cells could differentiate into several cell types. It is not confirmed if a single adipose-derived cell can differentiate into all of these lineages; however, Zuk et al. generated ADSC clones from single cells capable of expressing characteristics of four cell lineages (adipo-, chondro-, osteo-, and neuro-) (1, 23, 48), thereby demonstrating the presence of multipotent and oligopotent cells within adipose tissue.

As an example of its differentiation potential along cell types of the mesenchymal lineage, two lineages are chosen here for discussion, adipogenic and osteogenic.

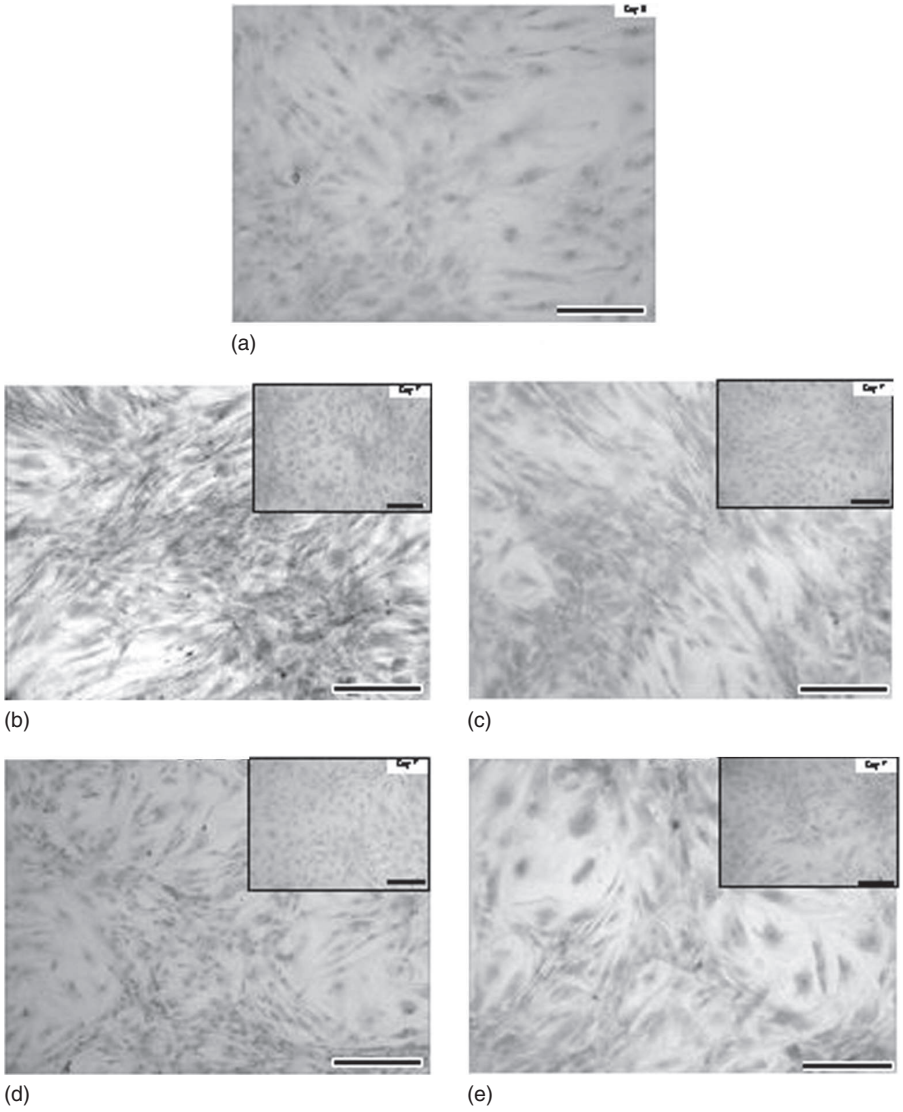


Figure 24.6. Immunoassaying of ADSC cultures for osteocalcin. (a) Uninduced day 0. (b) $VD3^{+}/BGP^{+}/AA^{+}$ Group 1. (c) $VD3^{+}/BGP^{-}/AA^{-}$ Group 2. (d) $VD3^{-}/BGP^{+}/AA^{+}$ Group 3. (e) Uninduced day 28 Group 4. Group 1 showed the presence of osteocalcin at d28. Osteocalcin tended to be found in clusters of cells in the extracellular matrix region. Group 2 also expressed comparable levels of osteocalcin to Group 1. There was no observable osteocalcin staining in Group 3 and Group 4. Scale bar represents 200 μm. Cell nuclei were stained with hematoxylin.

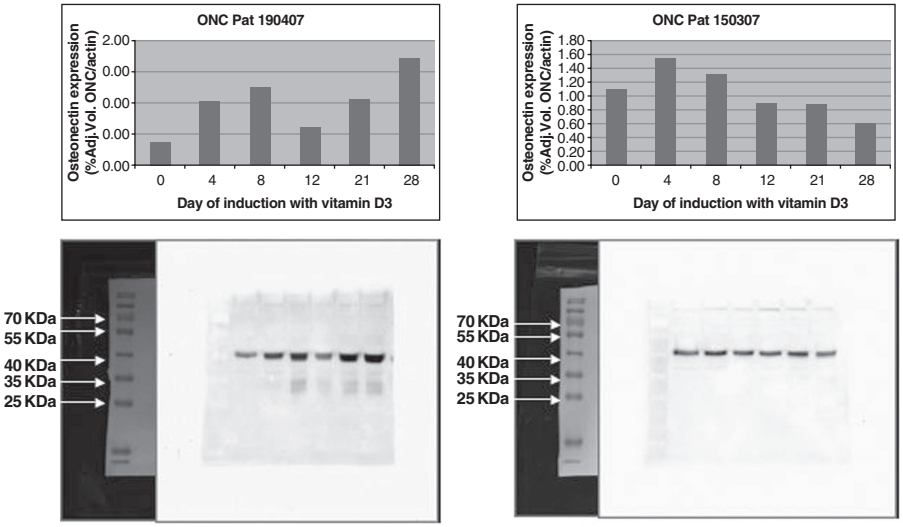


Figure 24.7. Western blots of ADSC cultures for osteocalcin.

ADIPOGENIC LINEAGE

Given the origin of ADSCs, it is not surprising that when cultured in adipogenic medium, ADSCs express several adipocytic genes, including lipoprotein lipase, aP2, PPARγ2, leptin, GLUT4, developed prominent lipid filled intracellular vacuoles—the definitive marker of adipogenesis (1, 24, 37, 49). Despite certain donor-to-donor qualitative differences in adipogenic potential (49), the pattern of adipocytic gene expression is similar to that of BM-MSCs (50–52). The *in vivo* potential of ADSC to differentiate into cells of the adipocytic lineage has also been demonstrated in studies involving the implantation of ADSCs seeded in scaffolds made from natural biomaterials like collagen (53, 54) and hyaluronic acid (55); or synthetic bioresorbable polylactic acid (56) or polyglycolic acid scaffolds (57). These studies generally agree that robust ectopic *in vivo* adipogenesis requires prior *in vitro* predifferentiation of ADSCs.

OSTEOGENESIS

The ability of BMSCs to give rise to osteoblasts is well known (58–60). A disorder called progressive osseous heteroplasia, where calcified nodules form in subcutaneous adipose depots, provides physiological evidence that cells capable of mineralization exist in adipose tissue (61, 62). In the past decade,

several groups have isolated cells from adipose tissue of humans and other species capable of differentiating into osteoblasts *in vitro* (1, 63–70). Under osteogenic conditions, ADSCs are observed to express genes and proteins associated with an osteoblastic phenotype, including alkaline phosphatase, type I collagen, osteopontin, osteonectin, osteocalcin, bone sialo protein, Runx-2, BMP-2, BMP-4, BMP receptors I and II, PTH-receptor (37, 64).

Using a variety of supportive scaffolds, human ADSCs can form bone in immunodeficient rodent ectopic bone models (57, 63, 71). In a murine critical size calvarial defect model, ADSCs were able to regenerate cranial bone, which spanned the defect site, within 8–12 weeks of implantation (72). The authors delivered ADSCs on an apatite-coated, resorbable scaffold into a critical size (4 mm) calvarial defect and demonstrated about 80% closure of the defect within 12 weeks, with radio opacity equivalent to 90% of uninjured bone at 8 weeks (72). With a lack of chondrogenic matrix, Cowan et al. proposed that bone formation occurred via intramembranous ossification. This thought is further supported by the finding that the calvarium normally develops through this mechanism. Cui et al. (73) investigated the feasibility of using ADSCs in combination with coral scaffolds to repair a bilateral full thickness cranial bone defect (20×20 mm) in a canine model. ADSCs isolated from canine subcutaneous adipose tissue were expanded in culture, osteogenically induced, and seeded onto coral scaffolds. The defects were either reconstructed with ADSC coral constructs or with coral alone as a control. At week 12 post implantation, three-dimensional CT scan showed that new bone was formed in the experimental group, while coral scaffolds were partially degraded in the control group. By radiographic analysis at 24 weeks post transplantation, it was shown that an average of 84.19+/–6.45% of each defect volume had been repaired in the experimental side, while the control defect had only 25.04+/–18.82% of its volume filled. Histological examination revealed defect repair by typical bone tissue in experimental side, while only minimal bone formation with fibrous connection was observed in the control group.

Work on ADSCs was carried further to the clinic when a seven-year-old girl suffering from a large, bilateral calvarial defect was treated with a mixture of autologous adipose-derived cells, iliac crest bone and fibrin glue, combined with a resorbable mesh (74). Computer tomography scans taken 3 months post surgery showed marked ossification throughout the defect. In this study, the isolated effect of the autologous ADSCs was not known as iliac crest bone can contribute significantly to the healing process. Nonetheless, this anecdotal report suggested that ADSCs may have contributed to healing of a critical-sized calvarial defect.

Transduction of ADSCs with the gene encoding bone morphogenic protein 2 (BMP2) resulted in the generation of ectopic bone production *in vivo* (63). In addition, ADSCs transduced with adenovirus-BMP-2 and seeded into standard collagen type I sponges formed a radio-opaque, bone-like material when implanted, intramuscularly, into immunocompromised mice (63).

POTENTIAL APPLICATIONS IN REGENERATIVE MEDICINE

Restoring blood flow to ischemic cardiac tissue has been proven instrumental in the treatment of patients with acute myocardial infarctions. Therapeutic angiogenesis, potentially from ADSC, has a broad range of clinical applications under investigation, such as ischemic cardiomyopathy, peripheral vascular disease, ischemic stroke, acute tubular necrosis, diabetic retinopathy, ischemic encephalopathy, traumatic spinal cord injuries, and transplant-related ischemia. ADSCs have been shown to increase angiogenesis to ischemic tissue; however, the underlying mechanism remains unclear. ADSCs can secrete substantial quantities of angiogenic growth factors under normoxic condition like vascular endothelial cell growth factor (VEGF), hepatocyte growth factor, transforming growth factor-β. However, there was a fivefold increase in VEGF under hypoxic conditions (11). ADSCs, besides secreting angiogenic factors, can also differentiate to cells of endothelial characteristics (75, 76). Miranville et al. have presented data supporting the presence of cells within adipose tissue that differentiated into endothelium (77). Among the population obtained from adipose tissue, $CD34^+/CD31^-$ cells expressed, after induction with VEGF and insulin-like growth factor (IGF), both CD31 and von Willebrand factor, markers of mature endothelium. Miranville et al. also demonstrated the ability of the $CD34^+/CD31^-$ ADSC to improve blood flow and capillary density in an athymic murine model of hind limb ischemia. These data are confirmed by another study showing that delivery of ADSCs to immunodeficient animals following induction of severe hind limb ischemia results in accelerated restoration of perfusion (11).

Up to the present, no effective therapeutic concept for patients suffering from steroid-refractory graft versus host disease (GVHD) is available. Alternative approaches such as antithymocyte globulin, mycophenolate mofetil, pentostatin, and the application of monoclonal antibodies have only shown modest success. To investigate the influence of ADSCs' immunomodulatory effects in GVHD, in an experimental clinical trial, Fang et al. (78) treated six patients afflicted with acute steroid-refractory GVHD grade III-IV with infusions of ADSC. The chosen ADSC dose was 1.0×10^6/kg. No side effects could be detected after administration of ADSC infusions. Five patients were treated once and one patient twice. Two patients received ADSC from haplo-identical family donors and four from unrelated mismatched donors. Symptoms of acute GVHD disappeared completely in five of six patients, suggesting that the application of ADSC might be a promising approach for the treatment of acute severe steroid-resistant GVHD.

Most corneal diseases affect corneal stroma and include immune or infectious diseases, ecstatic disorders, traumatic scars, and corneal dystrophies. Cell-based therapies are a promising therapeutic approach to overcome the current disadvantages of corneal transplantation. Therefore, in an experimental study in rabbits, Arnalich-Montiel and colleagues investigated the ability of human ADSC to regenerate corneal stroma (79). First, biosafety and the immuno-

genicity of ADSCs transplanted into the corneal stroma of rabbits were assessed. No immune response was elicited despite the use of immune-competent animals. ADSC survival was up to 10 weeks post transplantation, while the cells maintained their morphology, and remained intermingled in the stroma without disrupting its characteristic histological pattern. Transparency was preserved even 10 weeks after the transplant, when PLA cells formed a discontinuous layer in the stroma. In a different experimental setting, the regeneration of the corneal stroma by PLA cells was assessed, creating a niche by partial ablation of the stroma. After 12 weeks, human cells were disposed following a multilayered pattern and differentiated into functional keratocytes, as assessed by the expression of aldehyde-3-dehydrogenase and cornea-specific proteoglycan keratocan.

Duchenne muscular dystrophy is an inherited disorder, characterized by rapidly worsening muscle weakness that starts in the legs and pelvis, and later affects the whole body. To investigate possible applications of ADSCs in the therapy of Duchenne muscle dystrophy (DMD), Flk-1(+) MSCs were isolated from adult adipose tissue and induced to differentiate into skeletal muscle cells in culture (80). Within mdx mice, an animal model of DMD, adipose tissue-derived Flk-1(+) MSCs (ADSCs) homed to and differentiated into cells that repaired injured muscle tissue. This repair correlated with reconstitution of dystrophin expression on the damaged fibers. Flk-1(+) AD-MSCs also differentiated into muscle satellite cells. This differentiation may have accounted for long-term reconstitution. These cells also differentiated into endothelial cells, thereby possibly improving fiber regeneration as a result of the induced angiogenesis.

Peripheral nerve disorders are frequently a result of acute traumatic and chronic repetitive injury, but at times are caused by inheritable or acquired autoimmune disorders. To assess the potential of ADSCs in peripheral nerve injury and neurogenic disorders, it was investigated whether adult stem cells, isolated from adipose tissue, can be differentiated into functional Schwann cells (81). Rat visceral adipose tissue was enzymatically digested to yield rapidly proliferating fibroblast-like cells, a proportion of which expressed the MSC marker, STRO-1, and nestin, a neural progenitor protein. Cells treated with a defined combination of glial growth factors (GGF-2, bFGF, PDGF, and forskolin) adopted a spindle-like morphology similar to Schwann cells. Immunocytochemical staining and western blotting indicated that the treated cells expressed the glial markers, GFAP, S100, and p75, indicative of differentiation. When cocultured with NG108-15 motor neuron-like cells, the differentiated stem cells enhanced the number of NG108-15 cells expressing neurites, the number of neurites per cell, and the mean length of the longest neurite extended. Schwann cells evoked a similar response while undifferentiated stem cells had no effect.

The human skin represents one of the target organs most susceptible to environmental oxidative stress since it is in constant contact with oxygen and provides the outermost barrier protecting from various physicochemical

substances that may lead to the production of reactive oxygen species (ROS). ROS can be produced by various stimuli. However, the most important source directly related to cutaneous damage are photochemical reactions with UV light. UV light as well as ionizing radiation produce ROS such as superoxide anion radicals, hydroxyl radicals, and singlet oxygens. Kim et al. examined the protective effect of ADSCs on human dermal fibroblasts (HDFs) through antioxidation in a tert-butyl hydroperoxide (tbOOH)-induced oxidative injury model (82). The conditioned medium of ADSCs (ADSC-CM) was harvested and tested for antioxidant action. ADSC-CM had an antioxidant effect as potent as 100 μM ascorbic acid and various antioxidant proteins were detected in ADSC-CM by proteomic analysis. Morphological change and cell survival assay revealed that incubation with ADSC-CM aided HDFs to resist free radicals induced by tbOOH. In addition, activities of superoxide dismutase and glutathione peroxidase were enhanced in the ADSC-CM treated HDFs which confirmed the study hypothesis that ADSCs protect HDFs through antioxidant action. In a cell cycle analysis, ADSC-CM treatment reversed the apoptotic cell death induced by tbOOH and caused a decrease of sub-G1 cells with respect to untreated cells. The antiapoptotic effect of ADSC-CM was also reproduced by caspase-3 activity assay. The results suggest that ADSCs have potent antioxidant activity and protect HDFs from oxidative injury by decreasing apoptotic cells.

Myocardial infarction is the leading cause of congestive heart failure and death in the industrialized world. Current therapy is limited in preventing the progression of ventricular remodelling and congestive heart failure. Recently, interest has focused on the application of stem cells that can also differentiate into cardiomyocytes. Myocardial regeneration using stem-cell transplantation technologies is a possible treatment option to reverse the deleterious hemodynamic and neurohormonal effects that occur after myocardial infarction and can lead to congestive heart failure. Various preclinical animal studies show the potential to regenerate myocardium and improve perfusion to the infarct area to improve cardiac function but also suggest that stem cells may have proarrhythmic effects. Early phase I clinical studies indicate that stem-cell transplantation is feasible and may have beneficial effects on ventricular remodeling after myocardial infarction. However, so far little is known about molecular mechanisms contributing to the beneficiary effects of stem cell applicaton. In a study by Sadat et al., cytokines released by ADSCs were detected by ELISA and proangiogenic effects were assessed by tube formation assay (83). To define the antiapoptotic effect of ADSCs, neonatal rat cardiomyocytes were subjected to hypoxia condition in a coculture system. Data show that ADSCs secrete significant amounts of VEGF (810.65+/–56.92 pg/microg DNA) and IGF-I (328.33+/–22.7 pg/microg DNA). Cardiomyocytes apoptosis was significantly prevented by ADSCs and 62.5% of the antiapoptotic effect was mediated by IGF-I and 34.2% by VEGF. ADSCs promoted endothelial cell tube formation by secreting VEGF. In conclusion it was demonstrated that ADSCs have a marked impact on anti-apoptosis and angiogen-

esis and helps to explain data of stem cells possible benefit in treatment of cardiac infarction without transdifferentiation.

Up to present, the real-time distribution of stem cells *in vivo* after infusion in recipients is to a large extend not known. Historically, the evidence of engraftment has been obtained only later, either in the form of an analysis of peripheral blood or by evaluating tissue pathohistologically post mortem. As the goal of novel cellular therapies is their application to human disease, it has become increasingly important to complement tissue studies with the *in vivo* assessment of infused cells. To be able to use magnetic resonance imaging (MRI) to guide the transplantation of ADSCs in middle cerebral artery occlusion (MCAO)-injured mice, and to localize donor ADSCs in the injured brain using MRI+, Rice et al. labeled ADSC with superparamagnetic iron oxide (SPIO) particles (84). ADSCs harvested from mice inbred for green fluorescent protein were labeled with SPIO ferumoxide particles using poly-L-lysine. ADSC viability, iron staining, and proliferation were measured after SPIO labeling, and the sensitivity of MRI in the detection of SPIO-labeled ADSCs was assessed *ex vivo*. Adult mice ($n = 12$) were subjected to unilateral MCAO. Two weeks later, *in vivo* 7-T MRI was performed to guide stereotactic transplantation of SPIO-labeled ADSCs into brain tissue adjacent to the infarct. After 24 h, the mice were sacrificed for high-resolution *ex vivo* 7-T or 9.4-T MRI and histologic study. ADSCs were efficiently labeled with SPIO particles without loss of cell viability or proliferation. Using MRI, precise transplantation of ADSCs was guided. MR images of mice given injections of SPIO-labeled ADSCs had hypointense regions that correlated with the histologic findings in donor cells.

Gene Delivery

Owing to the high proliferation rate, ADSCs might be a source of cells capable of enhanced gene delivery. A number of investigators have transduced ADSCs in order to facilitate tracking or to engender a therapeutic effect. Thus, Leo et al. used Ad-CMV-luciferase to allow noninvasive, real-time tracking of ADSCs in rat spine (85). Similarly, Dragoo et al. (63) infected both BMSCs and ADSCs with E1A-deleted-type 5 adenovirus constructs containing the BMP-2 (bone morphogenic protein-2) gene or the bacterial beta-galactosidase (lacZ) gene. LacZ gene transduction efficiency was 35% for BMSCs and 55% for ADSCs. Ad-BMP2 infection resulted in levels of expression of BMP-2 protein that were three-fold higher than those derived from BMSCs. Ad-BMP-2 infected ADSCs exhibited *in vitro* osteoblastic differentiation in the absence of exogenous osteogenic factors. They also exhibited robust ectopic *in vivo* production of bone when cells were implanted into a collagen sponge within the subcutaneous space (63).

Given the success of unmodified MSCs in treatment of osteogenesis imperfecta (86, 87), these data support the potential for transplantation of allogeneic or gene-modified ADSCs for genetic disorders of the skeletal system. Kang

et al. (88) have also used an E1A-deleted type 5 adenovirus to infect ADSCs. As described above, these studies employed transduction of a tracking gene (lacZ) and a potentially therapeutic gene Brain-Derived Neurotrophic Factor achieving 100% and 94% transduction efficiency, respectively. Transduced cells were implanted into areas of the brain that had undergone transient (90 min) ischemia-reperfusion injury. Donor cells capable of continued expression of the transgene were retained to 30 d, the longest time point examined in that study. Published results of a study comparing infection of ADSCs with retroviral and lentiviral vectors (9) showed the lentiviral contructs resulting in 4–10-fold higher expression than the retroviral vector. The percentage of transduced cells was not high (10%–15%) but remained stable in culture over 100 d. Studies using lentiviral-infected cells in which transduction efficiency was 98% at day 3 and >95% at day 100 allowed examination of gene expression during *in vitro* differentiation. Retention of marker gene (EGFP) expression was observed following both adipogenic and osteogenic differentiation (5, 58).

ADSCs can be gene delivery vehicles for administering engineered genes that support tissue regeneration. Morizono et al showed the ability of ADSCs to express transgenes delivered by viral vector types (9). ADSCs, transduced by an adenovirus encoding the osteogenic factor bone morphogenic protein 2 (BMP-2), enhanced bone formation when transplanted subcutaneously into nude mice (63).

CONCLUSION

From the current literature it can be concluded indubitably that one of the promising adult stem cell sources is a culture plastic-adherent cell population from the human adipose tissue. In order to bring adipose derived stem cells (ADSCs) a step closer to the clinics, future work has to show that ADSC's fulfill the following:

- Reliable, sufficient, and possible procurement of autologus ADSCs;
- Characterization of cells in *in vitro* culture plastic environment;
- Characterization of cells in *in vitro* 3-D environment;
- Characterization of cells in *in vivo* animal models; and
- An in-depth analysis of the transcriptome signatures of ADSCs when induced.

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25

BRINGING MESENCHYMAL STEM CELLS INTO THE CLINIC

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ORIGINS

MSC is an acronym coined to describe adherent cells with fibroblastic morphology, originally isolated from bone marrow but subsequently found in a wide range of neonatal and adult tissues (1). Unlike terminally differentiated fibroblasts, MSCs have broader differentiation potential and are the progenitors for tissues of mesenchymal lineages. The most frequently characterized derivative tissue lineages are bone, fat, and cartilage although endothelial, smooth muscle, and stromal phenotypes can also be derived (2). These derivative tissue lineages can be identified using *in vivo* models, and can be induced *in vitro* using growth factors or metabolites consistent with normal embryological development patterns (3, 4).

The MSC acronym was originally an abbreviation of mesenchymal stem cells, although recently a standardization was accepted to refer to these populations as mesenchymal stromal cells (5). This change was made in part to comply with recent scientific criteria for stem cell self-renewal, and a failure to date to demonstrate the successive l adoptive transfer of MSC from one donor to the next, with preservation of the progenitor properties.

MSCs have been isolated from fetal lung and fetal blood (6, 7), neonatal tissues such as cord matrix (8) and placenta (9) as well as cord blood (10), and from a variety of adult tissues with primary emphasis on bone marrow and fat

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(11). Adherent stem cells within this class have also been isolated with multipotent or pluripotent tissue differentiation potential (referring to mesodermal, endodermal, and ectodermal germ layer potential) and are the subject of a recent review (12). Models have been proposed that this class of cell associates with the vasculature (e.g., pericyte) and therefore similar or identical cells could logically be isolated from multiple tissues (13).

Limited studies have been performed to identify the true progenitor to the *ex vivo* expanded MSC. From the McGonagle laboratory (14) a stepwise isolation procedure was developed which enriches MSC activity from human bone marrow to near homogeneity, and the phenotype of this precursor was identified and reported. These cells coexpress a fibroblastic antigen described as D7fib, as well as LFNGR (low affinity nerve growth factor receptor). Not surprisingly, a number of phenotypic markers on this precursor are gained or lost in the process of adherence and *ex vivo* expansion, most notably the expression of class II human leukocyte antigen (HLA) on the precursor and rapidly lost on plating. These precursors have not been studied in adoptive transfer experiments to demonstrate the progenitor or stem cell potential *in vivo*.

Other investigators have characterized murine and human adherent cell cultures that appear to contain a developmental precursor to the MSC, with these cultures exhibiting endodermal and ectodermal lineage differentiation potential as well. This is suggestive of a clonal multipotent or pluripotent adherent progenitor population but is equally consistent with a population of mixed germ layer progenitors. Cells of this class were initially termed multipotent adult progenitor cells (15), but other acronyms have been used to describe these cultures as isolated by other investigators or from tissues other than bone marrow. Again, these cell types have been the topic of a recent review (12).

Elegant proof that a precursor to the standard MSC exists in both *ex vivo* culture and *in vivo* was demonstrated by the laboratory of Dominic Bonnet in 2007 (16). Using mouse adherent stem cell cultures, it was possible to isolate an MSC precursor from an adherent culture using the phenotypic marker SSEA-1, which also marks primitive stem cell activity in murine embryonal stem cell cultures. These data showed that such precursors would default to an MSC fate when grown under standard MSC conditions, and that this was not reversible. These studies also demonstrated that this precursor could be prospectively isolated from mouse bone marrow, supporting that this developmental precursor to the MSC exists *in vivo* and that MSC are not a consequence solely of *ex vivo* adaption. This isolated precursor was able to reconstitute hematopoiesis *in vivo*, defining these cultures to contain a mesodermal progenitor developmentally upstream of mesenchymal tissue commitment shown by MSC.

An important and often overlooked consideration is that these adherent cultures should be evaluated as a dynamic hierarchy of cells, with a range of primitive development potential—that is, they are not clonal and homogeneous, and the heterogeneity is likely to vary between laboratories and according to individual isolation conditions. This is not only shown by the Bonnet

studies described above, but also reported by Pittenger et al. (17), in which characterization of individual MSC clones isolated using standard MSC isolation procedures was performed. Individual clones ranged from exhibiting bone, fat, and cartilage differentiation potential to being restricted to bone alone.

Therefore, when we study these classes of adherent cells and apply scalable development protocols for their clinical use, we must use the paradigm applied to bone marrow stem cell cultures. That is, the clinical cell product is likely a composition of primitive reconstituting stem cells among a progenitor population committed, in this case to a default mesenchymal fate. This is not complicating clinical use, but actually may comprise an optimal transplant product capable of providing multiple avenues of therapeutic benefit. Much basic research is being actively pursued to describe and prove the understanding of hierarchical relationships within MSC cultures and their *in vivo* significance.

PRODUCT DEVELOPMENT CONSIDERATIONS OF MSC

Limited tools have been developed to dissect subpopulations of adherent progenitor cells from bone marrow and other tissues. A profile of monoclonal antibodies such as those developed to discriminate hematopoeitic subsets has not been successfully identified. It is expected that new technologies driving transcriptome and proteome profiles will develop such valuable tools to characterize these cultures and will clarify distinctive subpopulations.

From the product development perspective, it is more important to address technical consistency than theorem with respect to these developmental biology relationships. A position paper was recently published from an ISCT (International Society of Cell Therapy) subcommittee, in which standard assay and phenotypic requirements for practicing MSC clinical development were recommended (18). These findings are summarized below.

- Phenotypically positive (>90%) for CD105, CD73
- Phenotypically negative (<5%) for CD45, class II HLA (hematopoietic contaminants)
- Cultures are capable of differentiation into at least two mesenchymal tissues

These guidelines are integral to the lot release assays applied by the United States and European Regulatory agencies for clinical product release, as described in a later section.

Immunological Properties

Ex vivo expanded MSC do not express class II HLA and do not express T cell costimulatory molecules. This property is consistent across adherent stem cell

cultures from all tissues (19, 20), and applies as well to those cultures having multipotent germ layer potential. This renders these cultures relatively inert in stimulating T cell responses, both antigen specific and allogeneic.

MSC are also actively immune inhibitory *in vitro*. Many labs have reported that this class of cells, again across all tissue types of origin, secrete factors which inhibit T cell activity *in vitro*. When exposed to allogeneic or activated T cell populations, MSC increase the production of cytokines such as TGF-beta or IL-10 which are known to exert immunosuppressive outcomes. In addition, production of prostaglandins has been linked to regulation of dendritic cell function (21). These findings are widely published and are the subject of two excellent recent review articles (22, 23).

These *in vitro* immunomodulatory properties have also correlated with *in vivo* preclinical studies, suggesting a pathway of mechanistic benefit supporting clinical cell use. MSCs have been shown to prolong acceptance of skin grafts in nonhuman primate models (24), and have been shown to persist, albeit at low levels, in long-term studies in the baboon (25). Allogeneic cells of this class have been used successfully in cardiovascular models of rat and pig without requirements for immunosuppression, or evidence of hypersensitivity reactions (15, 26). These are a few examples among many preclinical studies using allogeneic MSC, and are described in more depth in several recent reviews (27, 28).

CLINICAL PROOF OF CONCEPT

The connective tissue regenerative properties of MSC inspired their study in tissue engineering or orthopedic models and applications. Based on the observations of Friedenstein (1, 3), proof of concept for the *in vivo* creation of bone, fat, and cartilage using *ex vivo* expanded MSC had been sought. It was this commercial market that fostered the creation of Osiris Therapeutics, Inc., and the first corporate sponsored MSC clinical studies initiating in the late 1990s. A review of these early translational studies using MSC was published in 2001 (29).

However, first-in-man clinical studies using MSC were not in the orthopedic area, but rather were designed to exploit the stromal support properties of these cells as an adjunct therapy in bone marrow transplant. As has been the case with other progressive experimental therapies, a high-risk patient population with little alternative treatment was selected to receive an infusion of *ex vivo* expanded MSC. The therapeutic hypothesis was for adjunctive MSC to improve hematopoietic recovery rates in hematologic malignancy patients undergoing complete myeloablation prior to receiving autologous bone marrow transplants.

These proof of concept studies took place at Case Western Reserve University as physician sponsored Phase I clinical studies, reflecting the relationships with investigators at University Hospitals with the laboratory of Dr

Caplan. Hematopoietic stem cell transplant (HSCT) procedures and methodologies heavily influenced decisions on dosing, expansion technology, clinical lot characterization, and formulation. This influence remains today, and through the first 10 years of clinical experience, the primary translational thought leaders were drawn from hematology/oncology units within academic hospitals, with cell expansion and processing performed in the bone marrow transplant (BMT) centers. Translational research advances were guided primarily by hematology societies such as ASH (American Society for Hematology) and EBMT (European Bone Marrow Transplant) Society, with standards for nomenclature and characterization moderated by ISCT.

The first Phase I clinical studies initiated with the use of autologous *ex vivo* expanded MSC, and were regulated primarily by sterility testing of the expanded product. These studies were first performed in patients in remission from leukemia or lymphoma (30), and later in late stage breast cancer patients (31).

In a second physician-sponsored study, autologous MSCs were expanded and used as an adjunct clinical product in hematologic malignancy patients in complete remission. A total of 23 patients were enrolled, with successful MSC expansion reached in 15 patients. Patients received doses of MSC tiered at 1, 10, or 50 million cells/kg, with infusions safely tolerated. Unfortunately, a failure to expand sufficient MSC within the necessary HSCT transplant window resulted in unsuccessful patient treatment. No long-term complications attributed to the MSC infusion were found.

In the breast cancer patients, a single autologous bone marrow aspirate was used to generate a single clinical MSC product for infusion. Of 32 patients enrolled, clinical expansion to infusible dose was reached in 28 patients. Dosing was calibrated according to current standards for HSCT, and patients received between 1 and 2.2 million MSC/kg. Patients showed early hematopoietic engraftment within normal ranges, and infusions were safely tolerated. No ectopic tissue or calcification was detected at 100 d or 1 year. Long-term follow up showed no increased rate of tumor relapse or new tumor reports.

Dosing

In the HSCT field, clinical dose was limited based on total CD34+ cell isolation from bone marrow or apheresis products, whether autologous or allogeneic. In the context of hematopoietic recovery, a trend for improved recovery associates with higher HSC number, but complications from CD34+ cell quantitation as well as conditioning-related morbidity complicates further interpretation.

MSC were dosed accordingly, based in part on the safety profile for infusion volume and cell number, and also due to limitations in clinical expansion capacity. MSC have finite *ex vivo* expansion capacity, undergoing replicative senescence and loss of biological potency between 20 and 30 population doublings *ex vivo* (17), and in fact many patients on trial did not receive an MSC

dose due to a failure of these cultures to expand to adequate number within the clinical viable remission window for HSCT.

Variation in cell recovery in individual clinical expansions also contributed to variability in final delivered dose. Clinical dose ranging has not been performed in adequate patient numbers to date to establish a clear dose response relationship, both in the BMT setting and with use of the cells in the cardiovascular setting.

A paradigm shift occurred in the late 1990s with corporate sponsored studies by Osiris Therapeutics, demonstrating the safety and benefit for use of allogeneic *ex vivo* expanded MSC in the HSCT setting in leukemia/lymphoma patients (32). The progression from autologous to allogeneic Phase I testing was the standard progression advised by the Food and Drug Administration (FDA) in cell therapy. However, as discussed below, in the past 5 years, our understanding of the immunological properties of this class of cells has relaxed safety concerns for use of allogeneic cells and trials are initiating directly with use of allogeneic product.

MECHANISM OF BENEFIT

The therapeutic hypotheses in play at the time of first-in-man clinical studies with MSC were primarily targeting paracrine support of hematopoiesis, although it was implicit that infused MSC would engraft and persist as stromal elements in bone marrow. It was surprising to find that increased hematopoietic recovery was anecdotal and inconsistent between centers or cohorts. Safety for the infused MSC product was clearly shown, but the greatest clinical benefit trend was towards reduction of graft versus host disease (GVHD) morbidity (32). In addition, detection of donor infused MSC in bone marrow or blood was negligible to nonexistent. In an elegant study, Koc et al. (33) were able to track allogeneic MSC infused in (lysosomal storage disease) patients that had previously received allogeneic bone marrow transplant for treatment of the genetic disorder. Due to engraftment of the allogeneic HSCT, patients would recognize the allogeneic MSC infusion as self, allowing cell tracking in the absence of immune complications. These studies reported clearance of input MSC from circulation within 30 min, and detection of re-isolated MSC from bone marrow aspirates or biopsy.

Clinically, the pharmacokinetic distribution of MSC has not been evaluated. Preclinical studies in baboon models (25), supported by rodent and canine work show that the majority of cells delivered by intravenous infusion are trapped in the reticuloendothelial system in the lung, liver, and spleen, and are subsequently either lost to apoptosis or cell death, or distributed via the circulation to other tissue and organs. Even in bone marrow, the original tissue of isolation, less than 0.5% of an aspirate sample consists of donor material at best, and detection of donor cells *in situ* has been sporadic or more frequently impossible.

Excitement rose in the early part of this decade for use of MSC in regenerative therapy. The clinical safety proven from the adjunctive HSCT trials stimulated exploration for clinical use in other indications. The clear *ex vivo* regenerative capability of MSC prompted evaluation for use in tissue engineering indications (34). As well, early success in the MSC translational research arena led investigators to evaluate related cell types, or MSC derived from other tissue sources. A burst of preclinical studies reporting broad tissue plasticity across developmental boundaries was subsequently shown to be largely artifactual (faulty cell marking or detection procedures) or confused by cell fusion, and to a great degree, the adult stem cell community, including MSC, were left jaded regarding the use of these cells for regenerative therapy.

Immunomodulation

It was clear, however, that physiological benefit could be derived from MSC in preclinical models by paracrine influence. The early observations from use of MSC in the HSCT setting prompted a key observation linked to modulating GVHD. A seminal paper was published in 2004 by LeBlanc et al. (35) in which haploidentical MSC were expanded from the mother of a patient who had undergone allogeneic bone marrow transplant. The patient was undergoing a crisis of steroid refractory GVHD, and delivery of a single dose of MSC brought the several gut and liver morbidity down to normal levels. The patient was not cured, as removal of immunosuppressive regimens correlated with a flare of Grade III/IV GVHD, but this was again treatable using another dose of haploidentical bone marrow. This same laboratory had performed key immunological studies showing that MSC could downregulate allogeneic T cell responses (19), and thus a link between *in vitro* paracrine effects and clinical response was demonstrated.

This report stimulated wide use of MSC for the management of GVHD morbidity. While the first translational studies demonstrated infusional safety when delivered at time of HSCT, new clinical studies were initiated based on infusion of one or several doses of MSC at the onset of drug resistant GVHD symptoms. The striking consequences of this treatment has led to considerable support from the Regulatory agencies to accelerate testing and bring these modalities forward.

MANUFACTURING AND LOT RELEASE CRITERIA

MSC and related cell products are regulated through the FDA under the Cell and Gene Therapy branch of Center for Biologics Evaluation and Research. Specialists in the manufacturing and formulation aspects of clinical production provide guidance on individual clinical cell products, based on a common format (Table 25.1). These requirements apply equally to clinical production in academic or nonprofit institutions as well as for corporate investigations.

TABLE 25.1. Routine MSC Lot Release Testing of Clinical Product

Lot release Assay	Components of Testing	Requirements
Sterility	Frugal, bacterial presence	Negative
	Endotoxin detection	Negative
	Mycoplasma detection (14-d culture assay)	Negative
Viability, identity, purity	Flow cytometric analysis	>80% viable cell recovery >90% CD105+, CD73+ <5% CD45+, class II HLA+
Potency	Biological assay for differentiation	Positive for bone, fat, cartilage
	Biological assay for immunomodulation	*In vitro* immunosuppressive
Cytogenetic stability	G-banding karyotypic analysis of 20 metaphase spreads	No clonal abnormalities

Differences in testing requirements apply when producing a patient designated product (one product per donor), compared to production of many clinical products from the same donor (universal donor).

Originally, clinical MSC products were produced using an open cell expansion platform, that is, the cells are cultured in vessels and transferred during passing by using open tissue culture flasks and exposure to the atmosphere. Good Manufacturing Practices qualified reagents for cell processing were not completely available, and significant risk was posed for sterile breach or adventitious pathogen transfer. It is fortunate that no MSC-attributable adverse events occurred or were reported at early stages in this developing field.

Based on cell processing standards in the HSCT field, clinical production has been refined and can take advantage of closed sterile fluid transfer technologies, greatly reducing risk of pathogen exposure. An example of a prototype expansion platform is illustrated below in Fig. 25.1.

A bone marrow aspirate is collected from a healthy donor in anticoagulant and transferred to the cell processing facility. Elimination of the majority of granulocytes and erythrocytes can be accomplished by closed system continuous flow centrifugation, allowing collection of MSC precursors within the mononuclear cell fraction. Transfer of the gradient enrichment can be sterilely transferred to a single unit cell factory, allowing adherence of the MSC precursor. During this initial plating period between 1 to 2 weeks, a confluent monolayer of adherent MSC is obtained, with intermediate cell washing steps removing the majority of hematopoietic contaminants. At early stages a background adherence of monocytes can contaminate the culture, but are lost in subsequent stages. Repetitive passages can be derived by sterile transfer of trypsin solutions, inactivation, and transfer to large scale cell factories. This can

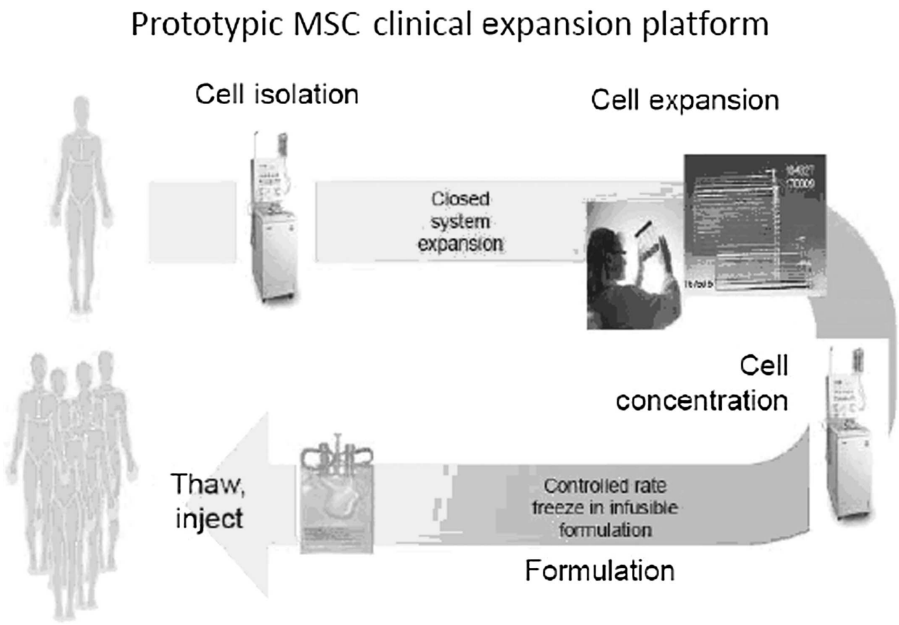

Figure 25.1. Prototype MSC clinical expansion platform. Proposed workflow for the use of MSC in a clinical setting. (See color insert.)

be repeated until cultures reach replicative senesence. Harvest can be accomplished by trypsinization and collection using an automated continuous flow centrifugation system, and collection achieved in the final cryoprotectant for storage.

Classical MSC expansion is performed in a basal media supplemented with selected lots of fetal bovine serum. This is currently the base procedure used in clinical expansion procedures in Europe organized by the EBMT Society, using standard protocols and serum lots (36).

Modifications to the base procedure have been made by investigators claiming retention of a more primitive developmental precursor to the MSC (12). The key alternative conditions involve culture of cells at low density avoiding confluence, and supplementation with growth factors such as basic fibroblast growth factor, epidermal growth factor, or platelet-derived growth factor (37). It appears that these conditions retain more primitive precursors but a lack of phenotype markers characterizing subsets of adherent stem cell culture limit more rigorous analysis of these cultures.

MSC cultures are ordinarily formulated for cryopreservation in a human serum albumin, dimethylsulfoxide (DMSO)-containing cryoprotectant. While details are not widely published, stability profiles of >3 years are understood in the industry.

Patient-designated Product

Patient-designated products are defined as clinical products from a donor (generally a healthy allogeneic donor) which are produced and used for a specific predesignated individual. This paradigm is similar to HSCT, in which an individual donor matched to the recipient patient is the source of a single clinical transplant product.

In clinical production of a patient-designated product, sterility and identity testing must be performed on the final product. Frequently, cell delivery to the patient is performed directly upon reaching expansion to clinical dose. In this case, long-term (>2 week) testing for potential mycoplasma contamination would complicate or prohibit timely delivery of product to patient, and hence the FDA has allowed cell delivery pending receipt of long-term sterility testing. In this circumstance, sponsors must have a corrective action plan in place to notify physician and patient should a positive finding be reported. In addition, the cell processing facility must have qualified the *ex vivo* expansion protocol and demonstrated that a sterile product is routinely achieved.

Potency assays (determination of the biological efficacy potential of the product) can frequently consume a significant fraction of the manufactured product when tested in animal models, and may not be required when producing a single clinical product, provided that sufficient qualification runs show that a routine potency is achieved, statistical confidence that clinical products manufacturing using this protocol routinely exhibit consistent potency. In some cases, potency can be correlated with a surrogate assay such that real time testing on the single product is feasible. For example, cell culture supernatants might be assayed for production of cytokines supporting hematopoiesis (2), when the therapeutic endpoint is postulated to be hematopoietic recovery in HSCT.

Similar arguments are applied to cytogenetics and tissue safety determinations, where the amount of sample required for testing may preclude clinical use. For example, a routine assay for absence of tumor potential is the test of human clinical product in an immunodeficient mouse model, which could require up to 20% of the expanded product. A surrogate for this endpoint might be an evaluation of cytogenetic stability by metaphase karyotypic analysis, provided that significant animal testing had been performed in the qualification of the *ex vivo* expansion protocol.

Universal Donor Products

Production of clinical material from a universal donor generally results in multiple cryopreserved doses pending patient assignment. This allows both the timing and cell requirements to perform sterility and other testing prior to patient delivery. It is also economically advantageous to use surrogate assays that have been validated relative to animal models. Significant value is

gained by implementing *in vivo* safety testing for tumorigenicity in process qualification.

As described, the immunology of MSC supports allogeneic cell therapy without immunosuppressive drugs. This is certainly substantiated by clinical experience when single doses of clinical product are used, or multiple doses delivered to patients undergoing hematopoietic recovery or immunosuppressive drug regimens (20). Single dose infusions of MSC were shown to be safe when infused to immunocompetent patients for treatment of acute myocardial infarction (J. Hare, unpublished data), with no acute infusional or immunological complications. Two risks are posed as a consequence of immune sensitization from an allogeneic MSC product. First, an allergic reaction might create severe acute toxicity risk to a patient. This has not been the case to date. The second immunological complication is the potential loss of efficacy as a consequence of donor cell rejection. It is not currently routine to prescreen patients for reactivity to donor MSC using either dermatological hypersensitivity reactions or screening for serum IgE or circulating anti-MSC antibodies. It is anticipated that this becomes standard of care as repeat administration of MSC increases.

Patient Delivery

Intravenous infusion of an MSC product poses minor risk for acute infusional toxicity related to the cryoprotectant formulation, notably DMSO (frequently constituting 5% of the final formulation). This is frequently addressed by thawing and diluting the final product with an infusible support media, and using standard blood cell infusion procedures for cell delivery. The alternative is the thaw and wash of final cell product using closed system bag washing kits. For universal cell products, a full repeat of sterility testing is not required provided a closed system patient delivery path is used. In this case, a viable cell count to determine accurate dosing is combined with a stat gram stain as a final sterility check prior to infusion.

It is also advised to store retained archival samples of the manufactured product for retrospective safety testing. This enables accurate identification of donor product and with precise molecular genetic typing for ectopic tissue or tumor occurrence, and for retrospective testing of new adventitious agents not discovered during manufacturing or patient treatment.

CYTOGENETIC INSTABILITY AND EXTENSIVE CLINICAL EXPANSION

Ironically, success using MSC in the clinical setting has resulted in production changes which may actually raise risk. As a consequence of achieving universal donor proof of concept, manufacturing campaigns have increased in lot

number, driven by business models to develop a sustainable and reimbursable therapy. The created risk is for overexpansion of cells and selective pressure for rapidly growing and abnormal populations. The risk is increased even greater when reductions in serum or growth factors for economic purposes increase selective advantage of abnormal cells.

MSCs are a somatic population with a finite expansion potential, limited by Hayflick's number and showing progressive telomere shortening during expansion *ex vivo* (17). As the populations enter replicative senescence, a crisis or lag in rates of population doublings occur. Investigators have shown that MSC selected for expansion beyond the routine 20–30 population doublings can contain cytogenetic abnormalities and can be tumorigenic *in vivo* (38). This places an additional requirement for careful cytogenetic monitoring, and adopting a stepwise manufacturing process in which intermediate master and working cell banks are used to allow methodical safety testing between short production campaigns. This is more feasible for some adherent stem cell populations expressing low levels of telomerase and greater linear expansion rates (37), and may not be feasible for MSC expansions, particularly those from aged donors.

Accordingly, it is valuable to apply new technologies that probe epigenetic change in cell culture in parallel with karyotypic analysis. Techniques have been developed for comparative genomic hybridization (CGH; [39]), in which proportional binding of test DNA to normal metaphase chromosomal spreads can detect loss or gain of chromosomal DNA from the clinical product within increasingly fine alleleic specificity. Similarly, assays have been developed using oligonucletoide hybridization probes representing single nucleotide polymorphisms (SNPs) and quantitative polymerase chain reaction (qPCR), to test for normal or abnormal gene copy numbers in a test genome. Such chromosomal SNP analyses can probe down to <50 kb of chromosomal segments across all chromosomes, yield fine structure gene copy number information capable of detecting loss or gain of genetic sequences.

The limitation of all of the described cytogenetic assays is the sensitivity. A standard karyotypic analysis examines 20 G banded metaphase spreads, for evaluation of clonal chromosomal abnormalities. This places statistical confidence for detection of abnormalities 1:20, or limits detection of an abnormal cell type when present at 5% or less of the total population. Testing even 50 metaphase spreads still limits detection of abnormal cells to 2% abundance or less. Unfortunately, the sensitivity of the molecular probes for detecting abnormal cells is similar, with a 2%–5% threshold of detection for abnormal cell types in a population.

A legitimate argument can be made for not using these assays altogether, particularly when added to the knowledge that a single proto-oncogene point mutation may confer abnormal tissue growth properties to a cell type. The corollary approach, *in vivo* animal testing, is similarly limited by the ability to test an adequately significant proportion of a clinical production run. A standard 12-week Nude mouse immunogenicity assay would use 20 animals per group receiving 2 million cells per animal. This constitutes a test of 40 million

cells, or between 40% and 4% of a clinical dose (configured at 1 or 10 million cells/kg). It is therefore very important to properly gauge patient risk benefit ratio in early stage MSC trials.

REGULATORY STRATEGIES

The original clinical development route for MSC cell therapy was initially a linear progression:

1. Phase I autologous safety trial, open label;
2. Phase I or I/II allogeneic safety trial, blinded and placebo controlled;
3. Phase II efficacy trial, blinded and randomized; and
4. Phase III approval trial, blinded and randomized.

Although the same manufactured MSC product might be used for different indications, the approval path would be repeated for each indication.

As the translational community gained experience in the safety for an infused MSC product, two development trends emerged. First, the FDA began to class bone marrow-derived MSC and related adherent cell types from other tissues together in terms of standardized preclinical data requirements and Phase I safety requirements. The composite experience within the translational research community had a collective impact on advancing confidence in safety for this class of cells.

Second, the FDA began to support an adaptive trial design, driven by the profile of an MSC cell product and the dose coupled with route of delivery. In other words, once the safety hurdle for infusion of MSC across a dosing range had been established, investigators were no longer required to start at the bottom of the ladder with a Phase I trial, but rather could initiate Phase II efficacy studies in a related indication based on prior experience. This is not broadly applicable across individual laboratories or between corporate sponsors of MSC development, but applies to the specific institutional manufactured MSC product. In addition, the FDA has allowed parallel tracking of Phase II exploratory efficacy trials.

The clinical development strategy used by Osiris Therapeutics is worth walking through as a case study.

- In 1998 Osiris initiated the first corporate MSC clinical trial, for use of MSC (Stromagen) as an adjunct in HSCT for hematologic malignancy patients. This study used autologous MSC expanded from patient bone marrow and returned at the time of HSCT.
- A second Phase I study was initiated in 1999 using allogeneic MSC (Allogen) from sibling donors, and in both studies acute infusional safety was demonstrated without interference with hematopoietic recovery.

- In 2000, a Phase II placebo-controlled clinical study was initiated and trends for improved hematopoietic recovery and reductions in GVHD morbidity were observed.
- These clinical data were later determined to be not statistically significant when compared across historical databases. In 2002 these studies were not continued.

In 2004, the clinical community was excited by the case study demonstration from LeBlanc et al. (35) that MSC could have striking benefit in treating GVHD crisis (as opposed to administration at the time of HSCT).

- In 2005 Osiris Therapeutics initiated a Phase II efficacy trial in steroid refractory acute GVHD. Dramatic improvements in morbidity were seen, and efficacy established in a patient base of about 45 patients.
- Based on these findings, in 2006 Phase II trial in Crohn's disease was initiated. The therapeutic premise was common in the HSCT trials to the Crohn's disease trial—that immunomodulation of gut inflammation could significantly diminish morbidity, enhance life quality and increase patient survival.
- In 2006 a Phase IIb study for use of MSC (now labeled Prochymal) in nonsteroid refractory GVHD was initiated.
- In 2007, based on striking efficacy endpoints, Osiris Therapeutics filed and initiated Phase III approval trials for treatment of GVHD.
- In 2008, Osiris Therapeutics filed and initiated Phase III approval studies in Crohn's disease.

This adaptive trial strategy was not limited to hematopoietic cell transplant or inflammatory bowel disease.

- In 2006, based on preclinical efficacy models in the pig, Osiris initiated a Phase I study using an intravenous route of delivery in acute myocardial infarct patients.
- Based on secondary endpoints for improvement in cardiac and pulmonary function, in 2008 Osiris Therapeutics filed to initiate studies a Phase II study in AMI.
- In 2008, based on secondary improvements in pulmonary function from the cardiac trial, Osiris Therapeutics initiated clinical studies in chronic obstructive pulmonary disease patients.
- In 2008, based on the efficacy improvements in the adult GHVD studies, Osiris Therapeutics received *approval to treat pediatric patients and receive reimbursement for therapy.*

Based on the accumulated safety data for an intravenously infused MSC product, Osiris Therapeutics was able to execute an aggressive development

campaign based on the common therapeutic hypothesis for MSC providing paracrine benefit in acute inflammatory and ischemic injury.

BUSINESS MODELS

There are three emerging business models evolving as MSC cell therapy moves forward into the clinic. It is critical that validation of a business model with economics for production, which meet acceptable health-care reimbursement thresholds, be defined early in the commercial development of MSC therapies. The cost of large Phase III approval trials is not feasible for the small to intermediate stage biotechnology companies currently bringing these technologies forward. It is the absence of a proven business model that contributes to the reluctance of the pharmaceutical and health-care industries to invest in clinical development.

Cell Therapy

The first is the obvious cell therapy model, in which an expanded cell product is produced in advance of patient identification, and is available for scheduled patient delivery. This applies to an infused or catheter based delivery route, and also applies to an acute versus degenerative injury or disease state. The lack of immunological consequences in MSC clinical experience to date supports this model. As well, the FDA has not been placing gender, ethnicity, or haplotype restrictions on donor selection. By large-scale manufacturing of the product, testing costs can be amortized over many products and economy of scale can be achieved which promise a viable reimbursable standard of care product.

Service and Reagent Model

The production of patient-designated MSC for clinical use drives an alternate business model. In this case the product is not the cell administered to the patient, but the media and expansion hardware, as well as reagents necessary for accurate release testing. In this case the *ex vivo* expansion can be accomplished in the stem cell processing lab of a hospital, with a donation of bone marrow from the patient, or a healthy donor, in advance of treatment and patient delivery. The stem cell laboratory purchases necessary materials and protocols and is reimbursed by the health-care system for labor and overhead in expansion.

This model carries the significant downside of being unable to treat acute injury. The *ex vivo* expansion requirements limit the time window for clinical product derivation and use, which is currently anywhere between 2 weeks and 2 months depending upon the procedure and testing requirements.

This model can work, and in fact is being practiced by an organization of investigators through the EBMT Society, for MSC treatment of acute GVHD

crisis. By purchasing common reagents and agreeing to common expansion protocols and treatment regimens, patients have been very successfully treated with very encouraging clinical outcome (ref).

Cell Banking Model

The logical extension from the service model and the patient-designated product is the cell banking model, in which individuals commit to tissue storage, frequently post natal, as an insurance toward cell therapy treatment in the future. This personalized medicine approach has proven successful in HSCT, particularly with banked cord blood for treatment of pediatric malignancies, and is likely to grow significantly with advances in *ex vivo* cell expansion or stem cell enrichment. It is a logical extension of this model to ex *vivo* expand a personalized MSC product for unanticipated use with availability for treatment of acute injury.

FUTURE THINKING

It has long been considered economically unfeasible to practice an individualized medicine approach in regenerative medicine, based primarily on the overhead costs of managing individual expansion suites, the high facility footprint for individualized incubators, and the inability to amortize costs over multiple clinical products. However, it is primarily the economic considerations and not clinical optimization that drive these models. As we continue our experience with MSC in the clinic, it is possible that we learn of immunological or safety considerations that drive this balance in favor of the personalized approach.

MSC clinical development is tightly linked to embryonic stem cell biology and therapeutic advances. On the one hand, the Regulatory considerations applied to the clinical use of MSC are the pioneering considerations that will be applied to cells produced from a pluripotent stem cell isolate such as an embryonal stem cell. The banking strategies that can be established for production of adult adherent stem cells are similarly a blueprint for isolation and production of similar cell types from an embryonic stem cell template. The ability to isolate a clonal MSC precursor from an embryonic stem cell source, enabling rigorous safety testing before clinical production, can duplicate the MSC isolation and *ex vivo* production capacity in place today, and can likely solve many issues related to donor variability and contribute to standardizing clinical production.

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