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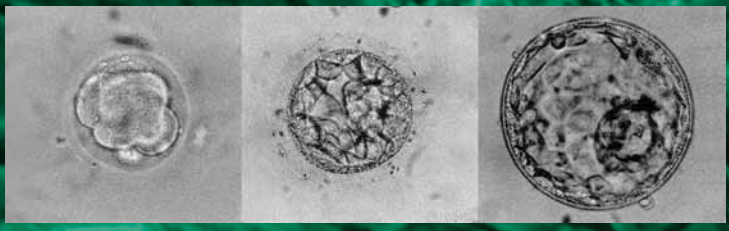
Human Embryonic Stem Cell Protocols

Edited by

Kursad Turksen



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Preface

Scientific research using human embryonic stem (hES) cells is one of the most controversial topics to come out of academic circles for some time. In fact, political and ethical controversies surrounding the study of hES cells have diverted many scientists from the field, thereby slowing its progress. Nevertheless, interest in understanding the regulation of their self-renewal capacity, commitment and differentiation along various lineages, as well as their potential utility in regenerative medicine applications, remains high. To facilitate the latter, there is a great need for the isolation of additional hES lines as well as the development of improved culture conditions to counter the view and practice that hES cells are difficult to maintain and use. I would therefore like to take this opportunity to thank all the contributors of this volume who have so generously shared their expertise and hard-won protocols.

I am grateful to Dr. John Walker for his support and encouragement during the process of compiling this protocol book. In addition, I would like to thank several others at the Humana Press for their support: initially Elyse O'Grady and Craig Adams and more recently Damien DeFrances. Also, I am grateful to Jennifer Hackworth for her wonderful support during the production of this volume.

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The tireless and always cheerful Tammy Troy has again been fantastic in helping to put together this volume.

Kursad Turksen

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1

Human Embryonic Stem Cells

Isolation, Maintenance, and Differentiation

Kursad Turksen and Tammy-Claire Troy

Summary

The isolation of pluripotent human embryonic stem (hES) cells having the capacity to differentiate in vitro to numerous cell types generated much excitement and promise in the field of regenerative medicine. However, along with great enthusiasm came hot controversy for stem cell research and researchers alike because available hES cell lines were isolated from "excess" embryos from in vitro fertilization clinics. Despite ethical and political debates, the methods and protocols to study diverse lineages are developing. Furthermore, strategies using specific growth factor combinations, cell–cell and cell–extracellular matrix induction systems are being explored for directed differentiation along a desired lineage. However, there is a great need to characterize the mechanisms that control self-renewal and differentiation and a necessity to improve methodologies and develop new purification protocols for the potential future clinical application of hES cells. After the scientific and political obstacles are overcome, it is anticipated that the hES cell field will make a tremendous difference in conditions, such as burn traumas and diabetic foot ulcers, as well a number of degenerative diseases such as Parkinson's disease, type 1 diabetes, rheumatoid arthritis, and myocardial infarction. In this introductory chapter, we will summarize and review recent progress in the field of hES cell differentiation protocols and discuss some of the current issues surrounding hES cell research.

Key Words: Embryonic stem (ES) cells; human embryonic stem (hES) cells; embryoid bodies (EBs); pluripotent; differentiation; in vitro differentiation; cell therapy; derivation; self-renewal; pluripotency.

1. Introduction

Embryonic stem (ES) cells possess two very important characteristics that have recently placed them at center stage for regenerative medicine: (1) the

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ability to proliferate without differentiation by a process of self-renewal and (2) the potential to form specialized cell types when induced to differentiation *(1)*. It has been a long-term objective of stem cell biologists to have a culture system whereby the ability to generate differentiated progeny from a continuously growing stem cell population in vitro would provide an arena for the study of stem cell/very early progenitor potential *(2,3)*. It would also make possible a comprehensive analysis of the underlying molecular mechanisms for the onset of cell lineage commitment and differentiation. With the increasing availability and utility of ES cells, some ground has been gained in this respect.

More than 20 yr ago, the first ES cells were derived from the inner cell mass (ICM) of 3.5-d-old mouse blastocysts *(4,5)*. When placed on a suitable fibroblast feeder layer in the presence of leukemia inhibitory factor (LIF), ES cells proliferate and remain pluripotent indefinitely *(4,5)*. It was also demonstrated that immortal stem cells could be manipulated in vitro, providing the opportunity to study early development as well as lineage potential of derived progenitors in vivo *(6,7)*. Since these pioneering studies, mouse ES cells have proved to be an excellent model system in which to study lineage commitment and progression in vitro *(8)*. Removal of ES cells from their feeder layer induces aggregation and differentiation into simple or cystic embryoid bodies (EBs). Simple EBs consist of ES cells surrounded by a layer of endodermal cells, whereas cystic EBs develop an additional layer of columnar ectoderm-like cells around a fluid-filled cavity, morphologically similar to embryos at the 6- to 8-d egg cylinder stage. The expression of markers for mesoderm (brachyury, activin), endoderm (collagen type IV), and ectoderm (α-fetoprotein) indicate that cells derived from all three germ layers occur in cystic EBs. At this stage, differentiation along several lineage pathways is possible with the appropriate inducing agents. Depending on culturing conditions, mouse ES cells have been shown to differentiate along a myriad of pathways including epidermal cells, type II alveolar epithelial cells, telencephalic precursors, osteoblasts, and cardiomyocytes, to name only a few *(9–14)*.

The very first established human ES (hES) cell culture was successfully isolated in 1998 from "unused" zygotes from an in vitro fertilization (IVF) clinic *(15)*. These and subsequent studies demonstrated that, as with mouse ES cells, hES cells have the capacity to self-renew without differentiation and are pluripotent in nature *(16–18)*. These characteristics impart great promise for tremendous impact on the future of regenerative medicine and medical research in general. In this overview chapter, we will provide a brief prelude to recent progress in the field of hES cell research before delving into the detailed protocol chapters within this volume.

2. Frontiers in hES Cell Derivation

IVF clinics assisting the reproduction of infertile couples have provided a source for the generation of several hES cell lines. In such clinics, it is common practice that cultured zygotes that are not transferred for pregnancy are either frozen for transfer at a later date or simply discarded at the request of the patients. Initial hES cells were derived by immunosurgery of 6-d-old blastocytes by complement-mediated removal of the outer trophectoderm of a blastocyst stage embryo leaving an intact ICM. The ICM was then plated on γ-irradiated or mitomycin C-treated mouse embryonic fibroblasts (MEFs) and cultured in high serum concentrations where ES cell colonies formed after several days in culture *(15,18)*. Since then, a number of other variations have been explored, including hES cell isolation from 8-d-old human blastocysts *(19)* or the feasibility of obtaining ES cell lines from human morule *(20)*.

To date, there are approx 19 "normal" hES cell lines in the National Institutes of Health (NIH) registry available to investigators (*see* http://stemcells.nih.gov/research/registry/). In addition, a repository of hES cell lines with various genetic abnormalities has been established from privately sponsored funding providing an unlimited source of "diseased cells" for research into the primary disturbances of cellular processes in genetic abnormalities. The hES cell lines of genetic disorders were derived from embryos unusable for transfer, deemed to be "mutant" by preimplantation genetic diagnosis; a common practice for IVF. There are 18 hES cell lines with genetic disorders including adrenoleukodystrophy, Duchenne and Becker muscular dystrophy, Fanconi anaemia, complementation group A, fragile-X syndrome, Huntington's disease (three lines), Marfan syndrome, myotonic dystrophy (two lines), neurofibromatosis type I (five lines), and thalassemia (two lines) *(21)*.

Another frontier in hES cell isolation for the study of disease and development was achieved by Hwang et al. *(22)*, who isolated the very first patient-specific, immune-matched hES cell line. It is anticipated that these ground-breaking studies will be important in the advancement of clinical deliberations for stem cell transplantation. Using this approach, Hwang et al. *(22)* generated 11 human embryonic stem cell (hESC) lines by nuclear transfer (NT) of skin cells from patients with disease or injury into donated oocytes. These lines (NT-hESCs) were grown on human feeders from the same NT donor or genetically unrelated individuals and were established at high rates, regardless of NT donor sex or age. NT-hESCs are pluripotent, chromosomally normal, and, most important, are an exact match to the NT patient's DNA. Furthermore, major histocompatibility complex identity showed immunological compatibility, an important milestone for their eventual transplantation and clinical application *(22)*.

3. Challenges in hES Cell Cultivation

Traditionally, the establishment and maintenance of hES cells has been finicky and arduous at best. Today, the manipulation of hES cells remains challenging, requiring intricate culturing skills; however, emerging protocols are more convenient and achievable. For instance, although both mechanical and enzymatic transfer methods for hESCs are being used, initially the mechanical transfer method for the maintenance of hES cells was thought to be fundamental. Mechanical transfer, a laborious and time-consuming procedure, was the method of choice primarily because of difficulties observed through enzymatic (e.g., trypsin) transfer where seeding hES cells in single-cell suspension was deemed problematic, in striking contrast to mouse ES cells. The infeasibility of hES cell passage in single-cell suspension suggests that undifferentiated hES cells are sensitive to cell density changes; implying that cell–cell interaction either between hES–hES, hES–ECM (extracellular matrix), or hES-feeder cells may be critical to hES cell signaling and survival. However, without generating a single-cell suspension, a successful enzymatic transfer technique using type IV collagenase was more recently shown to permit the efficient transfer of undifferentiated dissociated hESCs in similar clump sizes to a new feeder layer *(18,23)*, as opposed to mechanical transfer, in which a drawn micropipet is used to transfer hES cell clumps varying in size *(15,18)*. The beauty of having similarly sized clusters of hES cells obtained enzymatically ensures that individual colonies are derived from undifferentiated cells, whereas seeding larger hES cell clumps mechanically leads to the probability that both differentiated and undifferentiated hES cells are passaged. A combination technique for the passage of hES cells therefore allows for mass production by excluding differentiated colonies from passage by manual selection before enzyme treatment *(24,25)*.

3.1. Feeder Layer and Alternatives for hES Cells

Early successful derivation and maintenance protocols for hES cells were based on years of research using mouse ES cells, which have been shown to remain pluripotent when cultured on mitotically inactivated MEFs in the presence of LIF *(1)*. As with their mouse counterparts, hES cells exhibit a general requirement for MEFs to maintain their undifferentiated pluripotent state; however, they are LIF-independent *(26)*. The requirement of a feeder layer, either directly or indirectly as a source of conditioned medium in feeder-free culture systems, has posed serious limitations for the clinical use of hES cells because mice are known to harbor a variety of pathogens. Among these pathogens, endogenous retroviruses, including murine leukemia viruses, are of special importance. It is well known that some strains cause pathogenic (e.g., leukemic) effects and that xenotropic, polytropic, and amphotropic murine leukemia viruses are able to infect human cells. However, recent studies suggest that such infection of hES cells was not

observed in vitro *(27)*. On the other hand, the practice of using an MEF feeder layer contaminates the hES cells with *N*-glycolylneuraminic acid (Neu5Gc); a nonhuman cell surface sialic acid for which humans have naturally occurring antibodies. It was observed that hES cells contaminated with Neu5Gc were recognized as foreign and were attacked by human antibodies. Therefore, it is anticipated that any hES cells derived or maintained on MEFs would likely be rejected on therapeutic transplantation *(28)*. To circumvent this obstacle, the necessity to eliminate all nonhuman products is paramount.

In the very recent past a great undertaking has been embraced to establish human-derived feeder cells in order to circumvent this clinical roadblock. For instance, mitotically inactivated human bone marrow stromal cells *(29)* and human foreskin cells obtained from newborns after circumcision *(30)* have been described for use as hES cell feeder layers. An innovative approach using fibroblast-like cells derived from the spontaneous differentiation of hES cells has also been reported *(31)*, providing a novel feeder cell system. Isogenicity of the hES cells and their derived fibroblasts was confirmed by microsatellite analysis and the nature of the hES cell-derived fibroblasts was identified by the expression of specific markers. This resourceful feeder system permits for the continuous growth of undifferentiated pluripotent hES cells as demonstrated by the expression of specific markers, the formation of teratomas after injection into severely combined immunodeficient (SCID) mice, as well as by in vitro differentiation into the differentiated cells of ectodermal, endodermal, and mesodermal origin *(31)*.

Further efforts have been put forth to eliminate all together the feeder layer requirement of hES cells. The realization that MEF-derived matrix materials may have a role in the maintenance of undifferentiated hES cells has encouraged study into the effects of ECM such as Matrigel- or laminin-coated plates *(30)*, which can be readily sterilized. However, Matrigel itself is an animal product and therefore also presents complications. It is unknown specifically which factors produced by the MEFs are required for hES cell maintenance. However, in the absence of a feeder layer, or at least MEF conditioned media, there is spontaneous and random differentiation, with heterogeneous populations of differentiated cells appearing throughout hES cell colonies. Initial experiments indicate that an undefined soluble factor(s) released from the MEF feeder layer is an important element in maintaining an undifferentiated state in hES cells. Analysis of conditioned media of human neonatal fibroblasts using two-dimensional liquid chromatography-tandem mass spectrometry and two-dimensional electrophoresis followed by matrix-assisted laser desorption/ionization time of flight tandem mass spectrometry provided insights into the maintenance requirements of hES cells. A total of 102 proteins were identified using these approaches and were classified into 15 functional groups, including proteins involved in a variety of events such as cell adhesion, cell

proliferation, Wnt signaling, and inhibition of bone morphogenetic proteins (BMPs) *(32)*; however, these factors remain to be analyzed.

3.2. Serum Components and Replacement

To further the elimination of MEFs in hES cell maintenance, there is a requirement to remove nonhuman serum products and to find serum replacements. Human serum has been demonstrated to maintain undifferentiated hES cells in the absence of MEFs *(33)*. In efforts to define serum-free conditions, more recent protocols employ a serum-free alternative using serum replacement (SR) *(34)*; several new lines have been successfully isolated with SR *(35,36)*. The combination of noggin and basic fibroblast growth factor *(37)*, basic fibroblast growth factor alone, or in combination with other factors *(38,39)* (e.g., activin A) are capable of maintaining undifferentiated hES cells for an extensive number of passages without the need for feeder layers or conditioned media from MEFs *(40)*. In addition, it has been shown that hES cell lines can be successfully derived using SR medium and postnatal human fibroblasts as feeder cells. Inzunza et al. *(36)* derived two recent hES cell lines (HS293 and HS306) and 10 early cell lines using human neonatal fibroblasts as feeder cells as well as SR medium as an alternative to conventional fetal calf serum. The pluripotency of HS293 has been shown in vivo by teratoma formation in SCID mice and the karyotype is 46 XY: that of HS306 is 46 XX. This is a step toward xeno-free conditions and facilitates the use of these cells in transplantation. However, despite great efforts, the modified protocols for the routine maintenance of hES cells are suboptimal, with spontaneous differentiation regularly observed.

4. Characteristics of hES Cells

After injection into blastocysts, mouse ES cells are able to integrate into all embryonic germ layers, including the germ line. Their pluripotency is most evident through the generation of chimeric mice with ES cell contribution in all observable tissues, as well as the production of viable embryos derived entirely from ES cells *(6)*. Because such experiments are impossible for the hES cell model system, hES cell pluripotency is demonstrated as described previously by teratoma formation after injection into SCID mice *(15,18)*. Teratomas form cellular regions representative of all three germ layers including endoderm (gut and glandular epithelium), mesoderm (bone, smooth and striated muscle), and ectoderm (neural epithelium and stratified squamous epithelium) *(15,18)*.

As with mouse ES cells, hES cells exhibit two unique properties: self-renewal capacity and the ability to differentiate via precursor cells into terminally differentiated somatic cells. Morphologically, hES cells are distinctive, characterized by a high nuclear-to-cytoplasmic ratio with each cell typically having one or more prominent nucleoli; these characteristics have been ultrastructurally described.

Several studies have evaluated the undifferentiated state of hES cell cultures using variables such as normal karyotype and markers of pluripotency and they have been shown to express high levels of telomerase, a ribonucleoprotein responsible for maintaining the length of chromosomes. It has been reported that they express distinct markers including high levels of SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, and alkaline phosphatase, but do not express SSEA-1.

Several genes have been shown in hES cells indicating their pluripotent phenotype. For instance, as in mouse ES cells, hES cells express Oct-4 (a germ-line transcription factor) at high levels; expression is downregulated in the majority of murine adult tissues excluding the germ line. Although the role of Oct-4 in the maintenance of an undifferentiated fate appears to be conserved in hES cells, knockdown of Oct-4 by RNAi-based targeting forced differentiation of hES cells into cells including trophoblasts ***(41)***. Other genes having high levels of expression in undifferentiated mouse ES cells that are typically downregulated or lineage-restricted on differentiation appear to be conserved in hES cells, including Nanog (a recently identified homeobox containing transcription factor), Sox-2 (a transcription factor containing a SRY-related HMG box), FGF-4, and Rex-1 (a zinc finger transcription factor). Despite their similarities, accumulating data indicate that there are indeed differences between mouse and hES cells, suggesting the existence of alternate signaling pathways for self-renewal and pluripotency in human cells ***(26)***.

5. Transcriptome Profiling of hES Cells

Emerging studies are directed toward a complete transcriptomic profile of hES cells and their derivatives as compared with mouse ES cells ***(42,43)***. Wei et al. ***(43)*** used one murine and two hES cell lines to delineate their molecular characteristics using massively parallel signature sequencing (MPSS) technology. More than 2 million tags from each line were sequenced and validated by reverse transcription polymerase chain reaction and microarrays; results indicate that mouse and human ES cells share a small set of conserved genes. However, differences were shown in the LIF, transforming growth factor-β, Wnt, and FGF signaling pathways. Emphasizing the important transcriptional differences between the species implies that mouse ES cell developments regarding the role of various signaling pathways during very early development may not necessarily be directly translatable to hES cells.

Transcriptomic profiling of undifferentiated as compared to differentiated hES cells is of great interest and importance to the understanding of the signaling pathways involved in the differentiation process of hES cells. Miura et al. ***(26)*** performed gene profiling by expressed sequenced tag enumeration and MPSS using RNA samples from feeder-free cultures of undifferentiated (pas-

sages 40–50) hES cells (H1, H7, and H9) as compared with differentiated cells (d 14) of the same lines. MPSS and expressed sequenced tag scan analysis showed concordance and identified a large number of genes that changed rapidly as cultures transition from a pluripotent to a differentiated state. A subset of genes that were either up- or downregulated were selected and their differential expression confirmed by a variety of independent methods, including comparison of expression after further differentiation by reverse transcription polymerase chain reaction and immunocytochemistry. The analysis identified markers unique to the ES and EB stages as well as signaling pathways that likely regulate differentiation *(26)*.

6. Differentiation of hES Cells

The general curiosity vis-à-vis hES cell research surrounds their potential to differentiate into a diverse range of lineages. The conception is that hES cells will provide the fundamental building blocks for the purification of populations of specific cell types for direct transplantation-based clinical therapies. Further, the apparently unlimited proliferation capacity of hES cells make them a more viable source of large-scale specific cell type production when compared with adult stem cell types, which frequently show limited proliferation in vitro. As with mouse ES cells, the first step in the differentiation process of hES cells is the formation of EBs. Mouse EBs form spontaneously under specific conditions in suspension culture and comprise multiple lineages, rather similar to that of the early embryo. However, differentiation is "chaotic," often resembling a teratoma rather than precisely controlled as in the embryo. Despite the ease of murine EB formation, hEBs have proven to be much more difficult to culture with varying degrees of success amongst researchers. Nonetheless, hEBs may recapitulate certain aspects of early embryonic development *(44)*. One successful method involves hES cell disaggregation by collagenase or trypsin treatment and growth in suspension culture *(44)*. In addition, the use of specific growth factors has been shown to direct differentiation of hEBs toward defined germ layer representatives *(45)*. Furthermore EGF, FGF, retinoic acid, BMP-4, and transforming growth factor-β treatment induce hEBs to express both mesodermal and ectodermal markers, whereas activin-A treatment favors the expression of mesodermal derivatives *(45)*.

7. Summary

The number of laboratories worldwide using hES cells as a research tool is rapidly expanding, and numerous protocols have been developed for the differentiation of hES cells into various lineages. Yet, there are number of challenges to be resolved including elemental questions regarding basic concepts of hES cell biology before any of the astonishing potentialities of hES cells may come

to fruition. The "shopping list" of prerequisite issues to be resolved include (1) the definition of specific culture conditions for the differentiation of hES cells into defined lineages; (2) the elimination of the teratoma-forming potential of undifferentiated hES; and (3) the resolution of immune tolerance concerns. There is great optimism that these challenges will be resolved and that the potential of hES cells will be realized in the near future.

The overall intent of this introductory chapter was to highlight a number of controversial and practicable issues in the application of hES cells as a powerful in vitro model system for the delineation of differentiation mechanisms to specific cell lineages, a priori to their remarkable clinical potentials. Significant advancement in our understanding of the molecular mechanisms governing the maintenance of pluripotent hES cells and their directed differentiation must be achieved for their ultimate use as therapeutic agents. The scientific community is closely pursuing publications of several hES cell investigators, including the esteemed contributors of this volume to whom we are grateful to for sharing their hard-earned protocols. The world anxiously awaits the unfolding of hES cell developments that will have striking consequences on our traditional concept and practice of medicine.

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2

Blastocyst Culture for Deriving Human Embryonic Stem Cells

Ariff Bongso

Summary

Success in producing a human embryonic stem cell line depends largely on the quality of the 5-d-old embryo (blastocyst) used. Such blastocysts are grown from frozen-thawed d 1–3 surplus embryos left over from infertility clinics and donated for stem cell research with informed patient consent. Knowledge, therefore, of the culture conditions and embryo scoring systems that are used to generate high-quality blastocysts are crucial. This chapter describes an extended microdroplet culture protocol using two-stage sequential culture media with morphological and polarized optical parameters for embryo scoring for each day of embryonic growth to help produce high-quality blastocysts.

Key Words: Blastocyst culture; embryo scoring systems; human embryonic stem cell lines.

1. Introduction

The chances of success of producing a human embryonic stem (hES) cell line depends largely on the quality of 5-d-old embryos (blastocysts) used. Embryos provided for this purpose are usually surplus embryos left over from in vitro fertilization clinics and frozen at cleavage stages (two pronuclear, four-cell and eight-cell stages) on d 1–3 postinsemination. Institutional Review Board approval and informed patient consent must be sought before such donated embryos are used for hES cell derivation. There also appears to be a strong correlation between the stability of the ensuing hES cell line and the quality of the embryo used to derive it. Knowledge, therefore, of the various markers used for the daily scoring of embryos from fertilization to blastocyst formation is crucial to select the best quality embryo with the highest cumulative score for hES cell derivation. Morphological characteristics using polarized parameters *(1)* and

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Table 1
Embryonic Stages With Highest Scores Observed d 2–6[a]

Day	Embryonic stage	Description
2 (AM)	Cleavage stage	4–6 cells, regular blastomeres, no fragments
3 (AM)	Cleavage stage	8 cells, regular blastomeres, no fragments
	Compacting	Blastomeres fusing
4 (AM)	Compacting	Blastomeres fusing
	Compacted	Blastomeres fused
	Early cavitating	First signs of blastocoele
	Late cavitating	Distinct blastocoele ICM and TE not laid down
5 (AM)	Early blastocyst	Distinct ICM, TE, and blastocoele Embryo diameter same as d 4 or slightly larger
5 (PM)	Fully expanded blastocyst	Distinct ICM, TE, blastocoele. Thin zona pellucida Fully expanded diameter (~ 215 μm)
6 (AM)	Hatching blastocyst	ICM and TE completely hatched out from zona pellucida. Empty zona pellucida

[a]ICM, inner cell mass; TE, trophectoderm.

cleavage speed are the best markers correlating with quality of embryos *(2,3)*. The following are the most reliable descriptions of markers used for each day of human embryonic growth in vitro (**Table 1**).

1.1. Day 1 (Two Pronuclear and First Cleavage)

Hoffman's inverted optics are used to determine that normal fertilization has taken place by visualizing the presence of two distinct pronuclei; mono- or multipronuclear embryos also divide to the blastocyst stage and may thus have the potential of producing hES cell lines (parthenogenotes and polyploids). There is a strong correlation between two pronuclear (2PN) scoring and blastocyst development *(4,5)*. The markers for the best embryos on d 1 include (1) the abutment and proximity of the two pronuclei, (2) the alignment in vertical rows of the nucleoli of each pronucleus, (3) the appearance of a cytoplasmic clear halo around the pronuclei, and (4) the early appearance of the first cleavage (two-cell stage) *(6)* usually at 24–25 h postinsemination (**Fig. 1A**).

1.2. Day 2 (Four–Six Cell Stage)

Day 2 cleavage stage embryos (four- to six-cell stage) are usually scored according to three grades (grades 1, 2, and 3) based on (1) the size and regularity of the blastomeres and (2) the presence or absence of cytoplasmic fragments

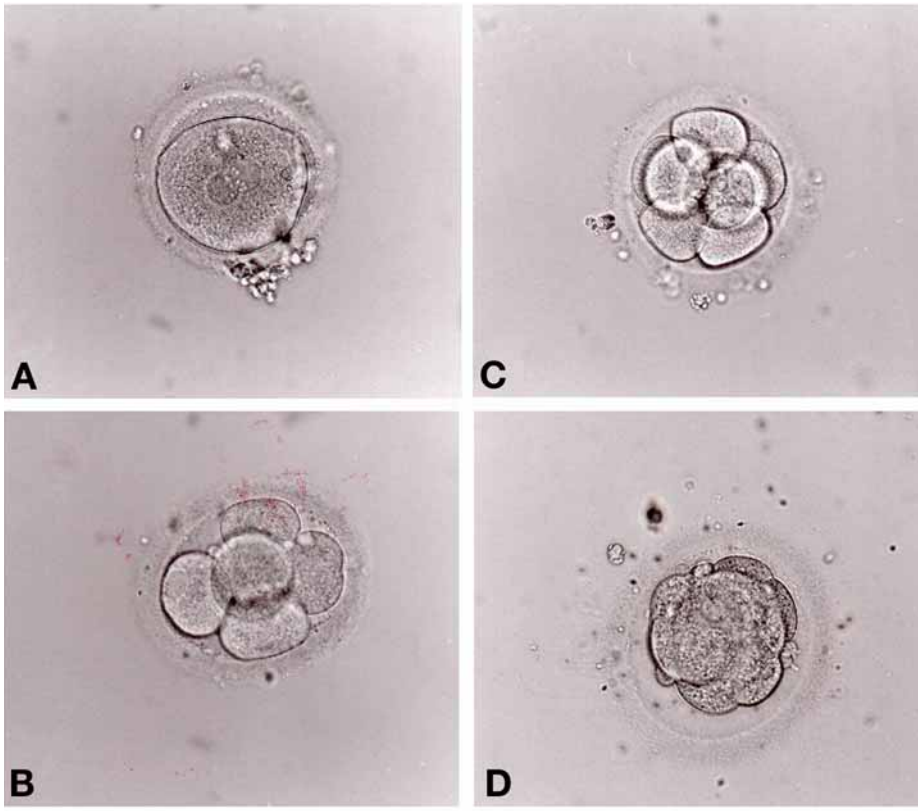

Fig. 1. Post-thawed d 1–3 human embryos. **(A)** 2PN (d 1, AM) embryo showing pronuclear abutment and nucleoli aligned in vertical rows. **(B)** Five-cell Grade 1 (d 2, AM) embryo with five regular-size blastomeres and no fragments. **(C)** Eight-cell (d 3, AM) embryo with eight regular-size blastomeres and no fragments. **(D)** Good-quality d 3 (AM) embryo showing compacting blastomeres and no fragments (×200). (Please *see* the companion CD for the color version of this figure.)

(**Fig. 1B**). Grade 1 embryos have regular size blastomeres, no cytoplasmic fragments, and receive the highest score. Grade 2 embryos have regular size blastomeres and a moderate number of cytoplasmic fragments, whereas grade 3 embryos have irregular blastomeres and many cytoplasmic fragments.

1.3. Day 3 (Eight Cell to Compacting Stages)

On the morning of d 3, embryos are observed for compaction as evidence of activation of the genome and the bypassing of the in vitro embryonic block between d 2 and 3. Embryos that have commenced compacting and have no cytoplasmic fragments (**Figs. 1D** and **2A**) receive the highest scores, whereas

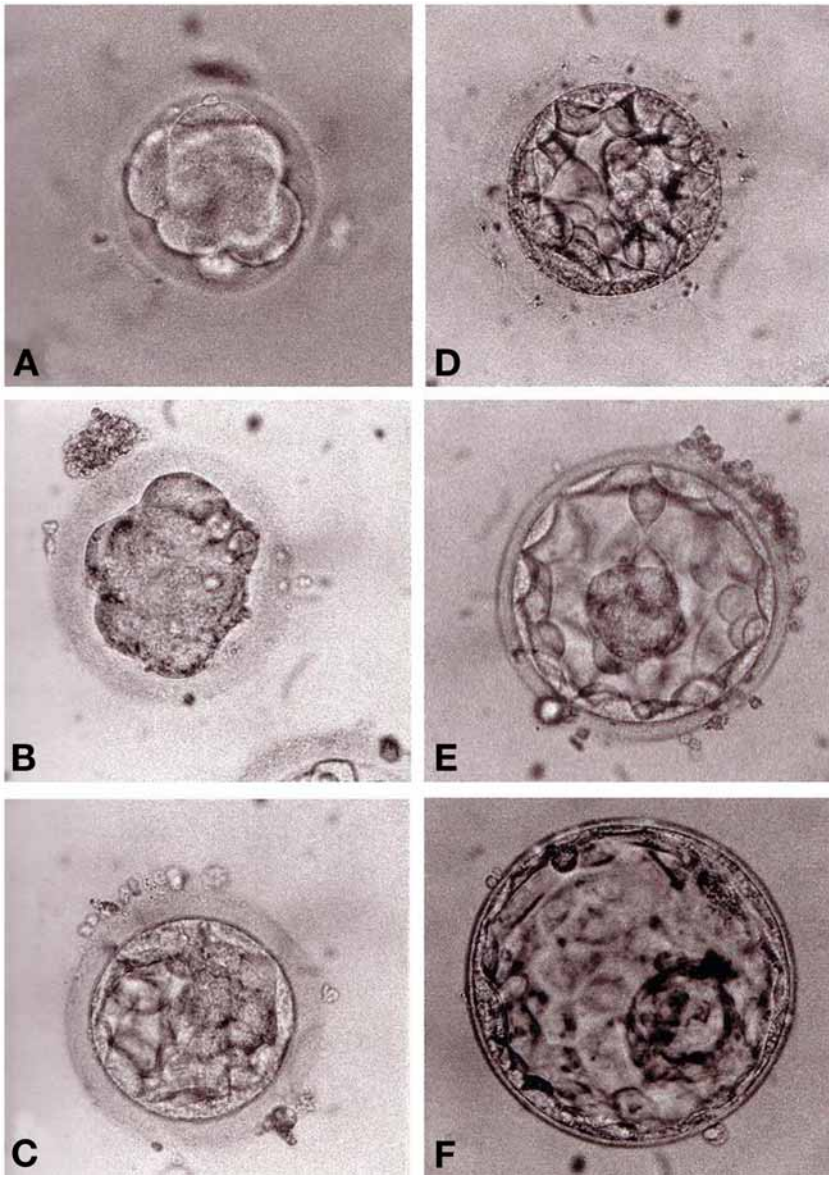

Fig. 2. Post-thawed d 3–5 human embryos. **(A)** Compacting embryo (d 3, AM). **(B)** Fully compacted embryo (d 4, AM). **(C)** Early cavitating embryo (d 4, AM). **(D)** Late cavitating embryo (d 4, AM). **(E)** Early blastocyst (d 5, AM) showing peripheral trophectoderm and inner ICM. Note increase in embryo diameter from cavitating stage in **D**. **(F)** Fully expanded blastocyst (d 5, PM). Note further increase in embryo diameter from early blastocyst stage in **E** (×200). (Please *see* the companion CD for the color version of this figure.)

embryos with eight equal-size blastomeres and no cytoplasmic fragments (**Fig. 1C**) receive the second highest scores. Poor-quality embryos have many fragments and lesser blastomeres.

1.4. Day 4 (Compacted to Cavitating Stages)

On the morning of d 4, embryos are scored for complete compaction and the appearance of an early blastocoele (early cavitation). Fully compacted (**Fig. 2B**) and early and late cavitating (**Fig. 2C,D**) embryos receive the highest scores, whereas those embryos with slow cleavage, no compaction, and no signs of a blastocoelic cavity receive low scores.

1.5. Day 5 (Blastocyst Stages)

On the morning of d 5, embryos are expected to have reached the blastocyst stage. An embryo is defined as a blastocyst if it has a clearly laid down inner cell mass (ICM), a trophectoderm, and a single blastocoelic cavity. Three categories of blastocyst can be recognized: (1) early (**Fig. 2E**), (2) expanding, and (3) fully expanded (**Fig. 2F**). The main differences between the three categories are increases in diameter and thinning of the zona pellucida. Usually on the morning of d 5, only early blastocysts and late cavitating stages are observed, and these receive high scores. Expanding and fully expanded blastocysts are commonly seen on the afternoon of d 5 or morning of d 6. The graduated embryo score *(7)* or highest cumulative score from 2PN to blastocyst stage is used to select the best blastocyst for hES cell derivation with a high hit rate. The reliable markers for good quality expanded blastocysts that are used for hES cell derivation are shown in **Table 2**. Approximately 70–75% of high-quality blastocysts are usually obtained when good-quality sequential culture media are used.

2. Materials

2.1. Thawing Materials for Frozen Cleavage Stage Embryos (Days 1–3)

1. THAW-KIT 1 (Vitrolife, Goteburg, Sweden; cat. no. 10067). Thaw solution 1 (TS1): 1.0 *M* PrOH and 0.2 *M* sucrose in Cryo-phosphate buffer solution (PBS containing 25 mg/mL human serum albumin [HSA]). Thaw solution 2 (TS2); 0.5 *M* PrOH and 0.2 *M* sucrose in Cryo-PBS. Thaw solution 3 (TS3): 0.2 *M* sucrose in Cryo-PBS.
2. GIII series medium (Vitrolife G-1 version 3; cat. no. 10091 and G-2 version 3; cat. no. 10092).
3. HSA solution (Vitrolife; cat. no. 10064).

2.2. Thawing Materials for Frozen Blastocysts (Days 5 and 6)

1. G-ThawKit Blast (Vitrolife; cat. no. 10076).
2. 8, 6, 5, 4, 3, 2, 1% glycerol. Incubation medium (G-2 version 3 and HSA medium, Vitrolife; cat. no. 10092).

Table 2
Markers for Good-Quality, Expanded Blastocysts[a]

Morphological characteristics	
	Large ICM
	Thin zona pellucida
	Single, large blastocoele
	"Sickle-shape" cells in TE
Cleavage speed	
	Cavitated, expanding —d 5 (AM)
	Fully expanded—d 5 (PM)
	Hatching/hatched —d 6 (AM)

[a]ICM, inner cell mass; TE, trophectoderm.

3. Four-well sterile plastic dishes (Nunclon, Roskilde, Denmark; cat. no. 176740).

2.3. Embryo Culture (Days 1–6)

1. G-1 version 3 medium (Vitrolife; cat. no. 10091).
2. G-2 version 3 medium (Vitrolife; cat. no. 10092).
3. HSA solution (Vitrolife; cat. no. 10064).
4. Ovoil (Vitrolife; cat. no. 10029).
5. Four-well sterile plastic dishes (Nunclon; cat. no. 176740).

3. Methods

3.1. Thawing Protocol for Frozen Cleavage Stage Embryos (Days 1–3)

1. Prepare all paperwork. Identify name of patient and location of straw in liquid nitrogen (LN_2) Dewar. Keep the straws immersed in LN_2 until actual thawing. Label dishes for thaw solutions and pipette appropriate amounts of TS1, TS2, TS3, and Cryo-PBS. Thaw straws containing embryos one at a time. Perform all steps at room temperature.
2. Remove straw and air thaw for 30 s (*see* **Note 1**).
3. Place straw in a 37°C water bath for 30 s. Remove and carefully wipe. Cut the plug end of the straw with sterile scissors and attach it to a 1-mL syringe. Then cut the other end carefully without shaking straw or making air bubbles.
4. Gently expel the embryos into TS1. Observe the embryos coming out of the straw; if you do not see them all, quickly refill the straw and gently flush, because occasionally the embryos will stick to the sides of the straw.
5. Incubate the embryos for 5 min in TS1.
6. Gently transfer the embryos to TS2 for 5 min (*see* **Note 2**).
7. Transfer the embryos to TS3 for 5–10 min (*see* **Note 3**).
8. Transfer the embryos to Cryo-PBS for 5–10 min (*see* **Note 4**).
9. Place embryos into G-1 version 3 and HSA medium if embryos were cryopreserved on d 1 or 2 and in G-2 version 3 and HSA medium if cryopreserved at the eight-cell

stage on d 3, and incubate at 37°C in a 5% CO_2 in air atmosphere (*see* **Note 5**). Assess and record survival and morphology compared to prefreeze. Score embryos according to markers described earlier for d 1–3 (*see* **Subheadings 1.1.–1.3.**).

10. Human embryos are cultured in G-1 version 3 and HSA medium on d 1 and 2 and in G-2 version 3 and HSA medium on d 3–6. The medium is changed every 48 h. Each embryo is monitored and scored daily in the morning. *See* embryo culture protocol described in **Subheading 3.3.**

3.2. Thawing Protocol for Frozen Blastocysts (Days 5 and 6)

1. Label each well of four-well dishes as 8, 6, 5, 4, 3, 2, 1% glycerol, and two wells as incubation medium.
2. Pipet appropriate volumes (0.5 mL) of the thawing solutions into each well. Prepare also a separate dish with incubation medium.
3. Equilibrate the dishes at 37°C in a 5% CO_2 in air atmosphere for 1 h.
4. Fill a large beaker with warm water (37°C). Identify name of patient and location of straw in LN_2 Dewar. Prepare all paperwork. Keep the straws immersed in LN_2 until actual thawing. Thaw straws containing blastocysts one at a time and perform all steps at 37°C.
5. Remove straws from LN_2, and wipe the straw quickly with a clean tissue. Air-thaw straw for 30 s and watch the straw for leaks.
6. Gently plunge the straw into the beaker of water (37°C) for 40–50 s.
7. Remove and gently dry the straw with tissue. Cut off the heat-sealed end with scissors and attach to a 1-mL syringe. Cut the cotton-plug end with a sterile scissors and expel the blastocysts into the 8% glycerol solution. If the embryos are not seen, flush the straw a few times. Leave the embryos in the 8% dish for 5 min.
8. Transfer the embryos to the 6% glycerol solution for 10 min at 37°C in 5% CO_2 in air.
9. Transfer the embryos to the 5% glycerol solution for 12 min at 37°C in 5% CO_2 in air.
10. Transfer the embryos to the 4% glycerol solution for 12 min at 37°C in 5% CO_2 in air.
11. Transfer the embryos to the 3% glycerol solution for 14 min at 37°C in 5% CO_2 in air.
12. Transfer the embryos to the 2% glycerol solution for 14 min at 37°C in 5% CO_2 in air.
13. Transfer the embryos to the 1% glycerol solution for 16 min at 37°C in 5% CO_2 in air.
14. Rinse the embryos twice in incubation medium.
15. Transfer the embryos to the dish with fresh equilibrated incubation medium (0.5 mL) and incubate at 37°C in 5% CO_2 in air.
16. Assess each embryo under the microscope. Compare the blastocyst quality with appearance at the time of freezing. Score the blastocyst according to the markers described earlier (*see* **Subheading 1.5.** and **Tables 1** and **2**).

3.3. Embryo Culture (Days 1–6)

3.3.1. Microdroplets Under Oil

Culture of human embryos in microdroplets of medium (20–100 µL) under oil can save on costs, help to maintain optimum osmolarity and pH, and control fluctuations in temperature. Daily monitoring and scoring of individual embryos are also easy, quick, and reliable. In vivo in the human fallopian tube, embryos grow in microdroplets of fallopian tubal fluid and because such in vivo conditions are simulated in vitro by using microdroplets of culture medium containing the optimal metabolic requirements for the embryo, high-quality blastocysts are generated *(8)*.

3.3.2. Sequential Culture Media

Knowledge of how coculture systems work by positive and negative conditioning *(9)* led to the evolution of sequential culture media. Human embryos are metabolically dynamic and their nutritional requirements change from day to day and from stage to stage. For example, early cleavage embryos require minimal glucose, whereas late blastocysts require plenty of glucose; for pyruvate, the requirements are the other way around. The antioxidant taurine is beneficial for blastocyst development, whereas hypoxanthine is detrimental. Late morula and blastocyst stage embryos require essential and nonessential amino acids, because transcription has commenced after genomic activation around d 3; these will be the building blocks for further protein synthesis. As such, to obtain optimal blastocyst development, early cleavage-stage embryos (2PN to four-cell stage) are grown in the first medium (G-1 version 3 and HSA), and from d 3 onward, when the genome is being activated, the embryos are grown in a more complex second medium (G-2 version 3 and HSA) that contain salts, energy substrates, proteins, amino acids, vitamins, and hormones *(10)*. It is crucial when transferring embryos from new to fresh or from G-1 to G-2 version 3 and HSA medium that drawn-out pipets are used to avoid the dilution of the second medium with the first.

3.3.3. Methods for Embryo Culture (Days 1–6)

Day 0: G1-version 3 and HSA dishes are made for thawed d 1 (2PN) embryos. Microculture droplets (20 µL) of G1-version 3 and HSA are placed in the wells of four-well or small Petri dishes and overlaid with sterile oil (Ovoil). Two separate rinse drops of G1-version 3 and HSA are made in the center of the same four-well dish or Petri dish. The dishes are incubated at 37°C in 5% of CO_2 in air.

Day 1: The thawed 2PN embryos are washed in the two rinse drops, before transfer to the microculture droplet. Each 2PN embryo is cultured individually in each microculture droplet and the dishes incubated at 37°C in 5% CO_2 in air.

Check the embryos once more at 24 to 25 h postinsemination to record the first cleavage (two-cell stage). 2PN and first cleavage embryos are scored according to the markers described earlier (*see* **Subheading 1.1.**).

Day 2: (1) Each embryo is assessed for cleavage on d 2 and scored further according to the three grades (1, 2, and 3) based on fragmentation and regularity of blastomeres. There is no change of medium and the embryos are grown in the same G-1 version 3 and HSA medium. (2) G-2 version 3 and HSA dishes are made up for the following day. Microculture droplets (20 µL) of G-2 version 3 and HSA are placed in each well of four-well dishes or in small Petri dishes and overlaid with Ovoil. Two rinse drops of G-2 version 3 and HSA are placed in the center of the same four-well or Petri dish. The dish is incubated at 37°C in 5% CO_2 in air.

Day 3: On the morning of d 3, embryos are scored and transferred from G-1 version 3 and HSA medium into the G-2 version 3 and HSA culture dish prepared the previous day. The embryos are rinsed in the two central drops of the culture dish before transfer to the microculture droplet (*see* **Note 6**). The embryos are incubated at 37°C in 5% CO_2 in air.

Day 4: (1) On the morning of d 4, embryos are scored. There is no change of medium and the embryos are incubated in the same G-2 version 3 and HSA at 37°C in 5% CO_2 in air. (2) Day 6 growth dishes containing fresh 20 µL microculture droplets of G-2 version 3 and HSA medium are prepared and incubated at 37°C in 5% CO_2 in air.

Day 5: (1) On the morning of d 5, embryos are scored using the blastocyst markers described earlier (*see* **Subheading 1.5.** and **Tables 1** and **2**). Good-quality expanded blastocysts with large ICMs generated from high 2PN scores are given priority when selecting embryos for deriving hES cell lines ***(11,12)***. (2) Remaining embryos that are slow in development are transferred to fresh 20 µL microculture droplets of G-2 version 3 and HSA medium made on d 4 and monitored until d 6 for further blastocyst development. Expanded blastocysts with large ICMs generated on d 6 can also be used for hES cell line derivation.

4. Notes

1. During this time, handle the straws carefully and examine them for air bubbles, cracks in the seal, and any leakage of LN_2.
2. Note that embryos are osmotically stressed and have to be handled very carefully.
3. Handle the embryos very gently because their membranes will be very fragile.
4. Do not place in the CO_2 incubator because the buffer capacity of Cryo-PBS is inadequate for the 5% CO_2 atmosphere.
5. Human embryos are usually not frozen at compacted and cavitating stages.
6. Care must be taken not to dilute the G-2 version 3 and HSA medium with G-1 version HSA medium.

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3

Propagation of Human Embryonic Stem Cells on Human Feeder Cells

Mark Richards and Ariff Bongso

Summary

Human embryonic stem (hES) cell lines are usually derived and propagated on inactivated murine embryonic fibroblast (MEF) feeders. The use of MEFs and culture ingredients of animal origin for hES cell support increases the risk of cross-contamination of the hES cells with infectious animal agents from the MEFs and animal-based culture medium. This thus makes such hES cells lines undesirable for clinical application. This chapter describes several protocols used in the propagation of hES cells on human fibroblast feeder cells. Two culture methods, the bulk enzymatic culture protocol and the microdissection "cut and paste" protocol are described. Only certain human fetal and adult fibroblast feeders support hES cell growth. Methods for the characterization of pluripotent undifferentiated hES cells grown on human feeders including cell surface marker staining and real-time polymerase chain reaction are also described.

Key Words: Bulk culture; "cut and paste" protocol; human adult fibroblasts; human embryonic stem cells; human fetal fibroblasts; nonsupportive feeder; supportive feeder.

1. Introduction

Human embryonic stem (hES) cell lines were first derived and serially propagated on inactivated murine embryonic fibroblast (MEF) feeder layers *(1,2)*. The use of MEFs and other components of animal origin in the culture media for hES cell support substantially increases the risk of contaminating these lines with infectious animal agents such as retroviruses and severely limits the potential of these lines for clinical usage as well. All National Institutes of Health registered hES cell lines approved for US federal government research funding have been derived on MEF feeder layers and have also been exposed to xenoproteins. This makes these hES lines undesirable for clinical application

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although they may suffice for most basic research studies. Therefore, many more new hES cell lines derived in "xeno-free" conditions need to be established for clinical investigation and application.

Several groups have shown it possible to derive and propagate hES cell lines on a variety of human feeder layers *(3–7)*. hES cells supported on human feeder layers form colonies that have angular and straight edges as opposed to hES colonies on MEF feeders, which appear circular. hES cell lines derived and serially propagated on human feeder layers remain pluripotent over extended passage; express Oct-4, Nanog, and telomerase; maintain stable karyotypes; individual cells retain a high nucleus to cytoplasm ratio, test positive for all molecular and cell surface markers associated with the pluripotent phenotype, form teratomas in severe combined immunodeficiency SCID mice; and are capable of multilineage in vitro differentiation *(8)*. Apart from differences in gross colony appearance, all other characteristics of MEF and human feeder supported hES cell lines appear to be similar.

In our laboratory, we have found that some human fibroblast feeders are an excellent substitute for MEFs, and we propagate our hES cell lines exclusively on human feeders. We have also been able to derive and maintain a human ES cell line in "xeno-free" conditions on human fetal muscle fibroblasts in culture media containing human based ingredients for more than 20 serial passages without recourse to any animal-based ingredients *(3)*.

Although many labs prefer using MEFs instead human feeder layers for hES support because of convenience, it is likely that the propagation of hES cells on human feeder layers will gain popularity especially with the push to transfer the science to a clinical setting. The production of a supportive human feeder line immortalized with telomerase is technically possible. If such a line were made widely available, this would certainly lead to the widespread use of human feeders for hES cell support.

2. Materials

1. High-glucose Dulbecco's modified Eagle's medium (DMEM) without sodium pyruvate, without L-glutamine 1X (GIBCO, Carlsbad, CA; cat. no. 11960-044), store at 4°C.
2. L-glutamine 200 m*M*, 100X liquid (GIBCO; cat. no. 25030-081), aliquot and store at –20°C (*see* **Note 1**).
3. Penicillin-streptomycin liquid containing 5000 U of penicillin and 5000 mg of streptomycin/mL (GIBCO; cat. no. 15070-63) aliquot and store at –20°C.
4. MEM nonessential amino acids 10 m*M*, (100X) (GIBCO; cat. no. 11140-050), wrap in foil to protect from light and store at 4°C.
5. Defined fetal bovine serum (Hyclone, Logan, UT; cat. no. SH30070-03) aliquot and store at –20°C.
6. Qualified fetal calf serum (GIBCO; cat. no. 10099) aliquot and store at –20°C.

7. Dulbecco's phosphate-buffered saline (PBS) (+) (GIBCO; cat. no. 14040-133), store at 4°C.
8. Dulbecco's PBS, Ca^{2+}, Mg^{2+} free, PBS(–) (GIBCO; cat. no. 14190-144), store at 4°C.
9. Sterile water, tissue culture grade (GIBCO; cat. no. 15230-162), store at 4°C.
10. Porcine gelatin (Sigma, St. Louis, MO; cat. no. G1890), store at 4°C.
11. Insulin-transferrin-selenium growth supplement (GIBCO; cat. no. 41400-045), store at 4°C.
12. 0.05% Trypsin-EDTA (1X) (GIBCO; cat. no. 25300-062), aliquot and store at –20°C.
13. β-Mercaptoethanol (GIBCO; cat. no. 21985-023), store at 4°C.
14. Dispase (GIBCO; cat. no. 17105-041), store at 4°C.
15. Collagenase IV (GIBCO; cat. no. 17104-019).
16. F12/DMEM, HEPES buffered (GIBCO; cat. no. 11330-057).
17. KnockOut Serum Replacement (GIBCO; cat. no. 10828-028).
18. Basic fibroblast growth factor, human recombinant (GIBCO; cat. no. 13256-029).
19. 1 *M* HEPES solution (GIBCO; cat. no. 15630-080), store at 4°C.
20. Ethylene glycol (Sigma; cat. no. E9129), store at room temperature.
21. Dimethyl sulfoxide, hybridoma tested, 5×10 mL in flamed sealed ampules (Sigma; cat. no. D2650), store at room temperature and protect from light.
22. Mitomycin C, cell culture grade (Sigma; cat. no. M4287), store at 4°C (*see* **Note 2**).
23. One-well dish, 60-mm diameter, well area: 2.89 cm^2 (BD, Franklin Lakes, NJ; cat. no. 353652).
24. Four-well plate, well area: 1.39 cm^2 (BD; cat. no. 353653).
25. Four-well culture slides, well area: 1.7 cm^2 (BD; cat. no. 354114).
26. 75 cm^2 canted neck, vented tissue culture flask (BD; cat. no. 353136).
27. 175 cm^2 canted neck, vented tissue culture flask (BD; cat. no. 353112).
28. 15-mL conical centrifuge tubes, high-clarity polypropylene (BD; cat. no. 352196).
29. 50-mL conical centrifuge tubes, high-clarity polypropylene (BD; cat. no. 352070).
30. 1-mL individually wrapped serological pipet (BD; cat. no. 357522).
31. 5-mL individually wrapped serological pipet (BD; cat. no. 357543).
32. 10-mL individually wrapped serological pipet (BD; cat. no. 357551).
33. 25-mL individually wrapped serological pipet (BD; cat. no. 357525).
34. 500-mL Stericup-GP filter unit (Millipore, Billerica, MA; cat. no. SCGP U05 RE).
35. Sterivex-GP 2000 filling bell filter unit (Millipore; cat. no. SVGP B10 10).
36. 33-mm Millex-GP filter unit (Millipore; cat. no. SLGP 033 RS).
37. Nunc System 100 cryogenic vials with silicon gasket (Nalgene, Rochester, NY; cat. no. 5000-1012).
38. Glass capillaries, 1.0-mm OD (Clark Electromedical Industries, Kent, UK; cat. no. GC100T-15).
39. Sterile 30-G hypodermic needles (BD; cat. no. 511252/511256).
40. Media for hES cells (hES media). hES cells are maintained in cell culture media comprising 80% high-glucose DMEM (v/v), 20% defined fetal bovine serum (v/v), 2 mmol/L L-glutamine, 50 IU/mL penicillin and 50 μg/mL streptomycin, 1X

nonessential amino acids, 1X insulin-transferrin-selenium G supplement, and 0.1 mmol/l β-mercaptoethanol. To prepare 400 mL media, combine 320 mL DMEM, 80 mL defined FBS, 4 mL L-glutamine, 2 mL penicillin-streptomycin, 4 mL nonessential amino acids, 4 mL ITS-G supplement, and 720 µL β-mercaptoethanol. Filter-sterilize before use. Wrap bottle with aluminium foil to protect from light and store at 4°C for up to 3 wk. The addition of antibiotics to culture media is optional (*see* **Note 3**).

41. Media for human feeder expansion (human fibroblast maintenance medium). Human fibroblast feeders are maintained in culture media comprising 90% high-glucose DMEM (v/v), 10% qualified fetal bovine serum (v/v), 2 mmol/L L-glutamine, 50 IU/mL penicillin, and 50 µg/mL streptomycin. To prepare 400 mL media: add together 360 mL DMEM, 40 mL qualified FBS, 4 mL L-glutamine, and 2 mL penicillin-streptomycin. Filter-sterilize media before use and store at 4°C for up to 3 wk. The addition of antibiotics is optional (*see* **Note 3**).
42. Mitomycin C solution (*see* **Note 4**). Use a 26-G sterile needle to vent the 2 mg mitomycin C ampoule. With an 18-G needle affixed to a 1-mL syringe, add 1 mL sterile PBS (–) to the ampoule, swirl ampoule gently until all powder is dissolved. Remove mitomycin C solution with the syringe and aliquot into sterile microcentrifuge tubes, wrap each tube with aluminium foil to protect from light, and immediately store at 4°C for up to 2 wk (*see* **Note 5**).
43. Gelatin solutions (*see* **Note 6**). To prepare 1% (w/v) gelatin stock solution: weigh 0.4 g of gelatin powder into a 50-mL conical tube and add 30 mL sterile tissue culture grade water, warm at 37°C for 1–2 h with intermittent pipetting to aid dissolution. After the gelatin has dissolved, top up to the 40-mL mark with sterile water. The 1% stock gelatin solution can be either autoclaved or sterile filtered with a Sterivex-GP 2000 filter bell before use. Stock solutions should be stored at –20°C and can be kept for 6 mo. To prepare 200 mL of 0.1% gelatin working solution: thaw and dilute 20 mL of 1% gelatin stock solution in 180 mL tissue culture-grade water. Aliquots of working solution can be stored at 4°C for up to 2 wk.
44. Dispase solution: dissolve 0.1 g of Dispase in 10 mL of hES media to give a final working concentration of 10 mg/mL of Dispase solution. Filter, sterilize, then warm to 37°C before use.
45. The sources of the monoclonal antibodies for the detection of the cell surface markers are as follows: SSEA-3 (MC-631), SSEA-4 (MC-813-70), Development Studies Hybridoma Bank (Iowa City, IA); Tra-1-60 (MAB-4360) and Tra-1-81 (MAB-4381) Chemicon; Oct-4 (SC-5279) Santa Cruz.
46. The sources of the secondary antibodies for the detection of the primary antibodies are as follows: rabbit anti-mouse immunoglobulin secondary antibody conjugated to fluorescein isothiocyanate (Sigma F2883) and anti-goat immunoglobulin secondary antibody conjugated to fluorescein isothiocyanate (Sigma F7367).
47. Vector Red Alkaline Phosphatase Substrate Kit I, SK-5100 (Vector Labs, Inc., Burlingame, CA; cat. no. SK-5100).
48. KaryoMAX Colcemid solution, liquid (10 µg/mL) in PBS (Invitrogen; cat. no. 15212-012).

49. TRIzol reagent (Invitrogen; cat. no. 15596-026).
50. Ambion DNA-free reagent (Ambion; cat no. 1906).
51. SuperScript III first-strand synthesis system for reverse transcription polymerase chain reaction (RT-PCR) (Invitrogen; cat. no. 18680051).
52. TaqMan probes were purchased from Applied BioSystems (ABI) Assay on Demand and Assay by Design service.

3. Methods

3.1. General Note on hES Culture Methodology

The most critical factor that is essential for success in culturing hES cells is the complete attention to detail in all preparative steps and in cell handling. Good aseptic technique is essential and some prior tissue culture experience will be helpful. The extensive daily observation of cells is important and may be particularly mandatory in the first few weeks of setting up stock cultures. In addition, the use of commercial high-quality reagents and disposable plastic ware in all procedures is highly recommended.

The techniques in this chapter incorporate a labor intensive, specialized subculture routine requiring manual colony manipulation, slicing, and microdissection under stereo optics. The key objective of this subculture routine is the passage of cells in clusters of approx 400–500 cells and the elimination of differentiated cells from the subculture. An obvious drawback of this method is the difficulty in generating sufficient numbers of cells for clinical application and some large-scale experiments.

Nevertheless, we feel that this technique will be particularly useful for the expansion of critical very early passage hES cell stock and for propagating hES cell lines that are not amenable to bulk culture protocols. In addition, manual colony slicing results in the growth of larger colonies, thus making colony selection and harvesting for experiments easier. It is also possible when harvesting hES cells for experiments to slice into the peripheral regions of the hES colony to avoid harvesting feeder cells and degenerating cells at the core.

Enzymatic dispersion and the bulk-culture of hES lines on human feeders is also possible (*see* **Subheading 3.5.2.**). Interestingly, this often leads to an acceleration of growth and an overall decrease in colony differentiation. A recent study has reported karyotypic instabilities in mid to late passage hES cell lines subcultured in this manner on MEFs ***(9)***. It remains to be seen to what extent culture conditions can influence and affect the karyotypic stability of these lines.

The procedures for the handling and subculturing of hES cells described in this chapter are conservative and it is likely that with future refinements, a less labor-intensive approach enabling the routine and massive scale-up of cells in defined culture conditions as well as the low-density clonal growth of cells will be possible.

3.2. hES Colony Morphology and Grading

hES colonies on human feeder layers are spindle-shaped with straight edges and appear to be thinner than the rounder hES colonies, which typically form on MEF feeders (**Fig. 1A–F**) *(3)*. Mature 6- to 8-d-old hES colonies approx 1–2 mm in diameter are serially passaged by slicing and microdissection. Colonies passaged by manual slicing are considerably larger than bulk cultured, enzymatically passaged colonies and often appear to contain a central darker "blob" of cells (**Fig. 1A**,**C**,**E**). This darker central region of the colony represents piled up differentiating hES cells and should be avoided during serial passage. Good undifferentiated regions of the hES colony are generally found at the colony periphery adjacent to the colony-feeder layer boundary (**Fig. 1G**).

With a little practice, it is easy to differentiate between morphologically differentiated and undifferentiated regions of the hES colony. Undifferentiated individual hES cells have a high nucleus:cytoplasm ratio (**Fig. 1H**), which is visible under high magnification. Undifferentiated cells are tightly packed together with very little intercellular space between cells (**Fig. 1H**). Differentiating cells are morphologically diverse, but in culture conditions containing serum, an epithelioid cell type appears rapidly during hES cell differentiation (**Fig. 2A**,**B**).

Good undifferentiated hES colonies have sharp defined colony boundaries (**Fig. 1G**), whereas colonies on the onset of differentiation have diffused and "fuzzy" colony boundaries (**Fig. 2C**). For serial passage harvest, the outer perimeter of colonies that appear at least 50% undifferentiated. A small amount of spontaneous differentiation approx 5–10% is usually present even in well-maintained hES cultures.

3.3. Serial Passaging and Transfer of hES Colonies on Human Feeder Layers

3.3.1. Microdissection and Slicing of hES Colonies Under Stereo Optics

1. A sterile 30-G hypodermic needle affixed to a 1-mL syringe is used for slicing hES colonies. Work with ×2.0–3.0 magnification under a stereomicroscope. Because colony slicing takes time, work on a 37°C warmed surface in a laminar flow hood to keep cells warm (*see* **Note 8**).
2. Remove hES media from organ culture dish and wash cells twice with warm PBS (+) solution.
3. Perform colony microdissection/slicing with 30-G needle under microscope control with the left hand holding the dish. It helps to support the wrist of the right hand with the needle on the bench top.
4. Avoid harvesting morphologically differentiated colony sections and the central region of the colony that contains piled up cells. As a rule of thumb, harvest only colony edges with good undifferentiated hES cell outgrowths. *See* **Fig. 1** for examples of undifferentiated and **Fig. 2** for examples of differentiated hES colonies.

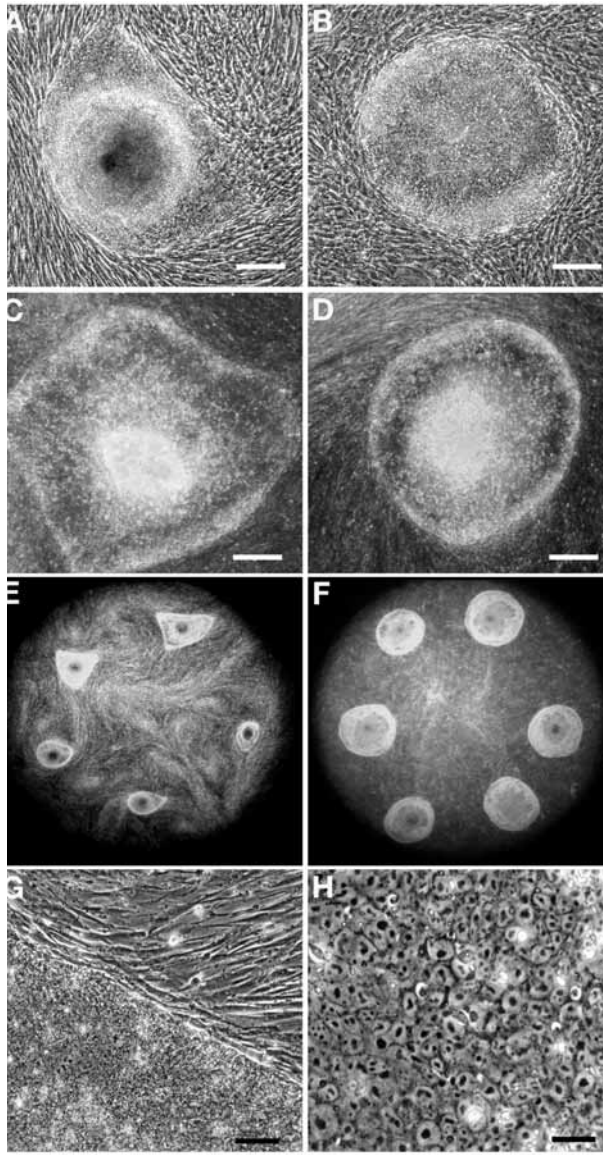

Fig. 1. Undifferentiated hES colonies on human and MEF feeder layers. **(A)** ×5 phase contrast image of a hES cell colony on a Detroit 551 human fetal feeder layer. **(B)** ×5 phase contrast image of a hES cell colony on a MEF feeder layer. **(C)** Bright field image of a hES cell colony on Detroit 551. **(D)** Bright field image of a hES cell colony on a MEF feeder layer. **(E)** Five 6-d-old hES cell colonies growing on Detroit 551. **(F)** Six 6-d-old hES cell colonies growing on MEFs. **(G)** ×20 phase contrast image of an undifferentiated well-defined hES cell colony edge on Detroit 551. **(H)** ×40 phase contrast image of individual undifferentiated hES cells in a colony on D551. Scale bars: **A–D**, 500 μm; **G**, 100 μm; **H**, 50 μm.

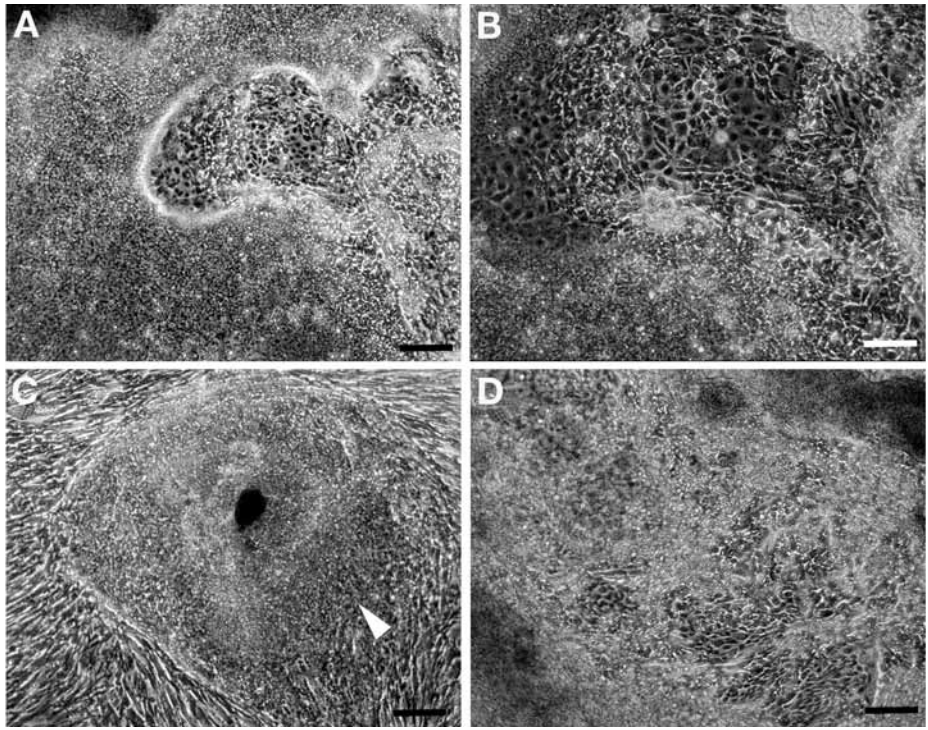

Fig. 2. Differentiating hES colonies on human fibroblast feeder layers. **(A)** ×10 phase contrast image of differentiated cells in a partially differentiated hES cell colony on Detroit 551. **(B)** ×20 phase contrast high magnification image of a common differentiated cell type. **(C)** ×5 phase contrast image of a whole partially differentiated hES cell colony on Detroit 551. Arrow indicates colony region that is differentiated; this area lacks a well-defined colony-feeder layer boundary. **(D)** ×10 phase contrast image of a hES cell colony showing extensive differentiation. More than two differentiated cell types are visible, and all cells have lost the high nucleus to cytoplasm ratio that is typical of hES cells. Scale bars: **A**,**D**, 250 μm; **B**, 100 μm; **C**, 500 μm.

5. Make cuts along the perimeter of undifferentiated colony edges well into the feeder layer then slice inwards into the colony.
6. After slicing is completed, aspirate off PBS (+) and add 1 mL of warm Dispase solution.
7. Incubate for approx 2–5 min at 37°C. Observe the dish carefully.
8. During the incubation period, you should be able to see the feeder layer disintegrating and retreating away from the hES colony fragments. After the feeder cells have detached from the colony, the sliced hES fragments also begin to round up and detach from the plastic; you will be able to see the hES fragments curl up slightly at the edges and begin to lift off from the dish. At this point, use a Gilson P20 to blow gently at the edge of a hES colony fragment. The fragment should lift off the dish very easily.

9. Dislodge all sliced fragments this way. As a rule of thumb, try minimizing the incubation period with Dispase.
10. Wash sliced colony fragments twice by picking up all colony fragments from the Dispase treated plate and transferring them to two new one-well dishes filled with warm PBS (+) sequentially.
11. Place four to five sliced fragments on a new human fibroblast feeder dish (**Fig. 1E**). After fragments have settled on the feeder layer, transfer dish very carefully to a humidified incubator at equilibrated at 37°C and 5% CO_2.
12. hES cells should be transferred to fresh feeder layers every 6–8 d; this is the duration of one serial passage.
13. Feeder layers perform optimally 24–48 h after mitomycin C treatment, so hES cells should be subcultured onto freshly inactivated feeders within this period.

3.3.2. Enzymatic Serial Bulk Culture of hES Cells on Human Feeder Layers

For bulk culture of hES colonies, cells are cultured in six-well dishes in a medium containing 20% knockout serum replacement, 80% knockout DMEM, 1X nonessential amino acids, 1 m*M* L-glutamine, 1X insulin-transferrin-selenium, and 0.1 m*M* β-mercaptoethanol supplemented with 6 ng/mL basic fibroblast factor.

3.3.2.1. Initiation of Bulk Cultured hES Cultures

1. Choose 10 colonies from dishes with microdissected colonies that are at least 85% undifferentiated.
2. Dissect colony peripheries; exclude harvesting the central colony core.
3. Detach sliced colony fragments from human feeder layer with Dispase treatment.
4. Wash hES colony fragments once in PBS (+).
5. Transfer fragment to a sterile 1.5-mL Eppendorf tube taking as little PBS (+) as possible.
6. Add 1 mL of warm Collagenase IV solution (1 mg/mL prepared in serum-free media-F12 DMEM).
7. Incubate Eppendorf tube at 37°C for 5 min.
8. After 5 min, dissociate colony slices into fragments of approx 30–60 cells with a 1-mL Gilson pipet. Triturate collagenase solution five times to aid fragment dissociation.
9. To dilute and inactivate collagenase enzymic activity, transfer cell suspension to 6 mL of bulk culture media.
10. Centrifuge at 170*g* (1000 rpm), remove supernatant, and resuspend pellet in 2 mL media.
11. Before plating hES suspension, aspirate media from feeders and seed entire 2 mL into one well of a six-well dish.

3.3.2.2. hES Bulk Culture Subculture Protocol

1. Bulk cultured cells grown in six-well dishes.
2. Human feeders are seeded at 180,000 mitomycin C treated cells per well of a six-well dish or 18,750 cells/cm^2.
3. Six-well dishes are coated with 0.5% gelatin before plating feeders.

4. Wash cells 1X with 2 mL PBS (+).
5. Add 1 mL of warm collagenase IV per well (1 mg/mL in serum-free F12 media, filtered sterile), return to incubator for 5–6 min.
6. Feeders may be detached from plastic and in cell suspension after incubation period.
7. Scrape with 5-mL serological pipet in a grid-like pattern.
8. Scrape monolayer with a cell scraper. Pay attention to edges of the well, colonies in this area are hard to remove.
9. Triturate large cell clumps with 1-mL Gilson set at 900 µL with a filtered tip and pipet up and down no more than five times.
10. Add 2 mL KO media per well to wash well and transfer cell clumps to a 15-mL Falcon. Add 1 mL more of KO media per well's worth of cells to effectively dilute collagenase activity.
11. Centrifuge at 170*g* (1000 rpm), remove supernatant, and resuspend pellet in appropriate volume of media. The optimal split ratio is usually 1:2 or 1:3.
12. Before plating hES-feeder suspension on fresh feeders, wash feeder layer 1X with KO and aspirate.
13. Resuspend pellet in appropriate volume (e.g., if pellet splits into four wells, resuspend in 8 mL KO).
14. Cells should be split every 5–7 d.

3.4. Characterization of hES Colonies

hES cells should be tested for markers of pluripotency, the ability to form teratomas, and karyotyped regularly.

3.4.1. Immunohistochemical Characterization of hES Cell Colonies With SSEA-3, SSEA-4, Tra-1-60, Tra-1-81, and Oct-4 Cell Surface Pluripotency Markers

1. For demonstration of stem cell surface markers, Tra-1-60, Tra-1-81, SSEA-3, and SSEA-4 hES colonies in four-well slide flasks are fixed with 4% paraformaldehyde in PBS for 15–20 min.
2. Wash three times with PBS (+) and let the slide dry.
3. For intracellular antigens, fixed cells can be permeabilized with 0.1% Triton X-100/PBS (+) for 15 min at room temperature. Wash three times with PBS (+).
4. Fixed cells are blocked with 5% normal goat serum in PBS (+) for 20 min on an orbital shaker or nutator.
5. Primary antibodies (MC-631, MC-813-70, MAB-4360, MAB-4381, and SC-5279) are diluted in PBS (+) according to manufacturer's instructions and incubated overnight or 2 h (minimum) on an orbital shaker or nutator.
6. Wash three times with PBS (+).
7. Antibody localization is performed using rabbit anti-mouse immunoglobulin secondary antibody conjugated to fluorescein isothiocyanate for Oct-4, SSEA-4 (**Fig. 3A–C**), Tra-1-60 (**Fig. 3F**,**H**), and Tra-1-81 (**Fig. 3E**,**G**).
8. SSEA-3 antibody localization is performed with anti-goat immunoglobulin secondary antibody conjugated to fluorescein isothiocyanate.

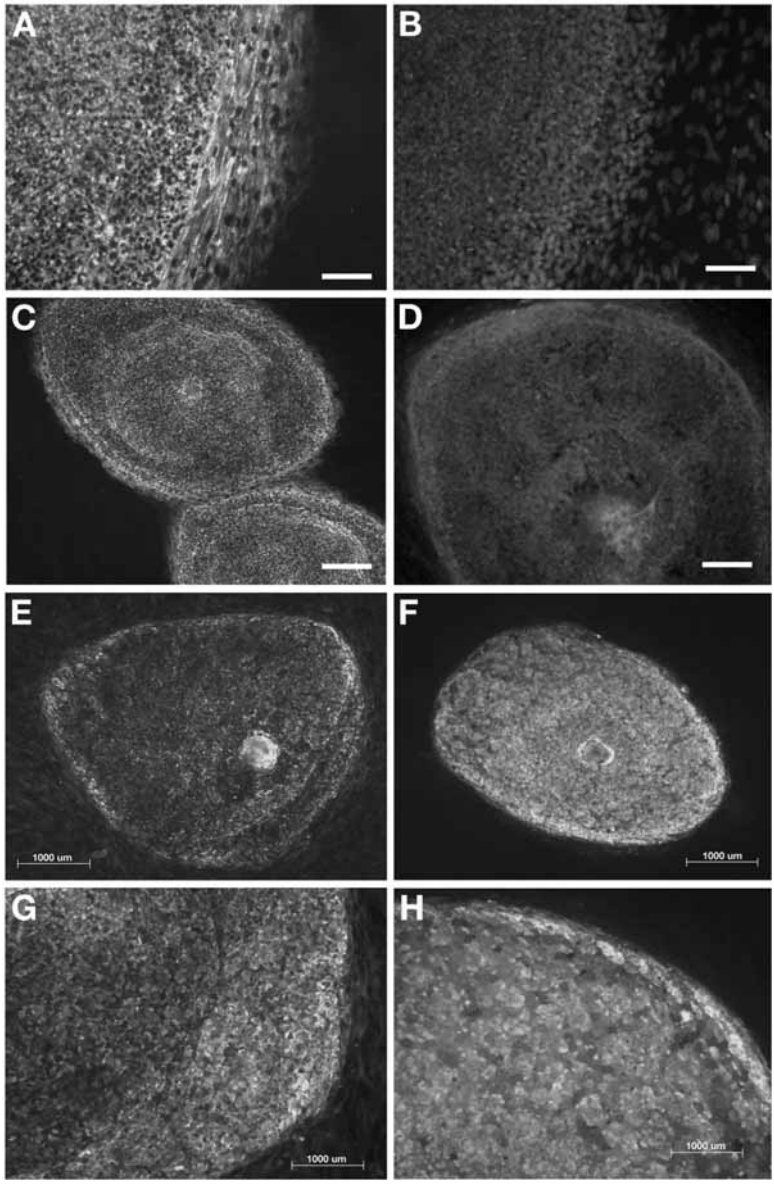

Fig. 3. Immunohistochemical cell surface marker detection of hES colonies on human fibroblast feeders. **(A)** High magnification picture of SSEA-4 cell surface antigen staining localized with FITC labelled secondary antibody. **(B)** DAPI counter stain of **A**. **(C)** Two SSEA-4-stained hES colonies. (**D**) Alkaline phosphatase activity visualized with rhodamine filters. **(E)** Tra-1-81 cell surface antigen staining localized with FITC-labeled secondary antibody. **(F)** Tra-1-60 cell surface antigen staining localized with FITC labeled secondary antibody. **(G)** High magnification picture of **E**. **(H)** High magnification picture of **F**. Scale bars: **A**,**B**, 250 μm; **C**,**E**,**F**, 1000 μm; **D**, 500 μm; **G**,**H**, 250 μm. (Please *see* the companion CD for the color version of this figure.)

9. Dilute secondary antibody according to manufacturer's specifications in PBS (+) and incubate for 30–60 min at room temperature in the dark on an orbital shaker or nutator.
10. Remove secondary antibody and wash once with PBS (+).
11. Cells can be counterstained with DAPI or Hoechst dye at this point if desired.
12. Wash three times with PBS (+).
13. Cells should be covered in PBS (+) for visualization or mounted on a slide with an anti-fade mountant if cells are stained on a cover slip.

3.4.2. Alkaline Phosphatase Activity Detection

Alkaline phosphatase activity is easily detected with the Vector Red Alkaline Phosphatase Substrate Kit I using the manufacturer's recommended protocol and viewed under bright field optics or with rhodamine excitation and emission filters (**Fig. 3D**).

3.4.3. Teratoma Formation in SCID Mice

Morphologically undifferentiated regions of postwarmed HES-2, HES-3, and HES-4 colonies grown on D551/CCL110 were sliced into clumps of approx 300–400 cells each. About 1×10^6 cells are injected with a sterile 27-G needle into the thigh muscle of anesthetized SCID mice (*see* **Note 9**). Tumors usually become palpable or visible by the 6th week and mice are sacrificed at the 8th or 12th week. Mice are sacrificed by cervical dislocation, tumors dissected and fixed overnight in 4% paraformaldehyde, then embedded in paraffin and examined histologically after hematoxylin and eosin staining (**Fig. 4**).

3.4.4. Karyotyping

Although general karyotyping protocols are straightforward, getting sufficient numbers of good metaphase spreads with hES cells is not easy. This is partly because hES cells have a low mitotic index. Thus metaphase spread preparation and karyotype interpretation is best done by a qualified cytogeneticist. To reduce ambiguity in interpreting karyotype data, it is best to karyotype a male hES cell line supported on a female human fibroblast feeder.

1. In general, hES colonies are incubated with 50 µg/mL Colcemid solution for 0.5–2.5 hr at 37°C and in a 5% CO_2 in air atmosphere.
2. Cells are removed from the feeder layer, trypsinized, washed with PBS, and pellets resuspended and incubated with 0.075 *M* KCl for 30 min at 37°C.
3. After treatment with hypotonic solution, cells are fixed with freshly prepared 3:1 methanol:glacial acetic acid three times and dropped onto precleaned chilled glass slides.
4. Chromosome spreads are digested with bacto-trypsin and banded with Leishman's or Giemsa stain and photographed digitally under ×100 magnification (*see* **Note 10**).

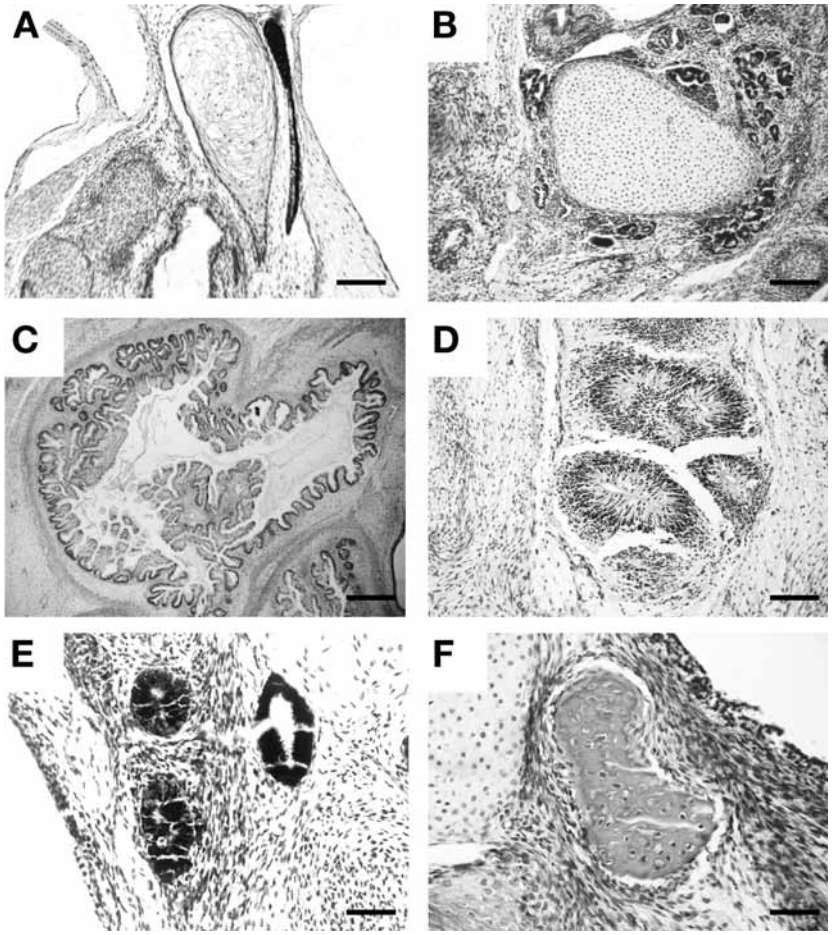

Fig. 4. Ectodermal, endodermal, and mesodermal components of teratomas derived from SCID mice injected with hES cells grown on human fibroblast feeders. **(A)** ×20 magnification of developing bone. **(B)** ×10 magnification of cartilage and glandular structures. **(C)** ×5 magnification showing developing gut and intestines. **(D)** ×10 magnification of hyaline cartilage. **(E)** ×10 magnification of a cluster of neural rosettes. **(F)** ×20 magnification of a cluster of pigmented epithelial cells. Scale bars: **A**,**F**, 100 µm; **B**,**D**, 250 µm; **C**, 1000 µm; **E**, 1000 µm.

5. At least 20 metaphase spreads and 5 banded karyotypes at 200-band resolution should be evaluated for chromosomal rearrangements (**Fig. 5**).

3.4.5. Semiquantitative RT-PCR Detection of Pluripotent Markers

hES cells can be tested with semiquantitative RT-PCR for several molecular markers of the pluripotent phenotype. This is in contrast to the limited array of antibodies against immunochemical cell surface markers.

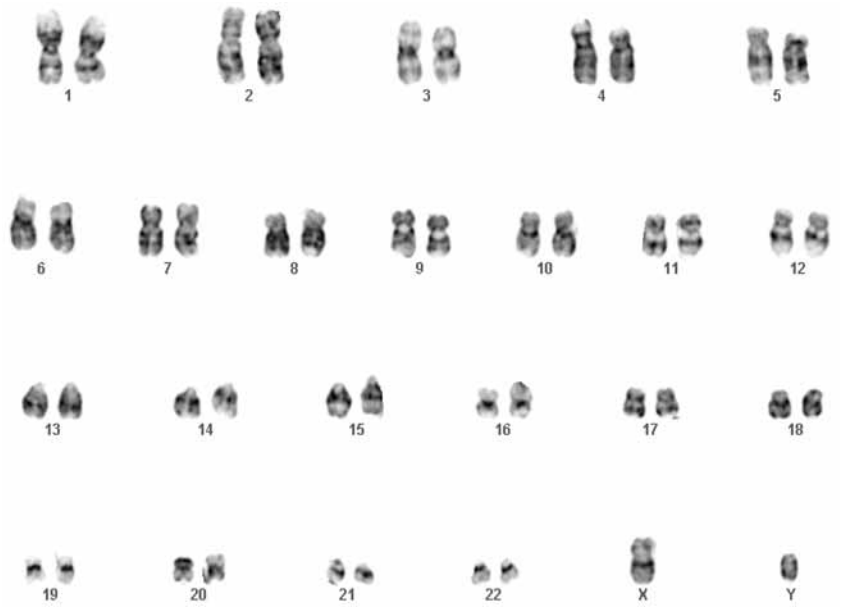

Fig. 5. Normal 46, XY karyotype of a mid-passage hES cell line grown on human fibroblast feeder layers.

1. Total RNA is routinely extracted from hES cells with TRIzol reagent following the manufacturer's protocol and treated with DNAse I using the DNA-free reagent from Ambion.
2. First-strand synthesis is performed using the SuperScript II first-strand synthesis system for RT-PCR. One microliter of first-strand reaction was used for each 50 µL PCR reaction together with 50 pmol of forward and reverse primers.
3. Initial denaturation was carried out at 94°C for 2 min, and followed by 35 cycles of PCR (94°C for 30 s, 55°C for 30 s, 72°C for 1 min) and a final extension cycle at 72°C for 5 min.
4. One-tenth of each PCR reaction was loaded on a 1.5% 1X TAE agarose gel and size fractionated (**Fig. 6**).

Primers used:

Gene	Forward primer	Reverse primer	Band size (bp)
ACTB	5′-tggcaccacaccttctacaat-gagc-3′	5′-gcacagcttctccttaatgt-cacgc-3′	400
NANOG	5′-ggcaaacaacccacttctgc-3′	5′-tgttccaggcctgattgttc-3′	493
DNMT3B	5′-ctcttaccttaccatcgacc-3′	5′-ctccagagcatggtacatgg-3′	433
REX1	5′-tctagtagtgctcacagtcc-3′	5′-tctttaggtattccaaggact-3′	418
SOX2	5′-ccgcatgtacaacatgatgg-3′	5′-cttcttcatgagcgtcttgg-3′	370
OCT4	5′-cgrgaagctggagaagga-gaagctg-3′	5′-caagggccgcagcttaca-catgttc-3′	247

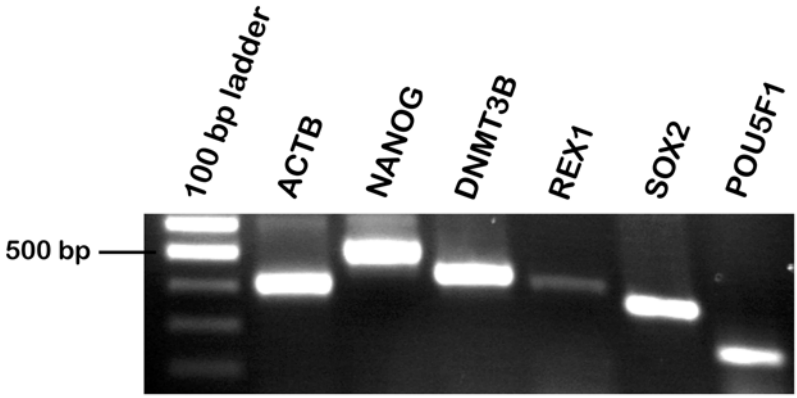

Fig. 6. Semiquantitative RT-PCR analysis for markers of pluripotency. Lane 1 = 100 bp DNA ladder, lane 2 = *ACTB*, lane 3 = *NANOG*, lane 4 = *DNMT3B*, lane 5 = *REX1*, lane 6 = *SOX2*, and lane 7 = *OCT4*. One-tenth of the PCR mix was loaded into each lane of a 1.2% 1 X TAE agarose gel and run at 100 V for 1 h.

3.4.6. Real-Time Quantitative RT-PCR Detection of Pluripotent Markers

Quantitative real-time PCR can be performed using TaqMan (*see* **Note 11**).

1. Total RNA is extracted from hES colonies using TRIzol reagent and first-strand synthesis performed using the SuperScript III first-strand synthesis system for RT-PCR.
2. Primer-probe sets for these target genes can be obtained from ABI's Assay on Demand service. Gene expression is usually normalized to endogenous housekeeping genes such as 18S rRNA, GAPDH, or ACTB levels (*see* **Note 12**).
3. Equal amounts of input cDNA (25 ng) must be used per reaction and all PCR reactions performed in triplicate (*see* **Note 13**).
4. Real-time PCR analysis is conducted using the ABI PRISM 7000 Sequence Detection System (ABI).
5. After an initial denaturation for 10 min at 95°C, real-time PCR was carried out using 40 cycles of PCR (95°C for 15 s, 60°C for 1 min).
6. Changes in gene expression levels are calculated using the $\Delta\Delta C_T$ method ***(10)***.

3.5. Classification of Human Feeder Layers

3.5.1. Supportive Human Feeder Layers

In a ranking study, we found that human fetal muscle fibroblasts derived from 14-wk human abortuses were the best supportive human feeders ***(4)***. The human fetal muscle feeder layer was capable of supporting both the prolonged undifferentiated growth of existing hES cell lines for more than 60 weekly serial passages and the derivation of a new hES line as well ***(4)***. A commercially available fetal skin fibroblast cell line Detroit 551 (CCL-110) from ATCC

(American Type Culture Collection, Manassas, VA) also performed very well as a supportive human feeder and was ranked second. Human fibroblast feeders derived from two adult skin biopsy samples from different patients also supported undifferentiated hES growth and were ranked third *(4)*. Other groups have reported that various isolates of human foreskin fibroblasts *(5,7)* and even human adult marrow cells *(6)* are capable of supporting undifferentiated hES cell growth.

3.5.2. Nonsupportive Human Feeder Layers

Not all human fibroblasts support the growth of hES cells. In particular, two widely used and well characterized human fetal lung fibroblast cell lines MRC-5 and WI-38 performed dismally *(4)*. The BJ foreskin fibroblast line also did not perform optimally *(4)*. Interestingly, data from our group and other published reports suggest that supportive human fibroblast feeders may be obtained from various isolates of fetal, neonatal, or adult skin.

It is important to note that not all human fibroblasts survive mitomycin C treatment well; this could be a chief consideration in evaluating the efficacy of a particular human fibroblast feeder for hES support. We have noticed that the plating efficiencies of some fibroblasts are dramatically decreased after mitomycin C treatment; this is often accompanied by extensive cell death. It is strongly recommended that different batches of human fibroblast feeder cells be tested for hES cell support before use.

3.5.3. Factors Secreted by Human Feeder Cells

An exogenous supply of the cytokine leukemia inhibitory factor (LIF) is sufficient to keep many mouse ES cell lines undifferentiated in culture *(11,12)*. hES cells, on the other hand, appear not to have a perceivable response to LIF *(1,2)*. Indeed, LIF appears not to elicit the same response of self-renewal in hES cells as it does in mouse ES cells *(1–3)*. Many hES cell lines have been derived and propagated without LIF supplementation as well *(3)*. Some recent reports also indicate that the LIF receptor and its cognate receptor, GP130 are expressed at very low levels in hES cells, suggesting that the LIF pathway may be inactive in hES cells *(13–15)*.

The identity of the growth factor or group of growth factors secreted by supportive human feeder layers or MEFs, which promotes the self-renewal and undifferentiated expansion of hES cells, is still unknown. Several transcription profiling studies have suggested that members of the Wnt, BMP4/transforming growth factor-β, and FGF family may act synergistically in providing the extracellular cues necessary for hES cells to divide and self-renew *(13–17)*. The role of direct hES cell and feeder contact though clearly of importance is also not well understood and warrants further study.

3.5.4. Thawing and Expansion of Human Fibroblast Feeders

1. Remove a vial from liquid nitrogen storage and let nitrogen vapor disperse for 30 s before performing a quick thaw in a 37°C water bath (*see* **Note 7**).
2. Carefully swirl the vial in a 37°C water bath, being careful not to immerse vial above the level of the cap.
3. When ice crystals have thawed, swab vial with 70% isopropanol, remove cells, and transfer to a 15-mL conical tube.
4. Add 10 mL human feeder media slowly dropwise to reduce osmotic shock.
5. Centrifuge cells at 250*g* for 5 min.
6. Remove supernatant and resuspend cell pellet in 5 mL media, transfer to a T75 flask with warm 15 mL media, and place in an incubator calibrated at 37°C, 5% CO_2.
7. Change media the next day and split cells in approx 2 or 3 d, when they become 90% confluent.

4. Notes

1. Make sure white precipitate visible when L-glutamine is thawed is completely dissolved before use. To do this warm L-glutamine for about 5–10 min in a 37°C water bath.
2. Mitomycin C solid is not light sensitive. Mitomycin C solution, on the other hand, is extremely light sensitive and aliquots should be wrapped in aluminum foil and stored at 4°C.
3. All stock solutions should be aliquoted and stored in the appropriate conditions as listed in **Subheading 2.**
4. Gloves and safety glasses should be worn when handling mitomycin C; it is carcinogenic and highly cytotoxic. Appropriate procedures for the disposal of this hazardous compound should be strictly followed.
5. If a black precipitate develops, discard the stock solution and prepare a fresh batch; the precipitate is toxic to cells. Mitomycin C solutions need not be sterile filtered.
6. Gelatin is used to coat one-well organ culture dishes before plating feeder cells to aid feeder cell and hES cell attachment.
7. Always wear protective face shield and cryogloves when handling liquid nitrogen. Nitrogen vapors escaping from cryovials at high pressure may cause occasional cryovial explosions. It is recommended that cryovial caps be loosened immediately to release pressure after removal from $N_{2(l)}$ storage then screwed tight again. Alternatively, wait 5 min for N_2 vapors to disperse before plunging into 37°C water bath.
8. All laminar flow cabinets in our laboratory have a heated base and a mounted stereomicroscope with a small footprint.
9. It is best to inject at least two SCID mice per cell line per experiment.
10. Karyotyping protocols need to be optimized for different hES cell lines at different passages.
11. We favor the use of robust TaqMan chemistry together with predesigned primer-probe sets from ABI, which are guaranteed to be optimally efficient *(18)*. This

saves time in designing PCR amplicons and optimizing reaction conditions. First-strand synthesis should be performed with random hexamers if using ABI designed TaqMan probes because most ABI TaqMan probes are designed to anneal in the 59 UTR of target genes.

12. Examples of markers of pluripotency include *OCT4, SOX2, NANOG,* and *REX1*; examples of early markers of differentiation include *AFP, ND1,* and *BMP4*.
13. Accurate pipetting is crucial to the success of the experiment and it is recommended that a dedicated set of pipets be reserved for quantitative PCR.

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4

Derivation and Maintenance of Human Embryonic Stem Cells

Michal Amit and Joseph Itskovitz-Eldor

Summary

Since their derivation in 1998, human embryonic stem cells (hESCs) have been the center of tremendous scientific efforts in improve the existing methodologies for their isolation and maintenance to exhaust the potential use of these unique cells in cell-based therapy and developmental research. To date, there are more than 50 reported well-characterized hESC lines worldwide. hESCs are traditionally isolated from the blastocysts on mouse embryonic fibroblasts. The most used method for isolating the inner cell mass from the human blastocyst is immunosurgery. This chapter focuses on the basic methods for the derivation and maintenance of hESC lines.

Key Words: Embryonic stem cells; teratomas; embryoid bodies; immunosurgery.

1. Introduction

Human embryonic stem cell (hESC) lines, as ESC lines from other species, are pluripotent cell lines derived from the inner cell mass (ICM) of the developing embryo. In addition to their ability to differentiate into any cell type of the adult body, hESCs are capable, in proper conditions, to proliferate indefinitely as undifferentiated cells, express high telomerase activity and typical markers sustain normal karyotype. Because of their unique features hESCs may have broad applications, both for developmental research including differentiation processes, lineage commitment, self-maintenance, and for clinical purposes, namely cell-based therapy.

ESC lines are traditionally isolated from blastocysts using immunosurgery. This process, developed by Solter and Knowles during the 1970s, initially served as a method for the research on early embryonic development *(1)*. The derivation

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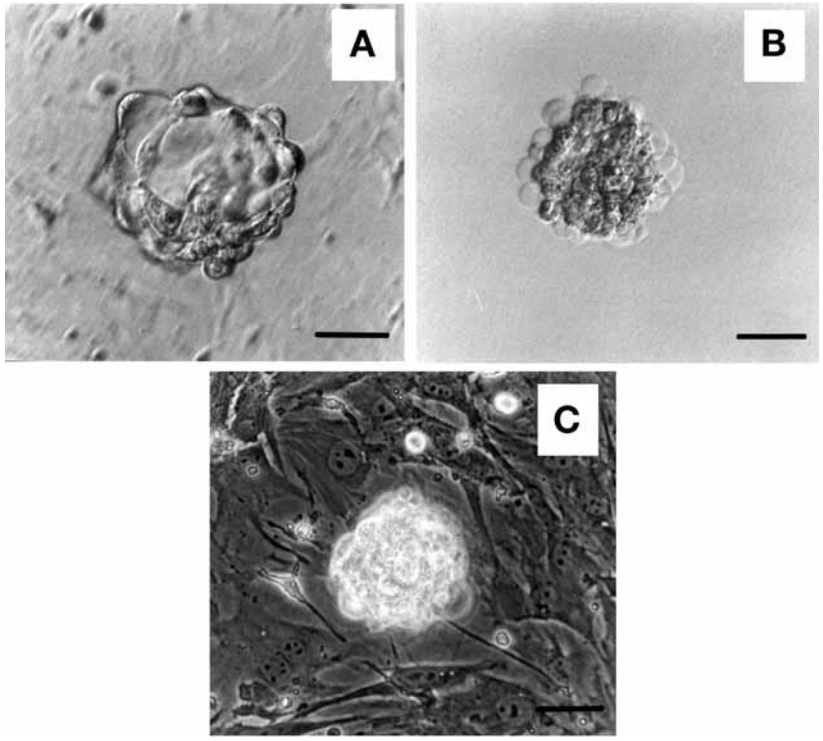

Fig. 1. Immunosurgery of a human blastocyst for the derivation of human embryonic stem cells lines. **(A)** Exposed human blastocyst after zona pellucida removal by Tyrode's solution during exposure to rabbit anti-human whole antiserum. **(B)** Embryo after exposure to guinea pig complement. **(C)** Intact inner cell mass immediately after immunosurgery on mitotically inactivated mouse embryonic fibroblast feeder layer. Bar = 50 µm. (Reproduced with permission from **ref.** *31*.)

and culture methods developed for embryonal carcinoma cells (ECCs) lay the base methodology for the first ESC line derivation from mouse *(2,3)*. The aim of immunosurgery is to selectively remove the trophoblast layer of the blastocyst, leaving an intact ICM, which will be further cultured on mitotically inactivated mouse embryonic fibroblasts (MEFs). The main steps of the immunosurgery process are illustrated in **Fig. 1**.

The goal of isolating the ICM can also be reached mechanically by removal of the trophoblast layer with 27-G needles or pulled pipets under a stereoscope. As with immunosurgery, the isolated ICM is then cultured with MEFs. ESC lines may also be derived without the removal of the trophoblast. The blastocyst is placed in whole within the MEFs, it attaches to the MEFs and continues to grow with the surrounding trophoblast. When the ICM reaches sufficient size, it

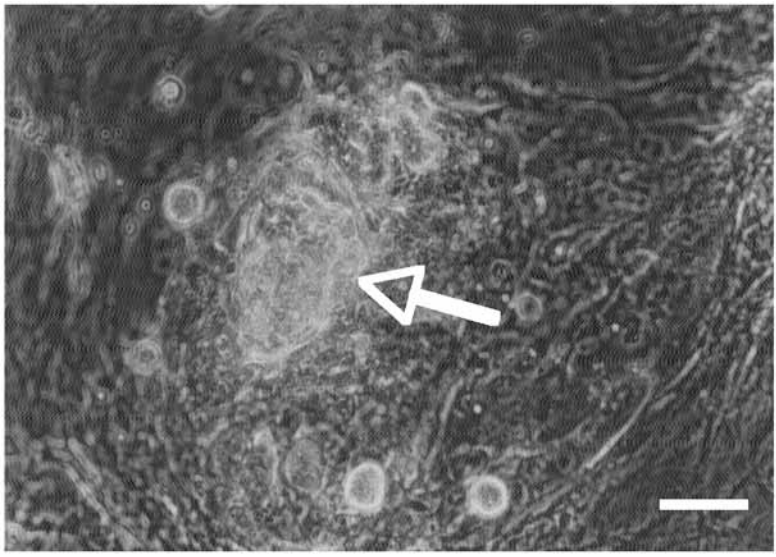

Fig. 2. Human embryo cultured as a whole embryo with mouse embryonic fibroblasts. The growing inner cell mass is marked with an arrow. Bar = 25 µm.

is selectively removed and propagated. An example of a proliferating ICM after plating an embryo in whole with MEFs is illustrated in **Fig. 2**.

The first ESC lines were derived from mouse blastocysts in 1981 *(2,3)*. Since these pioneering studies, ESC lines and ESC-like lines have been derived from other species including additional rodents, such as golden hamsters *(4)*, rats *(5)*, and rabbits *(6–8)*, domestic animals *(9–12)*, and three nonhuman primates *(13–15)*.

The first five hESC lines were reported by Thomson and colleagues in 1998 *(16)*. Despite their short life span, numerous studies have been published describing either additional culture methods developed for hESC such as feeder layer free and alternative feeder layers *(17–22)* or directed differentiation culture systems into specific cell types *(23–28)*. According to a list published by the National Institutes of Health in August 2001 there were 22 derived hESC lines *(29)*. Since then, other line derivations were reported independently of the National Institutes of Health registry. The increasing number of hESC lines indicate that the isolation of hESC lines is a reproducible procedure with reasonable success rates.

The main source of human embryos for the derivation of hESC lines is surplus in vitro fertilization embryos. Additional sources are discarded low-quality embryos, abnormally fertilized zygotes, and discarded genetically abnormal embryos that underwent preimplantation genetic diagnosis. The latter may

enable the isolation of hESC lines harboring specific genetic defects, leading to the development of culture systems designed to study the mechanisms of these genetic diseases and for drug screening.

2. Materials

2.1. MEFs

1. 0.1% Gelatin (type A of porcine) (Sigma-Aldrich, St. Louis, MO; cat. no. G-1890). All culture dishes should be covered with 0.1% gelatin at least 1 h before the plating of MEFs (*see* **Note 1**).
2. Freezing medium: 60% Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA; cat. no. 11995-065), 20% dimethyl sulfoxide (Sigma-Aldrich; cat. no. D-2650) and 20% fetal bovine serum (FBS) (HyCloce, Logan, UT; cat. no. SH30070.03) (*see* **Note 2**).
3. Splitting medium: trypsin/EDTA (0.05% trypsin, 0.2 g EDTA) (Invitrogen; cat. no. 17104019) (*see* **Note 3**).
4. Culture medium: 90% DMEM and 10% fetal calf serum (GibcoBRL Grand Island, NY; cat. no. 16170-078) (*see* **Note 4**). For MEF derivation, add penicillin-streptomycin (20 mL) (Sigma-Aldrich; cat. no. P-3539). For the preparation of 100 mL of MEF culture medium: add 87.5 mL DMEM into a filter unit, add 10 mL fetal calf serum and 2.5 mL penicillin-streptomycin and filter.
5. Mitomycin C: 8 µg/mL mitomycin C (Sigma-Aldrich; cat. no. M-4287) diluted in DMEM.
6. Watchmakers' forceps (Fine Science Tools Inc., Vancouver, Canada; cat. no. Dumont 5).
7. Iris scissors (Fine Science Tools Inc.; cat. no. 14060-09).
8. Nalgene freezing box (Nalge Nunc, Rochester, NY; cat. no. 5100-0001).

2.2. hESC Derivation

1. Tyrode's solution, acidic (Sigma-Aldrich; cat. no. T-1788) (*see* **Note 5**).
2. Antibodies: anti-human whole antiserum (Sigma-Aldrich; cat. no. H-8765) recommended dilution 1:30 in DMEM (*see* **Note 6**).
3. Complement proteins: guinea pig complement diluted 1:10 in DMEM or the solvent provided by the supplier (GibcoBRL; cat. no. 10723-013) (*see* **Notes 6** and **7**).

2.3. hESC Maintenance

1. Freezing medium (*see* **Subheading 2.1., item 2**).
2. Splitting medium: 1 mg/mL collagenase type IV (Invitrogen; cat. no. 17104019) in DMEM. For the preparation of 100 mL of splitting medium: add 100 mL DMEM into a filter unit, add 100 mg collagenase type IV, let set for 2 min for the powder to resolve and filter (*see* **Note 8**).
3. hESC-medium: 80% DMEM or knockout DMEM (Invitrogen; cat. no. 10829018), 20% FBS (HyClone; cat. no. SH30070.03), 1% nonessential amino acids (Invitrogen; cat. no. 11140-035), 1 m*M* L-glutamine (Invitrogen; cat. no. 25030-024),

0.1 m*M* β-mercaptoethanol (Invitrogen; cat. no. 31350-010). For the preparation of 500 mL culture medium: add 100 mL FBS, 5 mL nonessential amino acids, 2.5 mL L-glutamine, 1 mL β-mercaptoethanol, and 391.5 mL knockout-DMEM into a filter unit and filter (*see* **Note 8**).

4. hESC serum-free medium: 85% knockout-DMEM, 15% knockout serum replacement SR (Invitrogen; cat. no. 10828028), 1% nonessential amino acids, 1 m*M* L-glutamine, 0.1 m*M* β-mercaptoethanol, and 4 ng/mL bFGF (Invitrogen; cat. no. 13256-029). For the preparation of 500 mL culture medium: add 75 mL serum replacement SR, 5 mL nonessential amino acids, 2.5 mL L-glutamine, 1 mL β-mercaptoethanol, 2000 ng bFGF, and 416.5 mL knockout-DMEM into a filter unit and filter (*see* **Note 3**).

3. Methods

3.1. MEF Culture Methods

3.1.1. Derivation of MEFs From Pregnant Mice

1. Sacrifice one pregnant ICR mouse (*see* **Note 9**) on the 13th day of conception by brief exposure to CO_2 (*see* **Note 10**).
2. Wash abdomen with 70% ethanol and dissect the abdominal cavity to expose the uterine horns.
3. Remove the uterine horns into a 10-cm Petri dish and wash three times with phosphate-buffered saline (PBS).
4. Using two pairs of watchmakers' forceps, open each uterine wall and release all embryos carefully without touching the fur.
5. Wash retrieved embryos three times with PBS.
6. Use the same tools to dissect each embryo from the placenta and membranes and discard soft tissues as much as possible.
7. Transfer clean embryos into a new Petri dish and mince thoroughly using sharp Iris scissors.
8. Add 6 mL trypsin/EDTA and incubate for at least 20 min.
9. Neutralize trypsin using at least 6 mL MEF culture medium and transfer the cells into conical tubes. Use MEF culture medium to wash the plate.
10. Centrifuge 5 min at 300*g* (*see* **Note 11**).
11. Divide evenly into T-75 culture flasks (*see* **Note 12**).
12. Add 20 mL MEF culture medium to each flask (*see* **Subheading 2.1., item 4,** with the addition of antibiotics). Grow the MEFs up to 3 d or until confluent, changing the medium at least once during culture (do not vacuum the lumps).
13. Freeze the resulting MEFs (*see* **Subheading 3.1.3.**).

3.1.2. Splitting MEFs

1. Add 2 mL trypsin/EDTA and cover the entire culture-flask surface.
2. Incubate for 6 min.
3. Tap the side of the flask to loosen the cells and add 4 mL culture medium to neutralize the trypsin.

4. Remove cell suspension into a conical tube and centrifuge for 5 min at 300*g* (*see* **Note 11**).
5. Remove suspension, resuspend in 2 mL culture medium, and pipet to fracture the pellet.
6. Distribute cell suspension to desired number of culture flasks.
7. Add MEF culture medium to a final volume of 10 mL.

3.1.3. Freezing MEFs

1. Remove all lumps possible.
2. Add 2 mL trypsin/EDTA and cover the entire culture-flask surface.
3. Incubate for 6 min.
4. Tap side of the flask to loosen the cells. Add 4 mL culture medium to neutralize the trypsin.
5. Remove cell suspension into conical tube. Let remaining lumps sink and remove cell suspension into a clean conical tube.
6. Centrifuge for 5 min at 300*g* (*see* **Note 11**).
7. Remove suspension, resuspend in 2 mL culture medium, and pipet to fracture the pellet.
8. Drop by drop, add an equivalent volume of freezing medium and mix gently (*see* **Note 13**).
9. Place 1 mL into 2-mL cryogenic vials (*see* **Notes 14** and **15**).
10. Freeze vials overnight at –80°C in Nalgene freezing box (*see* **Note 16**).
11. Transfer vials into a liquid nitrogen container.

3.1.4. Thawing MEFs

1. Remove vial from liquid nitrogen and thaw briefly in a 37°C water bath.
2. When a small pellet of frozen cell remains, clean the vial using 70% ethanol.
3. Pipet the contents of the vial once, and transfer the cells into a conical tube.
4. Drop by drop, add 2 mL of MEF culture medium (*see* **Note 17**).
5. Centrifuge for 5 min at 300*g*.
6. Resuspend the pellet in culture medium.
7. Remove cell suspension into culture flasks and add 10 mL of culture medium.

3.1.5. Preparation of MEF-Covered Plates

1. Add 8 µg/mL mitomycin C into culture flask and incubate for 2 h (*see* **Note 18**).
2. Wash four times with PBS.
3. Add 2 mL trypsin/EDTA and cover the entire culture-flask surface.
4. Incubate for 6 min.
5. Tap the side of the flask to loosen cells and add 4 mL MEF culture medium to neutralize the trypsin.
6. Remove cell suspension into a conical tube.
7. Centrifuge for 5 min at 300*g*.
8. Remove suspension, resuspend in 10 mL culture medium, and pipet to fracture the pellet.

9. Count cells and resuspend in desired medium volume.
10. Add cell suspension into culture dishes (*see* **Note 19**).
11. Let MEFs set for at least 2 h before plating hESCs.

3.2. Isolation of hESCs

3.2.1. Immunosurgical Isolation

1. Culture donated surplus embryos according to standard in vitro fertilization protocols to the blastocyst stage.
2. Incubate the embryo for approx 30–60 s in previously heated Tyrode's solution under the scope (to 37°C). When the zona pellucida starts to dissolve, remove the embryo and wash three times in fresh ESC medium (an example of exposed blastocyst is illustrated in **Fig. 1A**).
3. Incubate the embryo in anti-human whole serum antibodies for 30 min. Immediately afterwards, wash three times in fresh ESC medium.
4. Incubate the embryo for up to 20 min in guinea pig complement (*see* **Note 20**). The intact ICM surrounded with lysed trophoblasts is demonstrated in **Fig. 1B**.
5. Wash the intact ICM three times in fresh ESC medium using a pulled pasture pipet to remove the lysed trophoblasts.
6. Plate the intact ICM on a fresh MEF-covered culture dish (*see* **Subheading 3.1.5**, illustrated in **Fig. 1C**) in ESC medium (*see* **Subheading 2.3., items 3** or **4**).

3.2.2. Mechanical Isolation

1. Expose the embryo from zona pellucida as described in **Subheading 3.2.1., step 2**.
2. If the ICM is clearly noticeable, remove as much trophoblast as possible using either a 27-G syringe needle or a blank pulled pasture pipet under a stereoscope. If the ICM is unrecognizable, plate the embryo in whole.
3. Plate the embryo on a fresh MEF-covered culture dish (*see* **Subheading 3.1.5.**) in ESC medium (*see* **Subheading 2.3., items 3** or **4**).

3.2.3. Culture of Recently Derived hESCs

1. Change half of the medium in the well on a daily basis.
2. Passage hESCs mechanically every 5–10 d directly onto fresh MEF-covered plates.
3. After five to seven passages, the newly isolated line can be treated as described in **Subheading 3.3.**

3.3. hESC Culture

3.3.1. Splitting hESCs

1. Remove medium and add hESC-splitting medium to cover the well. Incubate for 1 h (most colonies will float).
2. Add 1 mL culture medium (*see* **Subheading 2.3., items 3** or **4**) and gently collect cells.

3. Collect cell suspension and place into a conical tube.
4. Centrifuge for 3 min at 90*g* at a recommended temperature of 4°C.
5. Resuspend cells in media and plate directly on fresh MEF-covered plates.

3.3.2. Freezing hESCs

1. Add splitting medium and incubate for 1 h.
2. Add 1 mL culture medium, gently scrape the cells, and transfer into a conical tube.
3. Do not fracture the cells into small clumps.
4. Centrifuge 3 min at 90*g* at a recommended temperature of 4°C.
5. Resuspend cells in culture medium (*see* **Subheading 2.3., items 3** or **4**).
6. Drop by drop, add an equivalent volume of freezing medium and mix gently (*see* **Note 13**).
7. Transfer 0.5 mL into 1-mL cryogenic vials.
8. Freeze overnight at –80°C in a Nalgene freezing box (*see* **Note 16**).
9. Transfer to liquid nitrogen on the following day (*see* **Notes 21** and **22**).

3.3.3. Thawing hESCs

1. Remove a vial from the liquid nitrogen.
2. Gently swirl the vial in a 37°C water bath.
3. When a small pellet of frozen cell remains, wash the vial in 70% ethanol.
4. Pipet the content of the vial up and down once to mix.
5. Place the content of the vial into a conical tube and add, drop by drop, 2 mL culture medium (*see* **Subheading 2.3., items 3** or **4,** and **Note 17**).
6. Centrifuge for 3 min at 90*g* at 4°C.
7. Remove the supernatant and resuspend the cells in 2 mL medium.
8. Place the cell suspension on one well of a six-well plate, or on a four-well plate, covered with MEFs.

3.3.4. Routine Culture of hESCs

1. Change the medium on a daily basis (*see* **Note 23**).
2. Passage hESCs (*see* **Subheading 3.3.1.**) every 4–6 d directly onto fresh MEF-covered plates (*see* **Subheading 3.1.5.**).
3. Scrape differentiating colonies every five to seven passages.

4. Notes

1. Gelatin-coated plates can be prepared in advance, stored in a clean place at room temperature or in a 37°C incubator.
2. The freezing medium should be kept at 4–8°C for no more than 5 d. It is recommended to use high-quality serum, such as FBS from Hyclone.
3. All types of trypsin-EDTA will do.
4. FBS or heat inactivated newborn calf serum is also suitable. It should be kept at 4–8°C and should be used within 2 wk of preparation.
5. 0.5% pronase may be used instead of the Tyrode's solution.

6. There may be differences between batches; therefore, it is recommended to prepare a 10X stock solution and dilute to the appropriate working concentration before use.
7. The guinea pig complement may become toxic if stored longer than 8 mo.
8. All culture media but those mentioned should be kept at 4–8°C for no more than 2 wk.
9. Additional suitable mice strains are CD1 and C56J6. The time frame of 12–14 d of conception is acceptable.
10. Any other sacrifice method approved by your animal care committee including the use of Avertin can be used.
11. The centrifugation step is optional; the serum-containing medium can be used to neutralize the Trypsin.
12. We recommend a ratio of three embryos per flask.
13. Adding the freezing medium drop by drop is crucial for cell recovery.
14. It is recommended to freeze four vials from one confluent flask.
15. The cells can be counted and frozen at a recommended volume of 10^6 cells per vial.
16. The use of Nalgene freezing box increases the survivability rates.
17. Adding the medium drop by drop is crucial for cell recovery.
18. The MEFs can be mitotically inactivated by exposure to 3500 Ci gamma irradiation; the duration of the exposure should be calculated according to the source in use.
19. We recommend on 4×10^5 cells per well in six-well plates. The MEF number can also be calculated as 4×10^4 cells/cm^2. MEFs numbers can vary between 1.5×10^4 and 5×10^4 cells/cm^2.
20. It is recommended to monitor the procedure; if trophoblasts are lysed before the end of the incubation time, stop the incubation.
21. It is not recommended to leave the vials at –80°C for less than 24 h or more than a few days.
22. The recommended freezing ratio is 10 cm^2 per vial (one well in six-well plates).
23. For the cells to maintain their features, hESCs can be cultured and derived successfully using medium supplemented with either serum or serum replacement (for additional information, *see* **ref.** ***30***).

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5

Isolation and Characterization of Human Embryonic Stem Cells

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Summary

The derivation of human embryonic stem (hES) cells is a challenging procedure. The isolation and maintenance of hES is visually and manually complicated, involving mechanical or enzymatic passaging using either collagenase or trypsin. This chapter describes detailed protocols that have been used for the derivation, maintenance, and characterization of hES cells in vitro along with protocols to test their differentiation potential in vivo. When used as a guideline, these protocols will assist researchers in setting up a hES cell laboratory.

Key Words: Human embryonic stem cells; embryo; pluripotency; karyotype.

1. Introduction

Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass (ICM) of the preimplantation stage embryo, with the potential to develop to all three embryonic germ layers: mesoderm, ectoderm, and endoderm. Human ES (hES) cells were first derived by Thomson and co-workers in 1998 *(1)* and offer great potential for the treatment of diseases such as diabetes, Parkinson's, and Alzheimer's. hES cells can be maintained in vitro as undifferentiated cell lines when propagated on feeder layers. Under these conditions, they retain normal euploid karyotype and the capacity to be differentiated into multiple cell types in vitro and in teratomas in vivo. Their differentiation potential cannot, however, be tested in vivo in chimeras for ethical reasons. Derivation of hES cells is similar to that for primate ES cells *(2,3)*. Commonly, mouse embryonic fibroblasts have been used as a feeder layer to support the isolation and proliferation of hES cells in an undifferentiated state; also, there have been reports on isolation of hES cells using human cells as feeder source *(4)*.

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At BresaGen, Inc., in 2001, we isolated four hES cell lines (BG01, BG02, BG03, and BG04) *(5)*. These cell lines have been extensively characterized in vitro and in vivo in teratomas (unpublished data). hES cell lines cultured continuously by enzymatic treatment gain karyotypic abnormalities in vitro *(6,7)*. hES cell lines BG01 and BG02 were propagated by manual dissection of the hES cell colonies and have normal karyotypes at passages 105 and more *(8)*. These results confirm previous observations from Buzzard and colleagues *(7)*, indicating that the difficult and laborious manual passaging of hES cells will retain a stable karyotype even after 100 passages. Faster and easier means of passaging hES cells are available, but we found that they can promote chromosomal aneuploidy, especially trisomy 12 or 17 *(8)*. Abnormal karyotype was also associated with significant changes in expression of candidate genes implicated in maintaining pluripotency. We also found that, in addition to increased expression of pluripotent genes, abnormal cell lines also exhibit increased expression of early differentiation genes, implying that bulk passaging compromises the normal gene expression profile of undifferentiated hES cells. Our data suggest that the bulk passage methods that we describe in this chapter can perpetuate aneuploid cell populations after extended passage in culture, but may be used for shorter periods (at least 15 passages) without compromising the karyotypes. Our data, combined with other reports of karyotypic instability *(6,7)* in hES cells, suggest that bulk passaging can lead not only to abnormal karyotypes, but also to quantitative differences in gene expression. However, it may be possible to maintain a normal karyotype in hES cells under long-term manual propagation conditions followed by limited bulk passaging in experiments requiring greater quantities of hES cells than manual passage methods alone can provide. Additionally, our data underscore the need for simultaneous karyotypic and quantitative gene expression analyses for preserving the genetic integrity of hES cells in continuous culture *(8)*.

Techniques for derivation and characterization of hES cells have been described by many authors *(10,11)*. This chapter describes the derivation and maintenance and characterization of hES cells in detailed protocols. We also respond to questions about setting up the hES laboratory, preparation, and quality controls of reagents, passaging techniques, and help with defining morphologies of undifferentiated hES cells.

2. Materials

2.1. Tissue Culture

1. Acetic acid, glacial (Fisher Scientific, Pittsburgh, PA; cat. no. A-38-212).
2. 0.5% agarose in Dulbecco's modified Eagle's medium (DMEM)/F12: weigh 0.5 g agarose into a sterile glass bottle. Add 100 mL DMEM/F12 and replace the cap on the bottle, but do not tighten. Microwave and bring to boil. Confirm that all the agarose is in solution by swirling. Repeat microwaving until all the agarose

is in solution, then allow to boil for a further 15 s to ensure sterility. Allow to cool to room temperature before use. The solution can be stored at 4°C for 4 wk. Melt in the microwave and cool before each use.

3. Basic fibroblast growth factor (bFGF or FGF2) (1 mg; Sigma, St. Louis, MO; cat. no. F-0291).
4. Cell dissociation buffer (CDB), phosphate buffered saline (PBS)-based (100 mL; Gibco, Carlsbad, CA; cat. no. 13151-014).
5. Collagenase, type IV (Gibco; cat. no. 17104-019). To prepare a 1 mg/mL solution for manually passaged cells: dissolve 10 mg in 10 mL 15/5 hES medium (*see* **Subheading 2.1.1., item 2**) at 37°C. For trypsin passaged cells (and embryoid bodies [EBs]), prepare a 1 mg/mL solution in 10 mL of 20% KSR hES medium (*see* **Subheading 2.1.1., item 3**) at 37°C. Filter-sterilize and use within 1 wk; store at 4°C.
6. Distilled water (1 L; Gibco; cat. no. 15230-162).
7. Dissecting scissors, curved surgical (Fisher; cat. no. 08-935).
8. DMEM high glucose (500 mL; HyClone, Logan, UT; cat. no. SH30081.02).
9. DMEM/F12 (500 mL; Gibco; cat. no. 11320-033).
10. DMSO (100 mL; Sigma; cat. no. D-2650).
11. Dumont no. 55 forceps (Fisher; cat. no. NC9655963).
12. Dumont no. 3 forceps (Fisher; cat. no. NC9839169).
13. Ethidium bromide (EtBr), 10 µg/mL stock solution (Sigma; cat. no. E-1510). For a working concentration of 1 µg/mL: add 10 mL EtBr stock solution to 90 mL sterile distilled water. Filter with a 2.0-µm filter and store in dark bottles at −20°C. A 5-mL aliquot can be stored at 4°C for 1 mo.
14. Fetal bovine serum (FBS) characterized and screened for ES cell growth (HyClone; cat. no. SH30071.03) (*see* **Note 1**).
15. Fixative: 3:1 ratio of methanol: acetic acid. Combine 30 mL methanol with 10 mL acetic acid. Prepare fresh before use.
16. Guinea pig complement (Sigma; cat. no. S-1639).
17. Hanks balanced salt solution 1X without calcium and magnesium (Gibco; cat. no. 14170-112).
18. Human leukemia inhibitory factor (Chemicon, Temecula, CA; cat. no. LIF1010).
19. Hypotonic solution (0.075 *M* KCL). To prepare: dissolve 5.6 g KCL in a total volume of 1 L with distilled water.
20. Jewelers forceps, curved (Fisher Scientific; cat. no. 08-953F).
21. KaryoMax Colcemid solution (Gibco; cat. no. 15210-040). Working concentration: 10 µg/mL.
22. Knockout serum replacement (KSR) (Gibco; cat. no. 10828-028).
23. L-glutamine (Gibco; cat. no. 25030-081).
24. Methyl alcohol, anhydrous (Fisher Scientific; cat. no. A412-4, 4L).
25. Mitomycin C (2 mg; Sigma; cat. no. M4287). To make a stock solution 10 µg/mL: dissolve the content of a premeasured 2-mg vial in 200 mL complete MEF medium (*see* **Subheading 2.1.1., item 1**) (*see* **Notes 2** and **3**).
26. Monoclonal mouse anti-human placental alkaline phosphatase antibody (Dako, Glostrup, Denmark; cat. no. M7191).

27. Nalgene cryo 1°C freezing container (VWR, West Chester, PA; cat. no. 5100-0001).
28. Nonessential amino acids (Gibco; cat. no. 11140-050).
29. 1X phosphate-buffered saline (PBS–/–) without Ca^{2+} or Mg^{2+} (HyClone; cat. no. SH30028.02).
30. 1X phosphate-buffered saline (PBS+/+) with Ca^{2+} and Mg^{2+} (HyClone, cat. no. SH30264.02).
31. Penicillin/streptomycin (Gibco; cat. no. 15070-063).
32. Pronase (Sigma; cat. no. P-8811).
33. Trypan blue solution, 0.4% (Sigma; cat. no. T8154).
34. Trypsin 0.05%/EDTA (Gibco; cat. no. 25300-054).
35. Trypsin 0.25%/EDTA (Gibco; cat. no. 25200-056).
36. β-mercaptoethanol (Sigma; cat. no. M7522).

2.1.1. Media

1. Medium for mouse embryonic fibroblast cells (MEF media): high-glucose DMEM supplemented with 10% FBS, 1% penicillin-streptomycin and 2 m*M* L-glutamine. To prepare 100 mL of MEF media: combine 10 mL FBS, 1 mL L-glutamine, 1 mL penicillin-streptomycin, and 88 mL high-glucose DMEM.
2. 15/5 hES media: hES cells are maintained in DMEM/F12 supplemented with 15% FBS, 5% KSR, 1% NEAA, 1% penicillin-streptomycin, 2 m*M* L-glutamine, 1000 IU recombinant human leukemia inhibitory factor, 0.1 m*M* β-mercaptoethanol, and 4 ng/mL bFGF. To prepare 100 mL hES media: combine 10 mL FBS, 5 mL KSR, 1 mL penicillin-streptomycin, 1 mL L-glutamine, 100 µL hLIF, 1 mL NEAA, 16 µL bFGF, 10 µL β-mercaptoethanol, and 77 mL DMEM/F12.
3. 20% KSR medium for hES cells. 77% DMEM/F12, 20% KSR, 1% nonessential amino acids, 1% penicillin/streptomycin, 2 m*M* L-glutamine, 0.1 m*M* β-mercaptoethanol, 4 ng/mL basic fibroblast growth factor (from 25 µg/mL stock), and 10 m g/mL human leukemia inhibitory factor (from 10 µg/mL stock). For 50 mL: mix 38.5 mL DMEM/F12, 7.5 mL FBS, 2.5 mL KSR, 0.5 mL penicillin/streptomycin, 0.5 mL L-glutamine, 0.5 mL nonessential amino acids, 8 µL bFGF, 5 µL β-mercaptoethanol, and 50 µL leukemia inhibitory factor. Filter sterilize through a 0.22-µm filter to ensure sterility. Medium should be stored at 4°C and used within 1 wk.
4. Media for freezing human ES cells. Solution A: combine 50% medium (15% FBS, 5% KSR) and 50% FBS. Solution B: mix 80% medium (15% FBS, 5% KSR) and 20% DMSO. Filter-sterilize each solution. Make fresh each use.

2.1.2. General Comments and Required Equipment for Tissue Culture

As a general rule, all tissue culture protocols must be performed using sterile techniques with great attention given to using sterile disposable plastic ware and all media and solutions must be warmed to 37°C before use. The tissue culture facility for hES cell culture requires the following:

1. 37°C water bath.
2. Hemacytometer.
3. Pipets designated for tissue culture only (1, 2, 5, 10, 25, and 50 mL).

4. Humidified incubator at 37°C and 5% CO_2.
5. Inverted microscope with a range of phase contrast objectives (3 4, 3 10, 3 20, and 3 40) equipped with digital camera and fluorescent attachments.
6. Biosafety and laminar flow cabinet.
7. Liquid nitrogen storage tank.
8. Pipet aid.
9. Paster pipets.
10. Pipetmen (2, 10, 20, 100, 200, and 1000 µL).
11. Bunsen burner.
12. Dissection scope with high resolution (Nikon, Tokyo, Japan; model no. SMZ-1500 with the magnification range ×7.5 – ×12.5).
13. Aspirator assembly tube.
14. Tabletop centrifuge.

2.2. Immunocytochemistry

1. PBS +/+ (*see* **Subheading 2.1., item 30**).
2. 1 *M* sodium hydroxide: weight 40 g of sodium hydroxide (NaOH) and dissolve in 1 L distilled water.
3. Paraformaldehyde (PFA) solution. To prepare 4% PFA/4% sucrose in PBS: combine 4 g PFA and 4 g sucrose in a glass beaker. Add 75 mL distilled water and place on a heated stirrer to dissolve at a temperature of 56°C. Add two drops of 1 *M* sodium hydroxide; after all powder has gone into solution, add 10 mL 10X PBS (+/+). Using a pH meter (or paper), check that pH is between 7.2 and 7.4. Adjust accordingly. Make up volume to 100 mL with distilled water. Store at 4°C and use within 1 wk (*see* **Note 4**).
4. Blocking solution (for cell surface markers). For 10 mL: mix 9.7 mL PBS (+/+) and 0.3 mL goat serum in a 15-mL tube. Store at 4°C and use within 48 h.
5. Mounting media, ready to use Vectashield (Vector Labs, Burlingame, CA; cat. no. H-1000) or Aqua Polymount (Polysciences, Warrington, PA; cat. no. 18606).
6. High salt buffer (for intracellular markers). For 1 L: mix 14.61 g sodium chloride (250 m*M*), 50 mL 1 *M* Tris-HC1 pH 7.4 (50 m*M*), and distilled water to 1 L. Store at room temperature for several weeks.
7. Blocking solution (for intracellular markers). For 50 mL: weigh 0.5 g polyvinylpyrrolidone (1%) into a 50-mL tube and add 48 mL high salt buffer (*see* **Subheading 2.2., item 6**), 1.5 mL goat serum (3%), and 150 µL Triton X-100 (0.1%). Mix by inversion until completely in solution and store at 4°C. Use within 48 h.
8. Washing buffer (for intracellular markers). For 50 mL: mix 50 mL high salt buffer (*see* **Subheading 2.2., item 6**) and 25 µL Tween-20 (0.05%) in a 50-mL tube. Mix by inversion until completely in solution.
9. DAPI, dihydrochloride (VWR; cat. no. 80051-386).
10. Mouse α-OCT 4 IgG (Chemicon; cat. no. MAB4305).
11. Mouse α-OCT 4 IgG (Santa Cruz Technologies, Santa Cruz, CA; cat. no. SC-5270).
12. Mouse α-SSEA-1 IgM (Chemicon; cat. no. MAB4301).

13. Mouse α-SSEA-4 IgG (Chemicon; cat. no. MAB4304).
14. Mouse α-TRA-1-60 IgM (Chemicon; cat. no. MAB4360).
15. Mouse α-TRA-1-81 IgM (Chemicon; cat. no. MAB4381).
16. Goat anti-mouse Alexa Fluor 488 IgG (Molecular Probes, Eugene, OR; cat. no. A-11029).
17. Goat anti-mouse Alexa Fluor 488 IgM (Molecular Probes; cat. no. A-21042).
18. Rat α-SSEA-3 (Chemicon; cat. no. MAB4303).

3. Methods

3.1. Immunosurgical Isolation of Inner Cell Mass

Donated human embryos are cultured according to standard IVF protocols to the blastocyst stage. The method of immunosurgery is performed as described by Solter and Knowles *(9)*. It involves the removal of the zona pellucida followed by incubation of the embryo in antibody solution that binds to the proteins of trophectoderm cells but not to the inner cell mass cells; then lysing trophectoderm cells with guinea pig compliment.

1. Remove the zona pellucida by incubation in 0.5% pronase solution in embryo culture medium (*see* **Note 5**) for 30–40 s under microscope observation. When zona pellucida start to dissolve, remove embryos from the pronase solution and wash at least 7–10 times in embryo culture media.
2. Incubate the embryos in human anti-placental alkaline phosphatase antibody diluted 1:10 in embryo culture medium for 40 min. After incubation, wash the embryos five to seven times in embryo culture medium.
3. Incubate the embryos in guinea pig complement diluted 1:4 in embryo culture medium (embryos may collapse during this procedure).
4. Wash the collapsed embryos five to seven times in embryo culture medium using a pulled Pasteur pipet with a fire polished 100- to 120-μm diameter until lysing trophoblast cells are removed.
5. Plate the intact ICM onto an inactivated mouse embryonic fibroblast (MEF) monolayer in hES medium (for MEF monolayer preparation *see* **Subheading 3.2.**).
6. Change 50% hES medium every other day in ICM cultures. Start passaging 7–10 d after ICM growth will be observed (for further passaging of ICM, *see* **Subheading 3.4.2.**).

3.2. Isolation of MEF Cells

hES cells, as mouse ES cells, require a feeder layer of mitotically inactivated fibroblast cells or other cell types in order to remain pluripotent in culture. The hES cells that we routinely use, BG01 and BG02 cell lines *(5,8)*, were established on a MEF feeder layer. The MEF cells are made by standard procedures using 13.5-d post coitus mouse embryos and are frozen immediately after monolayer formation (*see* **Note 6**). The MEF cells are frozen immediately after they form a monolayer at passage one or two. The

performance of MEF as feeders will be compromised by multiple passage, so it is advisable not to expand MEF before use as feeders.

3.2.1. Removal of Uterus

1. Sacrifice pregnant mice by asphyxiation with CO_2. Place the mouse on its back. Pin the animal down onto a covered Styrofoam box lid by inserting needles through its legs.
2. Spray with 70% ethanol and swab with sterile gauze. Using scissors make a cut through the belly skin (at the median line just over the diaphragm).
3. Grasping the skin on both sides of the incision, pull in opposite directions to expose the untouched ventral surface of the abdominal wall (*see* **Note 7**).
4. Cut longitudinally along the median line of the exposed abdominal wall with sterile scissors, revealing the viscera. The uteri filled with embryos should be seen in the posterior abdominal cavity.
5. Dissect out the uterus with sterile forceps and scissors and place into a 50-mL screw-capped tube containing 20 mL sterile PBS +/+.
6. If more than one animal is being used, place the tube containing the uterus in the refrigerator or on ice until all uteri have been removed.
7. After the uterus has been isolated, place the dissected female carcass into an autoclave bag for proper disposal. Take the intact uteri to the tissue culture laboratory, and transfer them to a fresh dish of sterile PBS.

3.2.2. Dissection of Embryos

1. Place uterus in a 100-mm polystyrene Petri dish containing 10 mL PBS (+/+) (*see* **Note 8**).
2. Using watchmaker forceps and working under a dissection scope, tear the uterus with two pairs of sterile forceps, keeping the points of the forceps close together to avoid distorting the uterus and bringing too much pressure to bear on the embryos.
3. Release embryos from embryonic sacs and transfer to a new Petri dish containing fresh PBS (+/+) (*see* **Note 9**).
4. Under a dissecting microscope, remove the embryo heads and livers, intestines, heart, and all viscera using two watchmaker forceps.

3.2.3. Trypsinization of Cells

1. Transfer the embryo carcasses to a fresh Petri dish (no PBS). Carefully mince the embryos with a curved surgical scissors.
2. Add 5 mL 0.25% trypsin/EDTA per 10 fetuses and triturate through a 10-mL pipet.
3. Transfer the embryo/trypsin solution from the Petri dish with a 10-mL pipet to the barrel of a 10-mL syringe with an attached 18-G needle. Replace the syringe plunger and slowly and gently push the embryo/trypsin solution through the needle. Collect in a 50-mL conical tube.
4. Gently pass the embryo/trypsin suspension through the needle a second time.

5. Incubate the tissue suspension for 15 min at 37°C, triturating briefly every 5 min in a biosafety cabinet through a 10-mL pipet to dissociate the tissue.
6. Add an equal volume of MEF medium. Triturate the suspension vigorously with a pipet.
7. Plate one embryo equivalent per 175-cm^2 flask and add complete medium to make up a final volume of 30 mL per flask (*see* **Note 10**).
8. Incubate at 37°C with 5% CO_2 (*see* **Note 11**).
9. The next day, change the medium with an equal volume of fresh complete medium. On d 3–4, when the cells are 90% confluent, the MEF p0s are ready to be frozen.

3.2.4. Harvesting and Freezing of MEFs

1. Rinse the 90% confluent cells once with 20 mL PBS (–/–).
2. Detach cells using 3 mL 0.05% trypsin/EDTA solution per flask.
3. Tap the flask after 1 min to detach cells.
4. Inactivate the trypsin solution by adding 5 mL complete medium.
5. Pool cells from all flasks into 50-mL tubes ensuring even distribution among various tubes.
6. Count the number of viable cells using trypan blue and a hemacytometer (*see* **Note 12**).
7. Centrifuge the cell suspension at room temperature for 4 min at 200*g*.
8. Resuspend the cells in complete medium at two times the desired final freezing concentration (*see* **Note 13**).
9. Slowly and drop-wise, add an equal volume of 20% DMSO in FBS (*see* **Note 14**).
10. Dispense 1 mL cell mixture into prelabeled cryovials and place cryovials into a freezing container (which is prefilled with 250 mL room temperature isopropanol) and freeze at −80°C overnight (*see* **Note 15**).
11. After overnight incubation, rapidly remove the cryovials from the freezing container and place in liquid nitrogen (−196°C) for long-term storage.

3.3. Fibroblast Feeder Layer

3.3.1. Thawing MEFs

1. Remove vial of MEF p0 from the liquid nitrogen and briefly open the lid under a biosafety cabinet to release pressure. Close tightly again.
2. Thaw cells rapidly by hand, rotating the cryovial in a 37°C water bath and spray the vial with 70% ethanol and dry with a Kimwipe.
3. Working in a biological safety cabinet, transfer the thawed MEF cells to a sterile 50-mL conical centrifuge tube.
4. Swirling slowly, drop-wise add 10 mL MEF medium to the cell suspension. Add an additional 10 mL complete medium.
5. Centrifuge at room temperature at 200*g* for 4 min.
6. Resuspend the cells in 40 mL medium. Pipet well before removing aliquot for counting.
7. Count the viable cell number using trypan blue (*see* **Note 16**).

8. Seed T-175 flasks at approx 3×10^6 cells (*see* **Note 17**) per flask. Use 30 mL MEF medium per flask and swirl to ensure even distribution.
9. Incubate flasks at 37°C in a 5% CO_2 environment. MEFs should be 90–95% confluent, in the T175 flasks, 3 d after the initial thawing of the cells.

3.3.2. Inactivation and Plating of MEFs

1. After 3 d of incubation, check each flask individually by observing under a microscope to ensure cell growth and culture sterility (*see* **Note 18**).
2. In a biosafety cabinet, aspirate medium from T-175 flasks and add 16 mL mitomycin C solution (10 µg/mL).
3. Incubate treated flasks for 2 h at 37°C, 5% CO_2 (*see* **Note 19**).
4. After a 2 h inactivation of cells, aspirate off the mitomycin C solution (*see* **Note 20**).
5. Wash each flask five times with 20 mL PBS (+/+).
6. Aspirate the PBS (+/+) and wash the cells with 20 mL PBS (–/–) per flask.
7. Add 3 mL 0.05% trypsin/EDTA solution per flask.
8. Monitor, at room temperature, the degree of cell detachment. Rock and gently tap the flask (approx 1 min) (*see* **Note 21**).
9. When the cells are sufficiently detached from the flask (normally 1–2 min), add 5 mL MEF medium to each flask to inactivate the trypsin and rock to disperse.
10. Pool the cell suspensions from one to six flasks into 2X 50-mL conical tubes.
11. Add 15 mL MEF medium to the first flask and rinse out cells. Transfer this same 15 mL to the subsequent flasks and pool with cell suspension. Discard flasks.
12. Adjust the volume in each tube to 50 mL with MEF medium.
13. Centrifuge cells at 200*g* for 4 min at room temperature.
14. Resuspend cell pellets with MEF medium and pool into 1X 50-mL tube (*see* **Note 22**).
15. Centrifuge cells at 200*g* for 4 min at room temperature.
16. Resuspend cell pellet in 40 mL MEF medium. Use a 10-mL pipet and ensure to resuspend fully and adjust the volume to 50 mL with MEF medium.
17. Centrifuge cells at 200*g* for 4 min at room temperature (*see* **Note 23**).
18. Suspend the cell pellet first in 10 mL of complete medium and then add medium to final volume of 40 mL, mixing again vigorously before counting.
19. Count cells with trypan blue (*see* **Note 24**).
20. Centrifuge again if necessary to resuspend in a set volume to enable easier calculations for plating (*see* **Note 25**).
21. Plate MEF cells at the density described in **Table 1**.

3.3.3. Gelatin Coating for Staining

If plating MEFs on dishes or wells that will be used to stain ES cell colonies, it is advisable to gelatin coat before plating. This will enhance attachment of MEFs to the plastic during staining procedure.

1. Make a 0.2% gelatin solution in dH_2O.
2. Use 0.8 mL to cover a well on a four-chamber slide, or 2 mL for a 35-mm dish.

Table 1
MEF Plating Density

Culture container	Culture area cm^2	MEFs to seed for hES cell	Optimum volume (mL)
Dishes			
35-mm	10	1.2×10^6	2
60-mm	20	2.4×10^6	5
100-mm	60	7.2×10^6	10
Flasks			
T-25	25	3.0×10^6	5
Multiwells			
96well-	0.3	3.6×10^4	0.2
24-well	2	2.4×10^5	1
6-well	10	1.2×10^6	3
Chamber slides			
4	1.8	2.1×10^5	0.8

3. Incubate at room temperature for 1 h.
4. Wash once with PBS (+/+) before plating MEFs.

3.4. Culturing hES Cells

3.4.1. Thawing Manually Passaged hES Cells

1. Pre-equilibrate aged MEFs. Using at least 3-d-old MEF plates, aspirate off the medium, and replace with 2 mL 15/5 hES medium.
2. Place dish at 37°C until ready to plate out cells.
3. Wearing a protective face visor and cryo gloves, remove cryovials from liquid nitrogen. Briefly open the lid under a biosafety cabinet to release pressure and close tightly again.
4. Thaw the cells rapidly by hand by rotating the cryovial in a 37°C water bath until only small ice pieces remain. Spray vial with 70% ethanol and dry with a Kimwipe.
5. Working in a biological safety cabinet, transfer the thawed cells to a sterile 15-mL conical centrifuge tube.
6. Swirling slowly, dropwise add 10 mL of 15/5 hES cell medium to the cell suspension.
7. Centrifuge at room temperature at 200*g* for 4 min.
8. Resuspend pellet in 2 mL 15/5 hES medium.
9. Aspirate medium from the pre-equilibrated dishes and plate-thawed cells.
10. Check daily for colony formation. Colonies may take as long as 10–14 d to appear.

3.4.2. Manually Passaging hES Cells by Mouth Aspirator

3.4.2.1. Pipet Preparation

1. Prepare Pasteur pipets. This is done by heating a glass pipet on a low flame and pulling out the glass to the desired diameter (*see* **Note 26**).
2. Loop back the pulled glass, rub onto itself, and tap to break. The glass will break at the point of friction giving a straight edge.
3. Polish the end to remove ragged edges, by passing tip gently over a low flame.
4. Place pipet in the biosafety cabinet, ready to use (*see* **Note 27**).

3.4.2.2. Assemble Mouth Aspirator

1. Take two pieces of rubber tubing; join with a 13-mm sterile filter.
2. Attach a red mouthpiece to the tubing on the clear end of the filter.
3. Attach an aerosol barrier 1-mL tip to the other end of the tubing and insert a pre-pulled glass pipet.

3.4.2.3. Manual Passaging by Mouth Aspirator

1. Using at least 3-d-old MEF plates, aspirate off the medium and replace with 2 mL of 15/5 hES medium. Place dish at 37°C until ready to plate out cells.
2. Carefully cut hES colonies into pieces of 10–100 cells. Avoid any "bad" areas of differentiated cells and leave them attached to the dish.
3. After the colony has been cut into the desired pieces, transfer by mouth aspirator to a pre-equilibrated dish. Aim to have a minimum of 20 pieces per 35-mm dish.
4. Place the dish in 37°C, 5% CO_2. Once on the shelf move the dish up and down and sideways twice to evenly distribute colony pieces.
5. Change the medium daily with a 50% medium change.

3.4.3. Manual Passage of hES Cells by Hook

3.4.3.1. Pipet Preparation

1. Prepare Pasteur pipets. This is done by heating a glass pipet on a low flame and pulling out the glass, stretching it by 2 in.
2. Place the thinned area of the pipet above the flame and pull glass apart rapidly, forming a needle-like edge.
3. Quickly touch the needle end of the Pasteur into the flame allowing the glass tip to curve back on itself to form a hook.
4. Place pipet in biosafety cabinet, ready to use (*see* **Note 27**).

3.4.3.2. Manual Passage of Colonies by Hook

1. Using at least 3-d-old MEF plates, aspirate off the medium and replace with 2 mL of 15/5 hES medium. Place the dish at 37°C until ready to plate out cells.
2. Using a prepared hook, carefully cut colonies into pieces of 10–100 cells. Avoid any "bad" areas of differentiated cells and leave them attached to the dish.

3. After the colony has been cut into the desired pieces, transfer by pipet to a pre-equilibrated dish. Aim to have a minimum of 20 pieces per 35-mm dish.
4. Place the dish at 37°C, 5% CO_2. Once on the shelf move the dish up and down and sideways twice to evenly distribute colony pieces.
5. Feed cells daily with a 50% medium change.

3.4.4. Manual Passage of hES Cells by Chopping

3.4.4.1. Pipet Preparation

1. Prepare Pasteur pipet. This is done by heating glass pipet on a low flame and pulling out glass by 2 in.
2. Place the thinned area of pipet back above the flame and pull glass apart rapidly, forming a short flexible needle-like edge.
3. Place the pipet into a biosafety cabinet, ready to use (*see* **Note 27**).

3.4.4.2. Manual Passage of Colonies

1. Using at least 3-d-old MEF plates, aspirate off the medium and replace with 2 mL 15/5 hES medium (*see* **Subheading 2.1.1., item 2**). Place the dish at 37°C until ready to plate out cells.
2. Carefully cut colonies using a hook into pieces of 10–100 cells. Avoid any "bad" areas of differentiated cells and leave them attached to the dish. This is easiest achieved if done methodically. Make four to eight parallel cuts; rotate dish 90° and make another set of cuts.
3. Gently slide a glass needle under the colony and lift up the pieces.
4. Transfer by pipet to a pre-equilibrated dish. Aim to have a minimum of 20 pieces per 35-mm dish.
5. Place dish at 37°C, 5% CO_2. Once on shelf, move the dish up and down and sideways twice to evenly distribute the colony pieces.
6. Feed cells daily with a 50% medium change.

3.4.5. Freezing Manually Passaged hES Cells

Use plates of 3-d-old "good" undifferentiated colonies for freezing. If undesirable colonies are present remove these from the plate before proceeding.

1. Aspirate off the medium and wash the plate with 2 mL PBS (–/–).
2. Aspirate off the PBS and add 1 mL 1 mg/mL collagenase.
3. Incubate at 37°C for 3 min.
4. Aspirate off collagenase and add 1 mL 15/5 hES medium.
5. Under dissection scope, immediately use a cell scraper and gently scrape the bottom of the dish to break the feeder layer with colonies into pieces.
6. Remove cell suspension to a 15-mL tube.
7. Repeat the above steps with subsequent plates until all cells have been harvested.
8. Spin cells at 140*g* for 4 min.

9. Aspirate the supernatant and gently resuspend cell pellet in solution A at half the desired volume (*see* **Note 28**).
10. Slowly add an equal volume of solution B over 2 min, with occasional swirling.
11. Dispense 1 mL of cell mixture into prelabeled cryovials.
12. Place cryovials into a freezing container prefilled with 250 mL room temperature isopropanol and freeze at –80°C overnight (*see* **Note 29**).
13. After overnight incubation, rapidly remove the cryovials from the freezing container and transfer to liquid nitrogen (–196°C) for long-term storage.

3.4.6. Thawing Manually Passaged hES Cells

1. Pre-equilibrate aged MEFs. Using at least 3-d-old MEF plates, aspirate off medium, and replace with 2 mL of 15/5 hES medium for CDB or 2 mL 20% KSR medium for trypsinized cells.
2. Place dish at 37°C until ready to plate out cells.
3. Wearing protective face visor and cryo gloves, remove cryovials from the liquid nitrogen.
4. Briefly open the lid under a biosafety cabinet to release pressure. Close tightly again.
5. Rapidly thaw cells by hand by rotating the cryovial in a 37°C water bath until only small ice pieces remain.
6. Spray vial with 70% ethanol and dry with a Kimwipe.
7. Working in a biological safety cabinet, transfer the thawed cells to a sterile 15-mL conical centrifuge tube.
8. Swirling slowly, dropwise add 10 mL 15/5 hES medium to manually and CDB-passaged cells or 20% KSR medium to the trypsinized cell suspension.
9. Centrifuge at room temperature at 200*g* for 4 min.
10. Resuspend pellet in 2 mL medium and count.
11. Aspirate medium from pre-equilibrated dishes and plate-thawed cells at 150,000 per 35-mm dish.
12. Check daily for colony formation. Colonies may take as long as 3–5 d to appear (*see* **Note 30**).

3.4.7. Passage of hES Cells With CDB

1. Pre-equilibrate aged MEF feeder plates by aspirating off MEF medium and replacing it with 2 mL per 35-mm dish of 15/5 hES medium. Replace plates at 37°C 5% CO_2 until hES cells are ready for plating.
2. Aspirate the medium from hES cells and wash the dish with PBS (–/–).
3. Repeat wash step with PBS (–/–).
4. Aspirate off the PBS and add CDB to the cells. For a 35-mm dish, use 1.5 mL of CDB.
5. Over 1.5 min, using a 1-mL Eppendorf-style pipet and tip, gently pipet the cell aggregates up and down several times until most of the hES cells become detached from the MEFs, leaving the hES cells in suspension.

6. Transfer the supernatant containing the hES cells to a 15-mL sterile centrifuge tube containing 10 mL of 15/5 hES medium.
7. Using a bench top centrifuge, pellet the cells by centrifugation at 200*g* for 4 min at room temperature.
8. Discard the supernatant and resuspend the cell pellet in fresh 15/5 hES medium, 1 mL per 35-mm dish harvested.
9. Count cells (*see* **Note 31**).
10. Aspirate medium from pre-equilibrated dishes and plate cells at 150,000 cells per 35-mm dish in 2 mL medium.
11. Replace plates at 37°C, 5% CO_2. Move plates crosswise once placed on the shelf to ensure even distribution.
12. Change the media every day. Aspirate 50% medium and replace daily until ready to passage again around d 4.

3.4.8. Trypsin Passage of hES Cells

1. Using at least 3-d-old MEF plates, aspirate off medium and replace with 2 mL 20% KSR hES medium. Place dish at 37°C until ready to plate out cells.
2. Add 1 mL collagenase per 35-mm dish. Place on a 37°C stage for 2–3 min. Colonies can be observed rounding under the dissection scope.
3. Aspirate off the collagenase and add 1 mL 0.05% trypsin solution.
4. Allow trypsin to contact cells for no more than 40 s, aspirate off.
5. Add 1 mL 15/5 hES medium to the dish. Under the scope, begin to gently pipet to knock off and break up cell clumps.
6. Place harvested cells in a 15-mL tube containing 8 mL of 20% KSR medium.
7. Add fresh medium to the dish and wash off any remaining trypsinized cells, add to the 15-mL tube (*see* **Note 32**).
8. Spin harvested cells for 4 min at 200*g* at room temperature.
9. Resuspend the pellet in 20% KSR medium (2 mL per 35-mm plate trypsinized).
10. Count the cells (*see* **Note 33**).
11. Aspirate the medium from the pre-equilibrated dishes and plate cells at 150,000 cells per 35-mm dish in 2 mL medium. Place at 37°C, 5% CO_2. Move the plate crosswise on the shelf to evenly distribute the cells.
12. Change the media everyday with a 50% medium change until ready to passage again in 3–4 d.

3.5. Characterization of hES Cells

3.5.1. Agarose Plates for Embryoid Body Culture

3.5.1.1. Preparation of Agarose Plates

1. Working in a biosafety or laminar flow cabinet, cool the agarose solution and lay out 100-mm Petri dishes (*see* **Note 34**).
2. Using a 25-mL pipet, cover the bottom of one Petri dish at a time. Swirling the agarose to evenly distribute, then pipet up excess agarose. Continue onto subsequent Petri dishes.

3. Allow dishes to set for 10 min at room temperature. Replace dishes into plastic sleeve and store at 4°C until ready to use.

3.5.1.2. Equilibrating Agarose Plates

When EBs are ready to be grown in suspension, prepare and equilibrate agarose plates.

1. Remove desired number of plates from plastic sleeve at 4°C and add to each plate 10 mL DMEM/F12.
2. Place plates at 37°C and allow at least 40 min to warm.
3. Aspirate off DMEM/F12 and replace with prewarmed medium of choice for EB culture.

3.5.2. Formation of EBs

3.5.2.1. Formation of EBs by Hanging Drops

1. Prepare 100-mm Petri dishes by adding 10 mL sterile PBS (+/+) and prewarming at 37°C.
2. After you have desired colonies to convert to EBs, dissociate cells as if passaging.
3. After cells have been centrifuged after wash, count cells.
4. Resuspend at 2.5×10^5 cells/mL in 15/5 hES medium (*see* **Note 35**).
5. Open the lid of the Petri dish and place on a level surface. Onto the inner side of the lid pipet drops of 5000 cells in 20 μL.
6. Carefully replace the lid onto the Petri dish containing PBS (+/+). Do not allow drops to roll together (*see* **Note 36**).
7. After 2 d of culturing hanging drops, follow protocols for desired the pathway of EBs.

3.5.2.2. Formation of EBs by Cutting Colonies

1. After you have desired colonies to convert to EBs, equilibrate 100-mm 0.5% agarose-coated plates at 37°C.
2. Prepare a glass cutter by melting a Pasteur pipet under a small flame. Before the glass cools, pull to stretch about 2 in. Allow the glass to cool. Placing this stretched portion of the glass pipet into a flame, again melt and sharply pull apart to give a sealed and tapering glass tip. This tip should be no more than 1 in. long and extremely fine.
3. Work with one plate of ES colonies at a time under a dissection scope in a biosafety cabinet and aspirate the medium and rinse with PBS (+/+). Add 1 mL of chosen medium for EB culture.
4. Carefully use a glass cutter to chop each colony into 16–30 pieces, depending on colony size. This is easiest if done methodically, make four or five parallel cuts then rotate dish 90° and perform another four or five cuts perpendicular to the previous ones.
5. Transfer chopped colony pieces by pipet to pre-equilibrated 100-mm agarose dishes containing prewarmed medium of choice (*see* **Note 37**).

6. Place cells at 37°C with 5% CO_2. Move the plates in a crosswise manner to distribute evenly and prevent mass aggregation. EBs will grow in suspension.
7. Change the media every 3 d or as required per protocol.

3.5.2.3. Formation of EBs by Collagenase and Scraping

1. After you have desired colonies to convert to EBs equilibrate 100-mm 0.5% agarose plates at 37°C.
2. Prepare glass scrapers by melting a Pasteur pipet under a small flame. Before glass cools, pull to stretch about 2 in. Allow the glass to cool and placing this stretched portion of glass pipet into flame, again melt and sharply pull apart to give a sealed and tapering glass tip. This tip should be no more than 2 in. and extremely fine and flexible.
3. Working with one plate of ES colonies at a time, under a dissection scope in a biosafety cabinet, aspirate medium and rinse with PBS (+/+).
4. Add 1 mL of 1 mg/mL collagenase, place on a heated stage (37°C) and observe colonies for 4 min (colonies should be seen to round and curl at the edges).
5. Aspirate collagenase and rinse with 1 mL PBS (+/+).
6. Aspirate PBS and gently add medium of choice for culturing EBs (*see* **Note 38**).
7. Delicately rub glass scraper over the colonies, trying not to tear up the feeder layer. The colonies should break into clumps and readily detach.
8. After scraping is complete, pipet colonies into a 15-mL tube containing 10 mL medium of choice and centrifuge at 140*g* for 4 min at room temperature.
9. Aspirate supernatant and gently resuspend the colony pieces in chosen medium.
10. Transfer cell clumps into a prewarmed 100-mm agarose Petri dishes containing medium of choice.
11. Place cells at 37°C with 5% CO_2. Move plate in a crosswise manner to distribute evenly and prevent mass aggregation. EBs will grow in suspension.
12. Change the media every 3 d or as required per protocol.

3.5.3. Preparation of hES Cells for Karyotype Analysis

1. Change medium in hES cultures 24 h before karyotyping.
2. Incubate hES cells with 12 µg/mL EtBr for 40 min at 37°C, 5% CO_2 followed by treatment with 120 ng/mL of Colcemid for 20 min.
3. Wash cells with PBS (–/–) or Hank's solution.
4. Incubate hES cells with 0.25% trypsin-EDTA solution for 2 min at 37°C and dislodge hES cells to single cells.
5. Harvest hES cells into the same tube that has washed supernatant and add 2–5 mL of serum to inactivate trypsin. Centrifuge hES cells at 200*g* for 8 min.
6. Carefully aspirate the supernatant off and discard without disturbing the cell pellet. Gently break up the cell pellet by flicking the tube with your finger.
7. Resuspend the cell pellet in 4–6 mL hypotonic solution (0.075 *M* KCl) prewarmed to 37°C. Incubate the tube at 37°C for 20 min.

8. Add six to eight drops of freshly prepared fixative and incubate for additional 10 min. Centrifuge the cells at 200*g* for 8 min.
9. Remove supernatant and add fresh fixative, gently resuspend the cell pellet and incubate at room temperature for 30 min.
10. Repeat **step 9** twice without incubation.
11. To ensure the presence of metaphase plates, drop one to two drops of the fixed pellet to wet slides, air-dry, and observe under inverted microscope (*see* **Note 39**). Before shipment to cytogenetic laboratory, the cell pellet can be stored at 4°C for short-term and at –20°C for long-term storage.
12. Send the fixed pellet of cells to cytogenetic laboratory for further analysis (*see* **Note 40**).

3.5.4. Formation of Teratomas

1. Scrape hES cells from three to five 35-mm plates using a cell scraper and centrifuge cells for 3–5 min at 140*g*.
2. Leave as little medium as possible and inject the cells into the rear leg muscle or peritoneum of 4-wk-old male SCID, beige mice using 18- to 21-G needle. Teratomas will appear after 6 wk and can be dissected for analysis 8–10 wk after injection (*see* **Note 41**).

3.6. Immunostaining of hES Cells

3.6.1. Fixing Cells With Paraformaldehyde

1. Working in a fume hood, wash cells once in PBS (+/+) (*see* **Note 42**).
2. Add PFA solution to just more than cover the bottom of the well or dish and incubate at room temperature for 15–20 min.
3. Wash cells three times in PBS (+/+) and store fixed cells at 4°C until ready to stain with chosen markers.

3.6.2. Immunocytochemistry–Cell Surface Markers

1. Wash wells one at a time in PBS (+/+) (*see* **Note 42**).
2. Add 0.8 mL block solution for cell surface markers to each well. Incubate at room temperature for 45 min.
3. Prepare cell surface marker (1° Ab) in block solution at the dilution recommended in **Table 2**.
4. Aspirate block solution from wells and add 300 µL of 1° Ab solution. Cover and incubate 1 h at room temperature (*see* **Note 43**).
5. Wash cells four times in PBS (+/+) for 5 min each wash.
6. While completing washes in **step 5**, prepare 2° Ab in block solution at the dilution recommended in **Table 2**.
7. Aspirate off the last wash from the wells and add 300 µL of 2° Ab solution. Cover and incubate 1 h at room temperature. During incubation, cover sample with foil to prevent fluorescence bleaching.

Table 2
Cell Surface Markers

1°Ab	Dilution	2°Ab	Dilution
Rat-α-SSEA-3 IgM	1:500	Goat-α-rat Alexa Fluor 594 IgM	1:1000
Mouse-α-SSEA-4 IgG	1:1000	Goat-α-mouse Alexa Fluor 488 IgG	1:1000
Mouse-α-SSEA-1 IgM	1:1000	Goat-α-mouse Alexa Fluor 488 IgM	1:1000
Mouse-α-TRA-1–60 IgM	1:1000	Goat-α-mouse Alexa Fluor 488 IgM	1:1000
Mouse-α-TRA-1–81 IgM	1:1500	Goat-α-mouse Alexa Fluor 488 IgM	1:1000

Table 3
Intracellular Markers

1°Ab	Dilution	2°Ab	Dilution
Mouse-α-OCT 4 IgG Santa Cruz	1:500	Goat-α-mouse Alexa Fluor 488 IgG	1:1000
Mouse-α-OCT 4 IgG Chemicon	1:50	Goat-α-mouse Alexa Fluor 488 IgG	1:1000

8. Wash the wells four times in PBS (+/+) for 5 min each wash.
9. Add 0.8 mL of a 1:10,000 dilution of DAPI in distilled H_2O to each well. Incubate for 5 min at room temperature. Cover with foil during incubation.
10. Wash cells three times in PBS (+/+) and verify staining has taken place under ultraviolet microscope before mounting.
11. Gently remove sides of the chamber and aspirate excess surrounding PBS. Place one drop of mounting media directly in center of each well area.
12. Place one drop of mounting media directly in center of each well area and at an angle gently lower a cover slip onto the slide trying to avoid air bubbles where possible.
13. Remove excess mounting media from the slide and seal with nail varnish on all four sides. Keep in dark storage until results are observed or documented.
14. Compare the staining results according to **Table 4**.

3.6.3. Immunocytochemistry–Intracellular Markers

1. Wash wells one at a time in PBS (+/+) (*see* **Note 42**).
2. Add 0.8 mL block solution to each well and incubate at room temperature for 45 min.

Table 4
Antibody Specificity[a]

	SSEA-1	SSEA-3	SSEA-4	TRA-1–60	TRA-1–81	OCT-4
Human ES cell	-	+	+	+	+	+
Murine ES cell	+	-	-	-	-	+

[a]ES, Embryonic stem; -, undifferentiated ES cells show negative immunostaining for this antibody; +, undifferentiated ES cells show positive immunostaining for this antibody.

3. Prepare intracellular marker (1° Ab) in block solution at the dilution recommended in **Table 3.**
4. Wash the wells two times with high-salt buffer.
5. Add 300 µL of 1° Ab solution. Cover and incubate 1 h at room temperature (*see* **Note 43**).
6. Wash wells three times in washing buffer for 5 min each wash. Meanwhile, prepare 2° Ab in block solution at the dilution recommended in **Table 3**.
7. Wash wells one at a time in high-salt buffer.
8. Aspirate off the last wash from the wells and add 300 µL of 2° Ab solution. Cover and incubate 1 h at room temperature. During incubation, cover the sample with foil to prevent fluorescence bleaching.
9. Wash cells three times in wash buffer for 5 min each wash. While completing washes, prepare DAPI solution by diluting 1:10,000 in distilled H_2O. Store at 4°C.
10. Wash wells one at a time in high-salt buffer.
11. Add 0.8 mL DAPI solution to each well. Incubate for 5 min at room temperature. Cover with foil during incubation.
12. Wash cells three times in PBS (+/+) and verify staining has taken place under ultraviolet microscope before mounting.
13. Gently remove sides of chamber and aspirate excess surrounding PBS. Place one drop of mounting media directly in center of each well area.
14. At an angle gently lower a cover slip onto the slide trying to avoid air bubbles where possible. Remove excess mounting media from slide and seal with nail varnish on all four sides. Keep in dark storage until results are observed or documented.
15. Compare the staining results according to **Table 4**.

4. Notes

1. FBS (or fetal calf serum) for hES cell growth varies from lot number to lot number, so we advise performing a test before purchasing the particular lot of serum.
2. Store in the dark at 4°C for up to 6 wk or at –20°C for long-term storage.

3. Mitomycin C is highly toxic. We purchase premeasured mitomycin C in glass vials. Read and understand the material safety data sheet and handle accordingly. Used mitomycin C must be neutralized by the addition of 15 mL Clorox per 500 mL mitomycin C solution. Swirl the waste solution and incubate 15 min before discarding.
4. Work in a fume hood and wear gloves; PFA is toxic, see material safety data sheet.
5. Embryo culture media used here as a base medium for dilution of primary and secondary antibodies during isolation of inner cell mass can vary from one in vitro fertilization laboratory to another. This is a reason we did not provide the catalog number for embryo culture medium.
6. Because of the limited lifespan of MEF cells, we recommend preparing in advance a stock of frozen vials that are capable of supporting hES cells.
7. This can be assisted with rat tooth forceps.
8. Work with one litter at a time.
9. Remove the entire litter before proceeding.
10. This density allows the cells to adhere but not become overly confluent before harvest at d 3–4.
11. MEFs will attach to the flask and begin to divide overnight.
12. Make a note of the dead cells for future reference.
13. For example: 2×10^7/mL.
14. The final cell concentration is 1×10^7.
15. This procedure ensures that a −1°C/min rate of cooling is achieved, a step critical to cell viability.
16. Record dead cell number for reference of freeze/thaw procedures. Viability is usually greater than 95%.
17. Depending on the doubling time of the cell stock.
18. MEFs should be at 90–95% confluency in the T-175 flasks 3 d after the initial thawing of the cells.
19. Work in sets of no more than six flasks at a time.
20. Neutralize waste with Clorox.
21. MEFs are trypsin sensitive; do not overexpose.
22. A maximum of $12 \times$ T175 flasks of cells per 50-mL tube.
23. This has given a total of nine washes for cells: six before trypsin, one at trypsinization, and two after trypsinization in MEF medium.
24. Mixing is critical to get an accurate cell count.
25. For example: 2×10^6 then calculate required volume to add to medium for plating requirements.
26. This will involve pulling the glass about 24 cm. The diameter of the pipet must be in relation to the colony size; this should be no larger than one-third or half of the colony size.
27. At no point should the center of the pulled glass be touched by anything. After flaming and pulling, the pipet is considered sterile.
28. Generally aim for approx 100 colonies per cryovial (i.e., if one plate had approx 50 colonies, resuspend cells from two plates in 0.5 mL solution A).

29. This procedure ensures that a –1°C/min rate of cooling is achieved, a step critical to cell viability.
30. Change the media daily. Always pass colonies at least once before setting up experiments.
31. Note this method of passaging will result in clumps of about 3–10 cells. This is normal; count accordingly.
32. After the feeder layer begins to roll or break, it is time to stop. The best cells seem to be those that come off first, so do not try hard to collect everything on the feeder layer or those that remain attached to plate.
33. Take 10 µL of the cell suspension and mix it with 10 µL trypan blue. Fill one chamber of a 0.1-mm deep hemacytometer. Count four corner boxes on the grid. Ensure minimum of 100 events counted. Divide the total by the number of boxes counted. Take this number to $\times 10^4$, then multiply by 2 for trypan dilution and finally by the total volume of cells in the 15-mL tube. This will give the total cell count—i.e., 200 cells counted over four boxes from a 12-mL starting cell volume = – $(200/4) \times 10^4 \times 2$ (dilution) $\times$ 12 mL.
34. Keep plastic sleeve to store prepared dishes.
35. This will allow for good quality EB formation. Depending on the pathway chosen for EBs to follow, this number may be altered.
36. The cells will now grow in suspension in hanging drops. Less than 50% will aggregate successfully.
37. Medium of choice depends on pathway you want EBs to follow.
38. EBs can be used for producing the three germ layers or for differentiation along many pathways. Each process requires different medium and culture conditions.
39. Make sure to see at least 5–10 metaphase plates per slide before sending the pellet of fixed cells to cytogenetic laboratory.
40. To avoid misinterpretation of karyotype data, it is not advisable to do G-banding in your laboratory. This requires professional analysis and needs to be done by cytogenetic labs with clinical diagnosis experience.
41. Greater amount of cells injected will ensure formation of teratomas, but will compromise the health of the mice. Injection into one leg is advisable to avoid unnecessary suffering.
42. Use an aspirator to remove and a transfer pipet to add PBS. Be very gentle; cells will dislodge easily.
43. This can be extended to overnight at 4°C if necessary.

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6

Routine Culture and Differentiation of Human Embryonic Stem Cells

Jim McWhir, Davina Wojtacha, and Alison Thomson

Summary

Human embryonic stem cells provide both an in vitro model of human development and a potential source of cells for treatment of degenerative, metabolic, or traumatic disorders. This chapter describes techniques for routine maintenance and differentiation of human embryonic stem cells in culture.

Key Words: Human embryonic stem cells; hES cells; feeder-free culture; karyotype; embryoid bodies.

1. Introduction

The first human embryonic stem (hES) cells were only isolated some 6 yr ago *(1,2)*. Although we have learned much about these cells in the intervening period, the key molecules that control their self-renewal remain unidentified and present culture systems are almost certainly suboptimal. As a consequence, there are a variety of protocols available for the routine culture of hES cells that variously require feeder layers, conditioned medium, disaggregation by several enzymatic regimes, or mechanical dissociation. It seems likely that the various lines isolated under different conditions have adapted to those conditions and the best advice to those who are new to hES culture is to begin with the precise protocols provided at source. But this is not to say that any single protocol is in a general sense "best." This chapter provides the protocols presently used in our laboratory to culture the hES lines H1 and H9 that are available through the University of Wisconsin (Wi-cell). They are not the protocols provided by Wi-cell, and the H1 and H9 cell lines have required a period of adaptation.

To date, hES lines have been established by mechanical dissociation of explanted inner cell masses or blastocysts on a fibroblast feed layer. At least in

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their early passages, hES cells are highly sensitive to the method of disaggregation and some lines are maintained routinely by mechanical dissociation. However, this is a very cumbersome method of handling hES cells and precludes many large-scale culture experiments. Other lines have traditionally been passaged using collagenase treatment, which leads to the release of cell clumps from the culture surface, but not to a single-cell suspension, and in our hands is associated with poor plating efficiency. We have therefore set out to develop passage procedures that provide for the simplified expansion of hES cell numbers within a shorter period. In doing so, we have benefited from the underpinning work of others in developing a conditioned medium system that allows culture of hES cells without direct contact with feeders *(3)*. In our hands, hES cells passaged with collagenase frequently do not recover well and cultures frequently contain a second, fibroblast-like, population (**Fig. 1A**). We have found this problem greatly reduced when using a disaggregation regime comprising trypsin and ethyleneglycol tetraacetate (EGTA) (**Fig. 1B**).

There must be an accompanying warning that goes along with these and all other hES protocols—we have no standard assay of normality for hES cells. Protocols optimized for experimental convenience are not necessarily those that best retain the pluripotential, wild-type nature of the cells. In this respect, hES cells are at a disadvantage to their murine counterparts where germ line transmission provides a convincing indicator of normality. Nevertheless, our presently favored protocol for routine hES maintenance has been shown to sustain normal karyotype at extended passage (**Fig. 1C**) and to sustain the potential to differentiate widely when hES cells are injected into severe combined immunodeficiency mice (data not shown).

This is not necessarily true of all passage regimes, and we urge caution in introducing modifications to culture conditions without careful characterization of the cells over time. **Figure 1C** reveals the appearance of the translocation (iso7q) when cells were disaggregated for 10 passages using ethylenediaminetetraacetic acid (EDTA) alone. This translocation had overtaken the population within 10 passages. The repeatability of this result is under investigation and it is premature to draw conclusions about the effect of EDTA. However, others have reported recurrent gain of chromosomes 17q and 12 *(4)*, suggesting that under certain conditions, there can be strong selection pressure for chromosomal abnormalities. Whether these phenomena arise as a result of enzymatic disaggregation or as a consequence of culture to high cell density has been the subject of some debate *(5)*. It is intriguing that different results appear recurrently in different labs such that Buzzard et al. *(5)* report no abnormalities over 80 passages, Thomson and Andrews *(4)* report different recurrent duplications in their respective laboratories while using the same cells and culture systems, and we, using the same line but disaggregating in EDTA alone see yet a third duplication. On balance, the advantages of enzymatic disaggregation make it

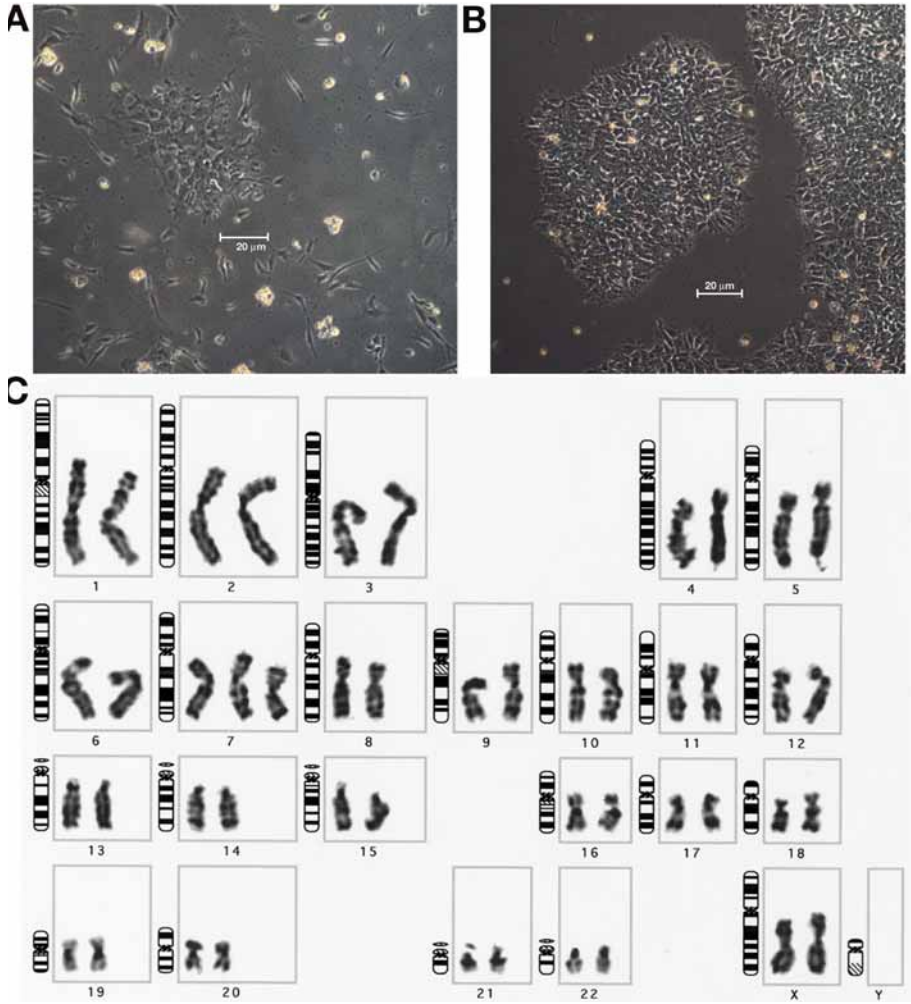

Fig. 1. Human embryonic stem cells 72 h after passage in **(A)** collagenase **(B)** trypsin/ethyleneglycol tetraacetate (EGTA), revealing the greater plating efficiency, growth rate, and reduced differentiation we observe with trypsin/EGTA. Cells maintain a stable karyotype for up to 54 passages with trypsin/EGTA (**C**, left karyotype), but abnormalities are observed when using ethylenediaminetetraacetic acid alone (**C**, right karyotype) (Please *see* the companion CD for the color versions of this figure.)

difficult to justify mechanical disaggregation for large-scale experiments, but the prudent researcher will avoid culturing hES cells to too high a density and will periodically assess the karyotypic stability of the cells.

Data are now accumulating regarding the patterns of gene expression that characterize hES cells *(6,7)*. Encouragingly, hES lines of differing origins do

show common expression patterns, suggesting that it may soon be possible to develop an expression "fingerprint" of normality for hES cells and this will go some way toward the further optimization of these protocols.

Experimental differentiation of hES cells often begins with the formation of embryoid bodies (EBs), structures that mimic the cellular interactions during embryogenesis and generate inductive signals. EBs are generated by partial disaggregation of hES cells and by plating onto nonadherent plastic in non-conditioned medium and in the absence of supplementary basic fibroblast growth factor (bFGF) (**Fig. 2**). For our own work with bone-forming cells, we have found that the prior formation of embryoid bodies is unnecessary for hES cells to respond to osteogenic-inducing agents. However, this method may be essential for other lineages and is a useful way of verifying the potentiality of cultures.

Finally, as an example of directed differentiation, we also include our protocol for osteogenic induction of hES cells *(8)*. Human ES cells enter the osteogenic pathway within a similar time scale to human mesenchymal stem cells, suggesting that the commitment of hES cells to something similar to a mesenchymal stem cell may occur rapidly. **Figure 3** shows the accumulation of alizarin red-stained areas of the culture well over time when hES cells are exposed to osteogenic factors (dexamethasone, b-glycerophosphate, and ascorbic acid).

2. Materials

2.1. Mouse Embryonic Fibroblast Reagents

1. Mouse embryonic fibroblast (MEF) medium: to 450 mL Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, Poole, England; cat. no. D-5796), add 50 mL fetal calf serum (FCS) (Globepharm, University of Surrey, Guildford, UK) and 5 mL 100X L-Glutamine (Invitrogen Life Technologies, Paisley, UK; cat. no. 25030-024). Filter-sterilize and store at 4°C for up to 4 wk.
2. MEF freezing mix: to 3 mL MEF medium, add 1 mL FCS and 1 mL dimethylsulfoxide (Sigma-Aldrich; cat. no. D-2650). Filter-sterilize (*see* **Note 1**).
3. Penicillin/streptomycin: a 2X solution is made by the addition of 2 mL penicillin/streptomycin (Invitrogen Life Technologies; cat. no. 15140-122) to 10 mL of phosphate-buffered saline (PBS) (*see* **Subheading 2.3., item 6**).

2.2. hES Culture Reagents

1. Gelatin: add 0.4 g of porcine skin type A gelatin (Sigma-Aldrich; cat. no. G-2500) to 400 mL H_2O, autoclave, and store at room temperature.
2. bFGF: for 100 mL of bovine serum albumin-supplemented PBS, add 1.3 mL 7.5% bovine serum albumin solution (Invitrogen Life Technologies; cat. no. 15260-037) to 98.7 mL PBS (*see* **Subheading 2.3., item 6**). Resuspend lyophilized bFGF (Sigma-Aldrich; cat. no. F 0291) at 1 µg/mL in PBS supplemented with bovine serum albumin. Filter-sterilize using a 0.2-µm syringe filter and store in 1-mL aliquots at −20°C.

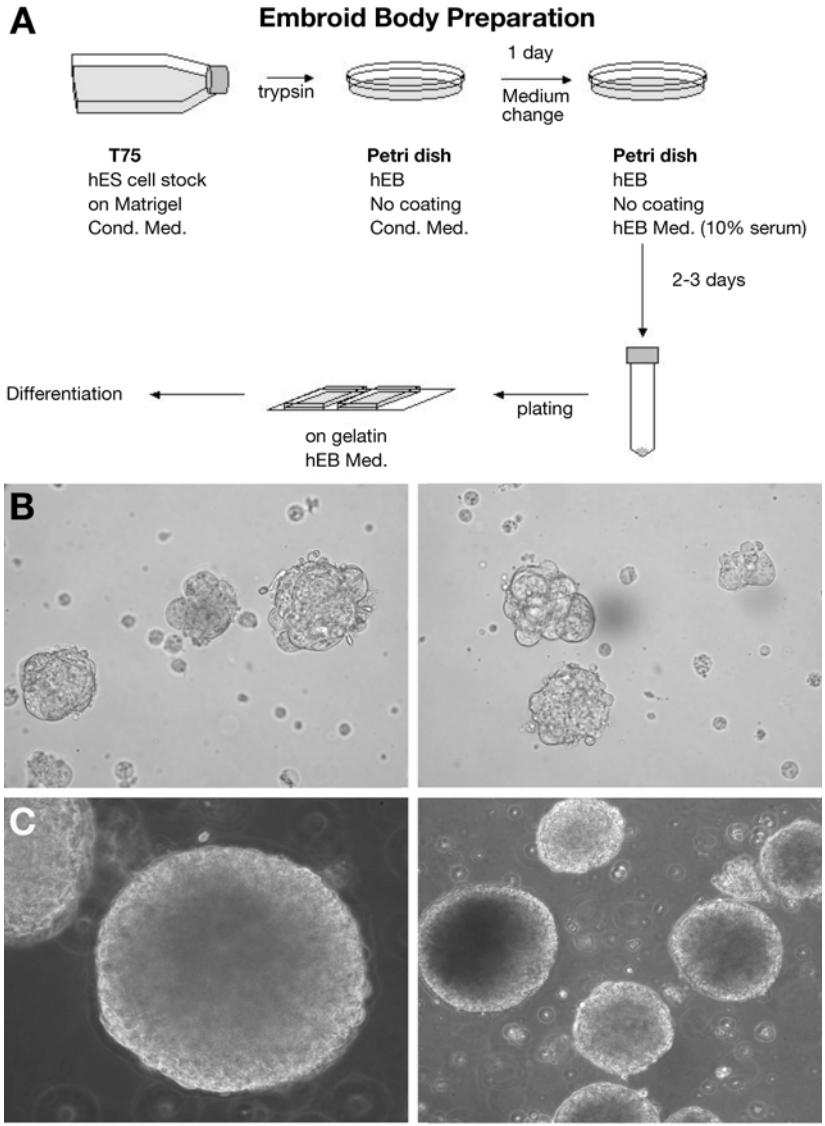

Fig. 2. Schematic showing production of human embryonic stem cell-derived embryoid bodies **(A)**, typical embryoid body morphology after 2 d of suspension culture **(B)** and after 5 d **(C)**.

3. hES medium: to 400 mL knockout (KO) DMEM (Invitrogen Life Technologies; cat. no. 10829-018), add 100 mL KO serum replacement (Invitrogen Life Technologies; cat. no. 10828-028), 5 mL 200 m*M* L-glutamine (Invitrogen Life Technologies; cat. no. 25030-024), 5 mL 100X nonessential amino acids (Invitrogen Life Technologies; cat. no. 11140-035), and 1 mL 50 m*M* β-mercaptoethanol

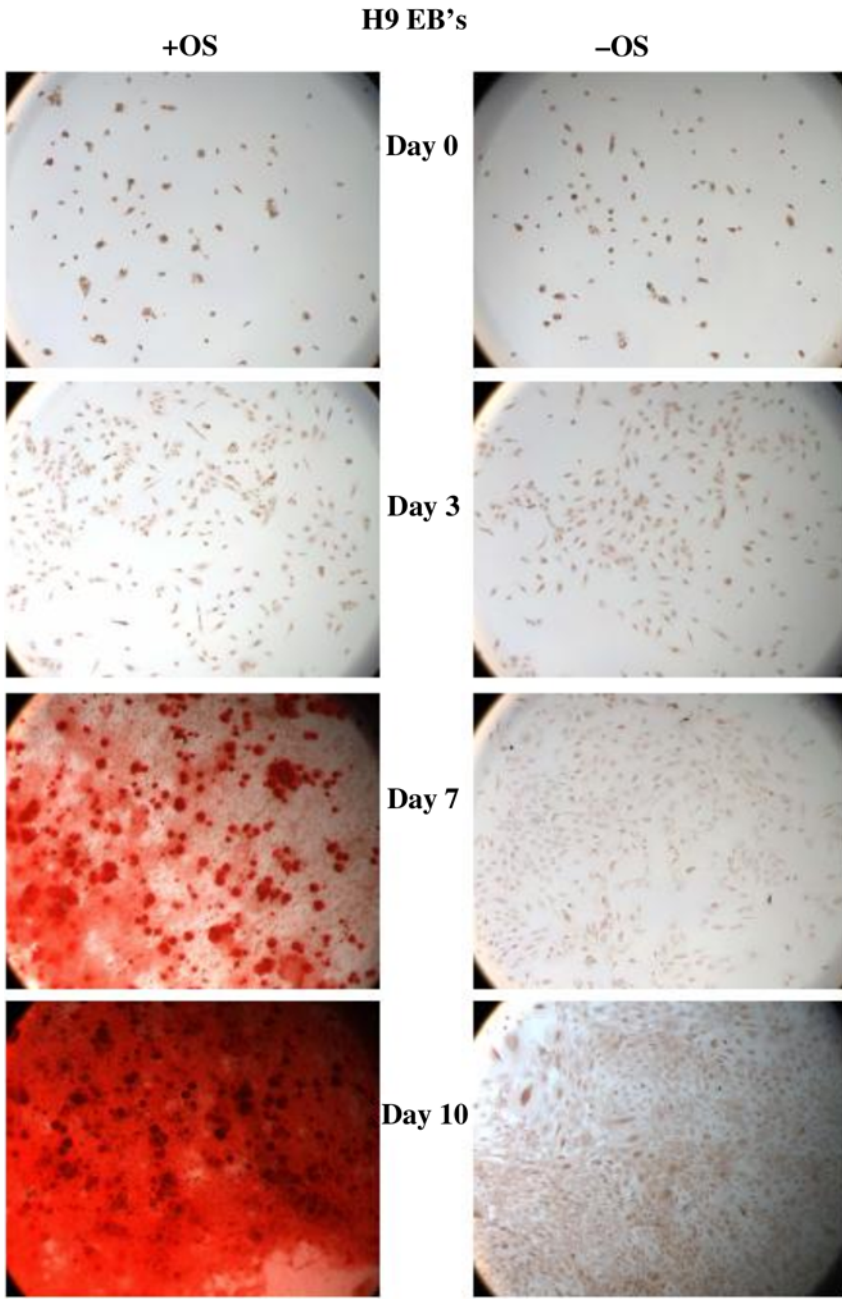

Fig. 3. Alizarin red staining for mineralisation in differentiating hES-derived embryoid body cultures in the presence (left panels) and absence (right panels) of osteogenic factors (dexamethazone, b-glycerophosphate and ascorbic acid) at 0, 3, 7, and 10 d of culture. (Please *see* the companion CD for the color version of this figure.)

(Invitrogen Life Technologies; cat. no. 31350-010). Filter-sterilize and store at 4°C for up to 4 wk.

4. hES freezing mix: to 7 mL hES medium, add 3 mL KO serum replacement and 2 mL dimethylsulfoxide (Sigma-Aldrich; cat. no. D-2650). Filter-sterilize (*see* **Note 1**).
5. Human EB medium: to 450 mL KO DMEM, add 50 mL FCS, 5 mL 100X L-glutamine, 5 mL 100X nonessential amino acids, and 1.1 mL β-mercaptoethanol.
6. Matrigel: thaw a 10-mL bottle of growth factor reduced Matrigel (Biotrace Fred Baker Ltd., Bridgend, UK; cat. no. 35-4230) at 4°C overnight on ice (*see* **Note 2**). Aliquot 0.5-mL amounts in prechilled tubes with a prechilled pipet. Store at −20°C.
7. Trypsin-EGTA (TEG) solution: weigh 0.90 g glucose (VWR International ltd., Lutterworth, England; cat. no. 101174Y), 0.12 g disodium hydrogen phosphate (VWR International Ltd.; cat. no. 444425M), 0.33 g potassium chloride (VWR International Ltd.; cat. no. 101984L), 0.22 g potassium dihydrogen phosphate (VWR International Ltd.; cat. no. 102032W), 6.30 g sodium chloride (VWR International ltd.; cat. no. 102415K), 2.70 g Tris (VWR International Ltd.; cat. no. 103156X). Make up to 800 mL with double distilled water and then add 0.40 g EGTA (Sigma-Aldrich; cat. no. E-4378), 0.10 g polyvinyl alcohol (Sigma-Aldrich; cat. no. P-8136), 100 mL 2.5% (10X) trypsin solution in saline (Invitrogen Life Technologies; cat. no. 15090-046), and 0.90 mL 1% Phenol red (Sigma-Aldrich; cat. no. P-3532). Adjust pH to 7.6 and make up to 1 L with double distilled water. Filter sterilize, aliquot, and store at −20°C.

2.3. Karyotyping Reagents

1. Bacto trypsin: add 500 µL Bacto trypsin (Biotrace Fred Baker Ltd.; cat. no. 215310) to 50 mL sterile distilled H_2O.
2. Gurr's buffer: add 1 Gurr's buffer tablet pH 6.8 (Invitrogen Life Technologies; cat. no. 10582-013) to 1 L distilled H_2O. Autoclave and store at room temperature.
3. 5% Giemsa solution: add 2 mL Gurr's R66 Giemsa (VWR International Ltd.; cat. no. 350864X) to 40 mL Gurr's buffer pH 6.8.
4. Hypotonic solution: dissolve 0.56 g KCL in double distilled water and make up to 100 mL. This solution should be made fresh on the day of use.
5. Karyotype fixative solution: add 3 vol of methanol to 1 vol of acetic acid. This should be made up immediately before use.
6. PBS: add 4 Dulbecco "A" PBS tablets (Mg^{2+} and Ca^{2+} free) (Oxoid Ltd., Basingstoke, England; cat. no. BR0014G) to 400 mL double distilled water. Autoclave and store at room temperature.
7. 2X sodium chloride/sodium citrate solution (SSC): dissolve 17.53 g sodium chloride (VWR International Ltd.; cat. no. 102415K) and 8.82 g trisodium citrate (VWR International Ltd.; cat. no. 102425M) in 800 mL distilled water. Adjust pH to 7.0 and make volume up to 1 L.
8. Depex mounting medium.

2.4. Osteogenesis Reagents

1. OS-supplemented human EB medium (OS+): to 100 mL human EB medium, add 294 µL 0.017 *M* ascorbic acid (50 µ*M* final concentration) (Sigma-Aldrich; cat. no.

A-8960), 1 mL 1 *M* β-glycerophosphate (10 m*M* final concentration) (Sigma-Aldrich; cat. no. G-9891), and 100 µL 0.1 m*M* dexamethasone (0.1 µm final concentration) (Sigma-Aldrich; cat. no. D-4902). A 0.1 m*M* dexamethasone stock solution is prepared by addition of 0.004 g dexamethasone to 95% ethanol and stored at −20°C.

2. Control human EB medium (OS–): to 100 mL human EB medium add 1 mL 1 *M* β-glycerophosphate (10 m*M* final concentration).
3. Alizarin red stain: for a 1% solution add 1.0 g alizarin red (Sigma-Aldrich; cat. no. A-5533) to 100 mL distilled water, allow to dissolve, and filter through Whatman 3M filter paper.
4. Calcium colorimetric kit (Randox Laboratories Ltd., Ardmore Antrim, UK; cat. no. CA590).

3. Methods

3.1. Isolation of MEFs

1. Sacrifice a pregnant mouse at d 13.5 of pregnancy by cervical dislocation.
2. Swab the abdomen with 70% alcohol, dissect out uterine horns, dip them briefly in 70% ethanol, and transfer into a bacterial Petri dish containing PBS and 2X penicillin/streptomycin solution.
3. Remove the embryos and place in a dish of PBS containing 2X penicillin/streptomycin solution.
4. Decapitate the embryos and transfer the bodies to a sterile capped tube for transport to a tissue culture hood.
5. Dissect and discard the soft tissues (liver, heart, and other viscera).
6. Wash each embryo carcass through three fresh Petri dishes of PBS (containing 2X penicillin/streptomycin solution).
7. Incubate each embryo in 2 mL TEG solution at 37°C for 5 min.
8. Vortex the embryo/TEG mix and return to 37°C for a further 5 min.
9. Repeat the vortex and incubation step sufficient times for the production of a cloudy cell suspension.
10. Add 3 mL MEF medium, vortex, and let larger clumps of debris settle.
11. For each embryo, remove the top 3.5 mL of cell suspension and transfer to a 75-cm^2 tissue culture flask with a further 15 mL MEF medium.
12. Incubate in a humidified 37°C incubator with 5% CO_2.
13. Change the medium daily until the cells are approaching confluency.
14. When the cells approach confluence, wash with PBS, aspirate, and incubate with 3 mL TEG until the MEFs can be easily knocked off the plastic.
15. Transfer cells into a 150-cm^2 tissue culture flask and add 30 mL MEF medium.
16. Incubate until almost confluent, wash with PBS, and treat with 5 mL TEG as before.
17. Centrifuge cells at 200*g* for 5 min. Resuspend in 1.5 mL MEF medium and add an equal volume of chilled MEF freezing mix, mix gently and place 1-mL aliquots in prechilled cryovials.

18. Place at −80°C overnight and then store under liquid nitrogen or at −150°C.

3.2. Generation of Conditioned Medium for hES Cells

1. Rapidly thaw a cryovial of MEFs by placing in a 37°C water bath.
2. Immediately after thawing, transfer the cells into 10 mL of prewarmed MEF medium and centrifuge at 200*g* for 5 min.
3. Resuspend the MEFs in 1 mL MEF medium, triturate a couple of times to break up any clumps, and then add 14 mL MEF medium. Transfer cell suspension to a 75-cm^2 tissue culture flask and incubate.
4. MEFs can be passaged or expanded with TEG as described in **Subheading 3.1., steps 14–15** and are used up to passage four for conditioning of medium.
5. Grow MEFs in MEF medium in an appropriately sized flask to approx 80% confluence. A 75-cm^2 flask of MEFs is used to condition 20 mL medium, and a 150-cm^2 flask of MEFs is used to condition 40 mL medium.
6. After the cells are approx 80% confluent, aspirate the medium and replace with hES medium supplemented with 4 ng/mL bFGF.
7. Leave the medium in the flask for approx 24 h for conditioning to take place, then harvest the medium and replace with fresh hES medium plus bFGF to be harvested the following day. This can be repeated up to four times (i.e., each flask of MEFs can be used to condition medium for 4 d).
8. Add another 4 ng/mL bFGF stock to the harvested medium and filter-sterilize through a low protein-binding filter unit. This medium can be used directly for the culture of hES cells. The conditioned medium can also be stored at −20°C for several months. In this case, neither add the second aliquot of bFGF nor filter sterilize until after thawing.

3.3. Growing hES Cells on Matrigel Matrix

1. Thaw an aliquot of Matrigel on ice.
2. Add to 50 mL cold KO-DMEM with a prechilled pipet and mix immediately by inverting.
3. Coat tissue culture plates or dishes by adding the diluted Matrigel at approx 0.2 mL/cm^2. Leave on a level surface at 4°C overnight or room temperature for 1 h.
4. Coated plates and flasks can be sealed with Parafilm and stored at 4°C up to 1 mo.
5. Before plating hES cells, aspirate the Matrigel/KO DMEM and rinse with prewarmed KO-DMEM.
6. Rapidly thaw a cryovial of human embryonic stem cells by placing in a 37°C water bath.
7. Once thawed, immediately transfer the cells into 10 mL of prewarmed KO-DMEM medium and centrifuge at 200*g* for 2 min.
8. Resuspend the cell pellet in 1 mL conditioned medium and triturate a couple of times to break up clumps.
9. Add an appropriate amount of conditioned medium for the size of flask or dish (*see* **Note 3**).

10. Transfer the cell suspension to the Matrigel-coated tissue culture flask or dish and place in incubator. Incubate at 37°C in a humidified incubator with 5% CO_2. Change the medium daily (*see* **Note 4**).

3.4. Trypsin-Based Passaging of hES Cells

1. Aspirate the medium and rinse with prewarmed KO-DMEM.
2. Incubate the cells in TEG, approx 0.04 mL/cm^2, at 37°C until all cells have rounded, then knock the flask to release the cells into suspension.
3. Add KO-DMEM to the flask and transfer cell suspension to a centrifuge tube.
4. Pellet the hES cells by centrifugation at 200*g* for 2 min.
5. While the cells are being centrifuged, prepare the new flask/dish. Remove the matrigel and rinse with prewarmed KO-DMEM. Replace the KO-DMEM with conditioned medium (*see* **Note 3**).
6. Resuspend the cells in 1 mL of conditioned hES medium. Gently triturate the suspension to ensure a single cell suspension. We usually passage a confluent culture 1:3 (*see* **Note 5**). Add 2 mL conditioned medium to the cell suspension, mix, and then transfer 1 mL to the new flask/dish.
7. Ensure the cells are well mixed in the fresh conditioned medium and return to the humidified incubator with 5% CO_2.

3.5. Cryopreservation of Human ES Cells

1. Incubate until almost confluent, wash with prewarmed KO-DMEM, and treat with TEG as in **Subheading 3.4., steps 2–3**.
2. Centrifuge cells at 200*g* for 2 min. Resuspend in KO-DMEM + 30% KO serum replacement at approx 0.02 mL/cm^2 of culture surface. Triturate gently to ensure there are no clumps.
3. Slowly add an equal volume of chilled hES freezing mix, mixing gently as you do so.
4. Place 1-mL aliquots into prechilled cryovials, place at −80°C overnight, and then store under liquid nitrogen or at −150°C.

3.6. Karyotype Analysis of hES Cells

1. Passage a confluent 75-cm^2 flask into 4 × 25-cm^2 flasks.
2. Exactly 25 h later, add Colcemid at 50 µL per 5 mL conditioned hES medium.
3. A further 2 h later, remove medium and store in a centrifuge tube.
4. Wash the flask with 2–3 mL PBS and add to the centrifuge tube.
5. Add 1 mL TEG to the cells and incubate until cells are ready to come off the flask. Add the medium/PBS stored in the centrifuge tube and transfer the cell suspension to a centrifuge tube.
6. Centrifuge cells at 300*g* for 5 min.
7. Discard the supernatant and disrupt the cell pellet using a vortex mixer.
8. Add 8–10 mL of hypotonic solution using a vortex mixer at a low-medium setting.
9. Incubate at room temperature for 10 min (*see* **Note 6**).
10. Centrifuge at 300*g* for 5 min and decant the supernatant as before.

11. Resuspend the cell pellet in FIX 3:1 methanol: acetic acid to a final volume of 8–10 mL (*see* **Note 7**).
12. Centrifuge at 300*g* for 5 min and then resuspend the pellet in fix to a final volume of 8–10 mL.
13. Repeat **step 12** a further two times.
14. Following the third centrifugation, remove the supernatant, carefully tap the cells into suspension, and add fresh fix until the suspension looks slightly cloudy.
15. Clean and polish a slide that has been previously washed with 95% ethanol and 5% HCl. Add one drop of suspension onto the slide from a fine-tipped pipet, allowing it to spread and air-dry (*see* **Note 8**). If required, the suspension can be stored indefinitely at −20°C. Transfer the suspension to an Eppendorf tube and fill the tube with fresh karyotype fixative solution. If making slides after storing at −20°C, the suspension should be allowed to warm up to room temperature and then centrifuged at 300*g* for 5 min and given two washes in fresh fixative solution before it is used to make slides.
16. View under phase contrast to check for number and quality of divisions present.

3.6.1. G-Banding

1. Store slides at room temperature for at least 3–5 d.
2. Incubate slides in 2X sodium chloride/sodium citrate solution (SSC) at 60°C for 2–4 h (minimum 2 h).
3. Remove slides and wash briefly in H_2O.
4. Place slides, one at a time, in 1% Bacto-trypsin solution for 30 s (*see* **Note 9**).
5. Wash slides individually in water.
6. Place slides in 5% Giemsa solution for 10 min.
7. Wash briefly in H_2O.
8. Blot dry between two sheets of filter paper, check for spreads, then leave to dry overnight at room temperature.
9. Place slides in xylene for 2–3 min.
10. Mount with Depex mounting medium and examine slides on a microscope at 100-fold magnification.

3.7. Generation of EBs

1. Trypsinize an almost confluent or confluent 75-cm^2 flask of hES cells as described in **Subheading 3.4.**
2. Resuspend the cell pellet in 1 mL conditioned medium. Triturate gently.
3. Add 39 mL conditioned medium and plate over two Petri dishes (*see* **Note 10**).
4. Incubate in a humidified incubator with 5% CO_2 overnight.
5. The following day, there should be clumps of cells floating in the medium.
6. Forty-eight hours after transferring to the Petri dishes, transfer the cells/medium to a 50-mL tube. Let the cell aggregates settle (approx 10 min) and then discard the medium and replace with hEB medium.
7. Plate the cell suspension out in new Petri dishes.

8. Forty-eight hours later, gelatin coat the wells/dishes that the hEBs are to be plated out on. Add sufficient gelatin solution to cover the plastic surface and leave at room temperature for at least 1 h. Immediately before use, aspirate the gelatin solution (leaving a thin film of gelatin) and replace with medium (*see* **Note 11**).
9. Collect the hEBs and transfer to a 50-mL tube and let settle (approx 10 min). Discard the medium and wash the hEBs in 10 mL PBS. Let the hEBs settle and then remove the PBS.
10. Add 0.5 mL prewarmed TEG and leave at room temperature for approx 5 min. Triturate the hEBs until they are disaggregated.
11. Resuspend in hEB medium and then seed on gelatin-coated plastic at approx 1×10^5 cells/mL. If the hEBs do not look big and mature enough on d 4, then, rather than disaggregating them, they can have the medium changed and left for a further 24 h before they are disaggregated.

3.8. Osteogenic Differentiation of hES Cells

1. Precoat the required number of plates with gelatin solution (*see* **Subheading 3.7., step 8**). A 96-well plate will be required for the calcium assay and 2 × 48-well plates for the Von Kossa and Alizarin red staining, respectively.
2. Plate out cells from dissociated hEBS at 1×10^5 cells/mL (*see* **Subheading 3.7., step 11**) as follows: for a 96-well plate, 100 µL cell suspension/well and for the 48-well plates, 250 µL cell suspension/well.
3. Incubate overnight in a humidified incubator with 5% CO_2 at 37°C.
4. Remove medium and replace with OS-supplemented hEB medium for test wells (OS+) and normal medium (OS–) for control wells. For a 96-well plate, add 200 µL/well, for a 48-well plate add 400 µL/well. Do not add OS-supplemented medium to the d 0 time points. Instead, these wells are fixed as described in **Subheading 3.8.1., steps 1–5**.
5. Replace medium every 3 d.
6. At each time point, fix three wells for OS+ and OS– treatments as outlined in **Subheading 3.8.1., steps 1–5**. Suggested time points are 0, 2, 4, 7, 10, 15, and 20 d.

3.8.1. Von Kossa Staining

1. Remove medium from the designated wells at each time point.
2. Wash twice with calcium and magnesium free PBS.
3. Fix with 200 µL 95% methanol for 15 min at room temperature.
4. Remove methanol and add 400 µL PBS.
5. Leave at 37°C until final time point.
6. After fixing the final time point, all time points are stained together (**steps 6–11**; *see* **Note 12**). Remove PBS and wash once with H_2O.
7. Add 200 µL 2% silver nitrate.
8. Leave under 70-W lamp for 1 h.
9. Wash once with H_2O.
10. Stop with 200 µL 5% Na thiosulphate for 5 min.
11. Wash with H_2O. Photograph before removing H_2O. Scan whole plate after wells have been allowed to dry.

3.8.2. Alizarin Red Staining

1. Fix as described in **Subheading 3.8.1., steps 1–6**.
2. Add 200 µL Alizarin red solution and leave for 5 min at room temperature.
3. Wash carefully three times with dH_2O.
4. Photograph soon after staining because color will fade with time. Plate can be scanned after wells have been allowed to dry.

3.8.3. Calcium Assay (see ***Note 13***)

1. Remove medium.
2. Wash wells three times with 0.9% NaCl.
3. Leave wells to dry out at 37°C.
4. To lyse cells add 50 µL 0.1 *M* NaOH and leave for 2–3 h.
5. After cells are lysed, neutralize with 20 µL 2 *N* HCl.
6. Dry plates in incubator at 60°C.
7. After plates are dried, they can be stored at room temperature until ready to perform calcium assay.
8. Perform analysis by the calcium colorimetric kit as per the manufacturer's instructions.
9. Prepare the calcium standard curve and optimize. Analyze wells in triplicate. The kit requires optimization for this protocol.
10. Read resulting color end point at 575 nm on a plate reader and calculate values using a standard curve.

4. Notes

1. Freezing solutions should be made fresh whenever possible. If necessary, they may be aliquoted and frozen at −20°C, but must be thawed only once.
2. Matrigel matrix will gel rapidly above 20°C and may occasionally gel in the fridge if the temperature is raised slightly. Keep on ice during preparation and use precooled pipets.
3. For a 25-cm^2 flask of hES cells, add approx 6 mL conditioned medium; a 75-cm^2 flask approx 20 mL; and a 150-cm^2 flask approx 35 mL. For a well of a 6-well plate use 3 mL and for a 10-cm plate approx 10 mL.
4. Human ES cells require passaging at (or just before) confluence and must be fed daily.
5. Human ES cell seeding rates are approx $2.5–5 \times 10^4$ cells per cm^2.
6. Incubation of hES cells in hypotonic solution longer than 10 min is not advised.
7. It is very important that addition of fixative is done carefully and slowly. The cells should be agitated gently on a vortex mixer while the first 2–3 mL of fixative is added *slowly*, dropwise, down the side of the tube. Adding the first fix is the single most important step.
8. Hold the slide at an angle as you drop the suspension onto it.
9. The time of exposure to trypsin is important. If left too long, the trypsin will overdigest the preps, and it is best to assess slides one at a time until the optimal period of exposure can be determined.

10. Tissue culture plastic must not be used for EBs because aggregates will attach to the surface and fail to form a suspension culture.
11. If embryoid bodies are small on d 4, they may be left a further 24 h before disaggregation.
12. **Steps 6–11** must be done very gently to prevent loss of cells, which are easily dislodged.
13. The purpose of the calcium assay is to determine the extent of matrix-associated mineralization after osteogenic treatment.

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7

Culture of Human Embryonic Stem Cells on Human and Mouse Feeder Cells

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Summary

This chapter describes the methods we use to maintain and expand undifferentiated human embryonic stem (hES) cells on human and mouse feeder cells. All of the available hES cells have been derived and propagated on primary mouse embryonic fibroblasts as feeder cells that have been mitotically inactivated. We found that hES cells can be successfully cultured on selected human feeder cells, such as marrow stromal cells derived from adult bone marrow and breast skin fibroblasts. Detailed protocols to use human and mouse feeder cells are described here, together with our method to split hES cells by trypsin/ethylenediaminetetraacetic acid-mediated dissociation. We also describe methods we use to characterize hES cells expanded on either human or mouse feeder cells, including alkaline phosphatase staining, immunostaining for cell-surface markers associated with undifferentiated hES cells, and teratoma formation in mice.

Key Words: Embryonic stem cells; human stem cells; mesenchymal stem cells; marrow stromal cells; MSCs; feeder cells; fibroblasts; immortalization; self-renewal; pluripotency; teratoma.

1. Introduction

All of the available human embryonic stem (hES) cells at this time have been derived and propagated on primary mouse embryonic fibroblasts (pMEFs) as feeder cells that have been mitotically inactivated *(1,2)*. Preparation of pMEFs from embryos is relatively easy; however, it must be done often because pMEFs after three to five passages display a reduction in proliferative rate and hES cell supportive activity. An additional disadvantage is that pMEFs may transmit known and unknown rodent pathogens to hES cells in coculture. We have shown that hES cells can be successfully cultured on selected human feed-

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er cells, such as marrow stromal cells (MSCs) derived from adult bone marrow *(3)*. Other studies showed that selected human feeders derived from tissues such as fallopian tubes, fetal muscle or fetal skin *(4)*, newborn foreskin *(5,6)*, and other cell types *(7)* can also maintain hES cells in an undifferentiated state. More recently, we identified a panel of postnatal human fibroblasts that are either capable or incapable of supporting undifferentiated hES cells over many passages *(8)*. In addition to MSCs, we found that normal fibroblasts from breast skin (ccd-1087sk) can also maintain the growth and pluripotency of hES cells in culture. The ccd-1087sk cells were subsequently immortalized by enforced expression of the human telomerase reverse transcriptase (*hTERT*) gene to produce a sustainable supply of feeder cells. The untransduced parental cells start to senesce at about passage 31, whereas the hTERT-transduced cells retained the same growth rate beyond 42 passages. We call the immortalized ccd-1087sk cells human adult fibroblasts, immortalized (HAFi). The HAFi cells are karyotypically normal and nontransformed. More important, the HAFi cells support the growth of hES cells at least as efficiently as pMEFs. In comparison, hTERT-transduced MSCs often grew faster, became sensitive to radiation (used to make mitotically arrested cells), and appeared transformed (thus discontinued).

In this chapter, we describe the routine culture of MSCs and HAFi cells and the culture of hES cells on human feeder cells in comparison with pMEFs. This system will be useful in maintaining hES cells more reliably by reducing variation from feeders and in determining the factors produced by the feeder cells and their interaction with hES cells.

2. Materials

2.1. Tissue Culture

1. Dulbecco's modified Eagle's medium (DMEM), high glucose (Invitrogen, Grand Island, NY; cat. no. 11995-065).
2. Minimum essential media with Earle's salts (Invitrogen; cat. no. 11095-080).
3. DMEM, low glucose (Invitrogen; cat. no. 11885-084) for MSCs.
4. Knockout D-MEM: optimized D-MEM for ES cells (Invitrogen; cat. no. 10829-018).
5. Fetal bovine serum, heat inactivated (Gemini Bioproducts, Woodland, CA; cat. no. 100-106) or (Hyclone, Logan, UT; cat. no. SH30071.03).
6. Na-pyruvate 100 m*M* (100X) in MEM (Invitrogen; cat. no. 11360-070).
7. Knockout serum replacement (Invitrogen; cat. no. 10828-028).
8. L-glutamine (200 m*M*) (Invitrogen; cat. no. 25030-081).
9. MEM nonessential amino acids (10 m*M*) (100X) (Invitrogen; cat. no. 11140-050).
10. 1X phosphate-buffered saline (PBS) Ca^{+2} and Mg^{+2} free (Invitrogen; cat. no. 14190-144).
11. β-mercaptoethanol (EM Science, Gibbstown, NJ; cat. no. EM-6010).
12. Penicillin-streptomycin (100X) (Invitrogen; cat. no. 15140-122).
13. Antibiotic-antimycotic solution (100X) (Invitrogen; cat. no. 15240-062) for MSCs.

14. Basic fibroblast growth factor (bFGF) (Peprotech, Rocky Hill, NJ; cat. no. 100-18B).
15. 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA) (Invitrogen; cat. no. 25300-054).
16. Trypsin inhibitor type I-S from soybean (Sigma, St. Louis, MO; cat. no. T-6522): used at 0.5 mg/mL in hES medium, filter-sterilized with Steriflip (Millipore, Billerica, MA; cat. no. SCGP00525).
17. Stericup (Millipore; cat. no. SCGPU11RE).
18. Trypan blue 0.4% solution (Invitrogen; cat. no. 15250-061).
19. Sigma FAST 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) (Sigma; cat. no. B-5655).
20. Mouse anti-human stage-specific mouse embryonic antigen (SSEA)-4 (mIgG3) antibody (Developmental Studies Hybridoma Bank, Iowa City, IA; Clone: MC-813-70).
21. Mouse anti-human TRA-1-60 (mIgM) antibody (Chemicon; cat. no. MAB4360).
22. Mouse anti-human TRA-1-81 (mIgM) antibody (Chemicon; cat. no. MAB4381).
23. Matrigel™ matrix (Becton Dickinson [BD], Bedford, MA; cat. no. 354234).
24. Polybrene (Sigma, cat. no. S2667).
25. Hygromycin B (Roche Diagnostics Corporation, Indianapolis, IN; cat. no. 843 555).
26. 15-mL (Sarstedt Inc., Newton, NC; cat. no. 62.554.002) and 50-mL (Sarstedt; cat. no. 62.547.004) centrifuge tubes.
27. 10% buffered formalin (Fischer Scientific, Fairlawn, NJ; cat. no. SF 100-4).
28. Trizma (Tris[hydroxymethyl]aminomethane) (Sigma; cat. no. T-6066).
29. Magnesium chloride hexahydrate (EM Science; cat. no. MX0045-2).
30. Bovine serum albumin (Fraction V) (Rockland Immunochemicals Inc., Gilbertsville, PA; cat. no. BSA-50).
31. Human immunoglobulin (Ig)G (to block nonspecific binding of IgG): Gamunex (10% human IgG) solution purchased from a pharmacy; made by Bayer Corporation (Pittsburgh, PA). Dilute 50 times to reach a working concentration 2 mg/mL.
32. Six-well tissue culture plate (BD; cat. no. 353046).
33. 24-well tissue culture plate (BD; cat. no. 353047).
34. Sterile Hank's balanced salt solution (HBSS) without phenol red (Invitrogen; cat. no. 14025–092).
35. 25-cm cell scrapers (Sarstedt; cat. no. 83-1830).
36. 1-mL sterile syringes (BD; cat. no. 309628).
37. Sterile 25-gage needle (BD; cat. no. 305122).
38. Gelatin (Sigma; cat. no. G-2500). Prepare 0.1% gelatin solution: add 1 g of gelatin to a 1-L capacity sterile glass bottle. Make up to 1 L with deionized water. Sterilize by autoclave solution at 20 psi for 15 min or using the liquid cycle of the autoclave. Allow to cool before using to gelatinize plates (*see* **Note 1**).
39. Medium for undifferentiated hES cells. For 1 L: 800 mL Knockout DMEM, 200 mL knockout serum replacement, 10 mL 100X MEM nonessential amino acids, 10 mL 200 m*M* L-glutamine, 0.1 m*M* β-mercaptoethanol (*see* **Note 2**), and 4 ng/mL of bFGF (*see* **Notes 3** and **4**).

40. Medium for mouse embryonic fibroblast (MEF) cells. For 1 L: 900 mL DMEM, high glucose, 100 mL fetal bovine serum (FBS), 10 mL 100X nonessential amino acids.
41. Media for human MSCs. For 1 L: 900 mL DMEM low glucose, 100 mL FBS, 10 mL 100X antibiotic-antimycotic solution, and 1 ng/mL bFGF (*see* **Note 3**).
42. Medium for CCD-1087sk cells and HAFi cells. For 1 L: 900 mL minimum essential medium with Earle's salts, 100 mL FBS, 10 mL 200 m*M* L-glutamine, 10 mL 100X Na-Pyruvate, 10 mL of 100X nonessential amino acids, and 10 mL 100X penicillin-streptomycin.

2.2. Cells

1. hES cells: hES cell line H1 (Wi-Cell, Madison, WI; NIH code WA01).
2. pMEF feeder cells: untreated Hygromycin resistant (Specialty Media, Inc., Phillipsburg, NJ; cat. no. PMEF-HL) or mitomycin C-treated (Speciality Media; cat. no. PMEF-CF [mitomycin-treated]) (*see* **Note 5**).
3. Normal breast feeder cells: CCD-1087sk (American Type Culture Collection, Manassas, VA; cat. no. CRL-2104).
4. Bone marrow mononuclear cells (AllCells; LLC, Berkeley California; cat. no. ABM010F). MSCs were derived from adult bone marrow as given below (*see* **Subheading 3.2.**).

2.3. Retroviral Vector

1. pBabe-hTERT-hygro: the retroviral vector was generously provided by Dr. Robert Weinberg at Whitehead Institute, MIT. The retroviruses were generated from PG13 packaging cells.

2.4. Immunocompromised Mice

1. Non-obese diabetic-severe combined immunodeficient (NOD-SCID) mice (Taconic, Germantown, NY; cat. no. NODSC-M) or the SCID-beige mouse (Taconic, cat. no. CBSCBG-MM).

3. Methods

3.1. Preparation of pMEF Feeder Layers

1. Thaw a frozen vial of MEF cells quickly in a 37°C water bath (*see* **Note 6**).
2. Transfer the cells into a 15-mL centrifuge tube. Add 10 mL of MEF media slowly and dropwise to minimize osmotic shock. Intermittently, shake the tube while adding media. Resuspend the cells gently in 10 mL MEF medium by pipetting up and down.
3. Centrifuge at 230*g* to pellet the cells. Aspirate out the supernatant.
4. Tap the tube on the palm of your hand to disperse the cells in the residual media left behind in the tube.

5. In the case of untreated pMEFs, add 5 mL MEF media to the tube and resuspend the cells gently by pipetting up and down.
6. Irradiate the cells at 50 Gy using γ-irradiation. After irradiation, centrifuge the cells, aspirate supernatant, and resuspend in 12 mL MEF medium for plating. In case of mitomycin C-treated cells, **steps 5** and **6** are not necessary; directly resuspend cells in 12 mL MEF media after performing **step 4**.
7. Plate the cells in six-well gelatinized plates (*see* **Note 7**). Place the plates in a 37°C incubator with 5% CO_2 (in air) and relative humidity set to 90%.
8. Observe the plates the next day for proper adherence of the pMEFs.
9. The cells can be used to plate the hES cells in the next 7 d. It is preferable that culture medium is changed to serum-free hES media (after 1X PBS wash), 1 d before plating the hES cells.
10. One day before seeding hES cells, add 2 mL fresh hES media/well and incubate overnight. On the next day, seed with hES cells directly into conditioned medium.

3.2. Human MSCs as Feeder Cells

1. Suspend bone marrow mononuclear cells in the MSC medium.
2. Plate the cells in 175-cm^2 flasks at a density of 6×10^7 cells/flask and culture in a 37°C incubator with 5% CO_2, and with relative humidity set to 90%.
3. Change the medium after 48 h and every 3–4 d thereafter. The cultures will reach 90% confluence in 10–14 d.
4. Once confluent, aspirate out the medium and wash once with PBS.
5. Add 3 mL 0.05% trypsin EDTA, and incubate at 37°C until the cells detach. Then stop trypsin action by adding the FBS-containing MSC media.
6. Collect the cells in a tube and centrifuge at 230*g* for 5 min.
7. Aspirate supernatant and resuspend cells in 10 mL of medium.
8. Irradiate cells at 50 Gy and resuspend in 10 mL of medium.
9. Perform a cell count using a hemocytometer and 0.4% trypan blue.
10. Plate 0.2×10^6 viable cells/well of a gelatinized six-well plate.
11. Allow cells to adhere overnight. The feeders are now ready for seeding with hES cells.

3.3. Culture of Feeder Cells Derived From Normal Breast Tissue

The ccd-1087Sk cells are cultured in appropriate media (*see* **Subheading 2.1.**) and maintained in 75-cm^2 or 175-cm^2 flasks.

1. Cells grow as a monolayer and show contact inhibition. Cells are passaged once per week at a 1:3 to 1:4 split ratio. For passing, cells are trypsinized with 0.05% trypsin EDTA. Stop trypsin action by adding complete medium containing 10% FBS.
2. Harvest cells, centrifuge at 230*g*, and discard supernatant. Resuspend in 5 mL of medium.
3. Perform a cell count using a hemocytometer and 0.4% trypan blue. We ideally seed 0.35×10^6 cells/75-cm^2 flask or 0.8×10^6 cells/175-cm^2 flask. Change media on d 4 and passage on d 7.

3.4. Immortalizing Feeder Cells

We use a retroviral vector encoding the catalytic domain of hTERT (*see* **Subheading 2.3.**) to immortalize the ccd-1087 Sk cells. The vector has a Hygromycin selection gene, which enables the selection of stably transduced cells based on resistance to Hygromycin B.

1. The viral vector is harvested as a supernatant from PG13 cells, stably producing the vector. The ccd-1087sk cells are transduced in a 75-cm^2 flask, with the vector, in presence of 8 µg/mL polybrene overnight. Cells should be 40–50% confluent for transduction.
2. The next day, aspirate the media and add fresh medium.
3. After 48 h, add 50 µg/mL Hygromycin B. Continue the Hygromycin B selection for three more days.
4. Passage the selected cells for a second round in the presence of Hygromycin B to select for stable Hygromycin B-resistant clones (*see* **Note 8**). These cells are the immortalized cells termed HAFi (**Fig. 1**) and are used to support the growth of undifferentiated H1 hES cells (**Fig. 2**).

3.5. Preparation of HAFi Feeder Layers

1. HAFi cells are cultured in 175-cm^2 flasks. Seed at a density of 0.8×10^6 cells/175-cm^2 flask.
2. Change media on d 4. At d 7, the cells are confluent and can be passaged and are used for making feeder cells.
3. Remove the medium from the confluent flasks and add 10 mL PBS to wash the cells.
4. Add 3 mL 0.05% trypsin EDTA and place the flask in the incubator until the monolayer detaches.
5. Add 6 mL complete MEM media to the flask and gently pipet the cells up and down.
6. Centrifuge, aspirate supernatant, and resuspend cells in 5 mL of medium.
7. Perform a cell count using a hemocytometer and 0.4% trypan blue.
8. Irradiate and plate cells at a density of 0.2×10^6 cells/well of a gelatinized six-well plate.
9. Allow cells to adhere overnight. The feeders are now ready for seeding with hES cells. Follow same protocol as described for hES cells plating on PMEFs.

3.6. Splitting hES Cells

1. Remove the medium from the plate containing confluent hES cells colonies and wash once with PBS (*see* **Note 9**).
2. Add 1 mL of 0.05% Trypsin/EDTA to each well of the six-well plate. Place the plate in the incubator for 3–5 min. Pipet the cells up and down with a 1-mL micropipet and 1-mL microtip to break up the cells into small clumps (*see* **Note 10**).
3. Add 1 mL soybean trypsin inhibitor (0.5 mg/mL) to each well. Pipet the cells again using a 10-mL pipet. Strain the cells through a 100-µm strainer into a 50-mL sterile collection tube (*see* **Note 11**).

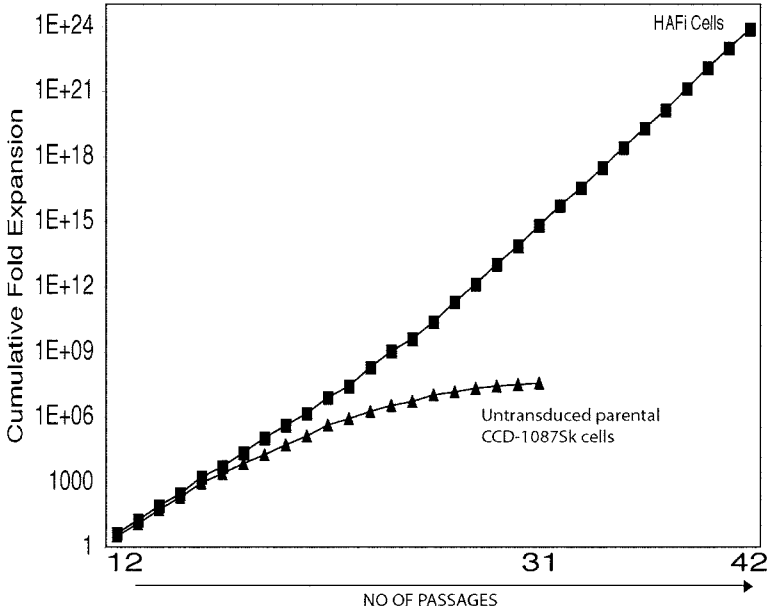

Fig 1. The cumulative fold expression of immortalized human adult fibroblasts, immortalized (HAFi) cells is shown as compared with the untransduced parental ccd-1087sk cells. The HAFi cells grow well beyond (42 passages) the untransduced cells, which senesced at passage 31.

4. Pellet cells at 230*g*; aspirate supernatant. Resuspend cells in hES medium and plate onto PMEF feeders already prepared preferably a day before.

3.7. Characterization of Undifferentiated hES Cells on HAFi Feeder Cells

hES and human embryonal carcinoma cells, which share a distinctive expression of cells surface antigens that are different from mouse ES and EC cells *(11–15)*. We typically characterize the growth of undifferentiated hES on HAFi feeder cells by; their ability to form compact colonies (**Fig. 2**), surface alkaline phosphatase staining (**Fig. 2**) *(9,10)*, and the expression of typical surface markers like SSEA-4, TRA-1-60, and TRA-1-81, known to be associated with undifferentiated hES cells (**Fig. 3**).

3.7.1. Alkaline Phosphatase Staining

1. Start with the plates containing compact colonies of hES cells on top of the HAFi feeder cells.
2. Aspirate out the medium from the wells and wash once with PBS.
3. Fix the cells with 10% buffered formalin at room temperature for 30 min.
4. Aspirate out the formalin and wash once with PBS.

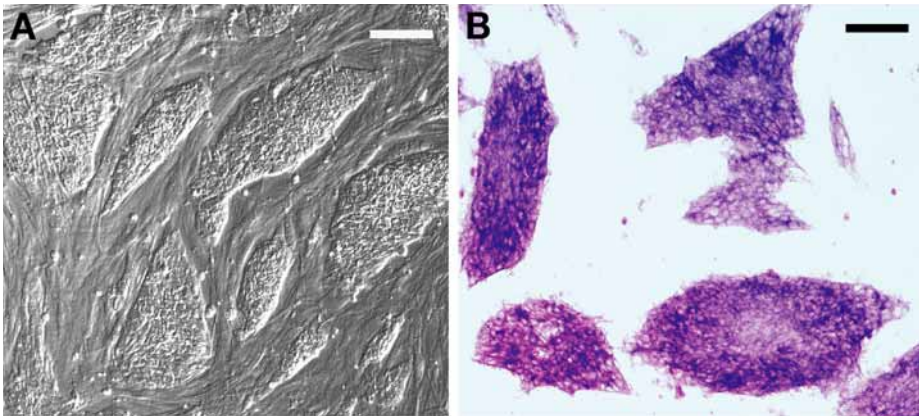

Fig 2. Compact human embryonic stem cell colonies growing on human adult fibroblasts, immortalized (HAFi) feeder layers **(A)**. Cells were fixed and surface alkaline phosphatase activity assessed using a substrate for the enzyme. Bright-field image shows surface alkaline phosphatase activity **(B)**. Bar represents 200 µm. (Please *see* the companion CD for the color version of this figure.)

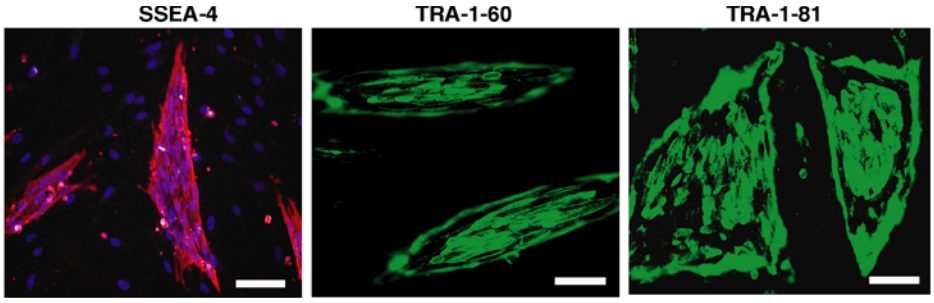

Fig 3. Human embryonic stem (hES) cells on human adult fibroblasts, immortalized (HAFi) feeders were fixed and stained for surface markers associated with undifferentiated hES cells. The illustration shows expression of stage-specific mouse embryonic antigen (SSEA)-4, TRA-1-60, and TRA-1-81 on the surface of hES cells. For SSEA-4, the nuclear staining was done using the Hoechst dye. Bar represents 200 µm. (Please *see* the companion CD for the color version of this figure.)

5. Wash the wells three times with Tris buffer solution (*see* **Note 12**).
6. Remove the Tris solution and add 1 mL/well of a six-well plate of alkaline phosphatase substrate BCIP/NBT (*see* **Note 13**). Incubate at room temperature and slowly rock the plate on a shaker (*see* **Note 14**).
7. Stop the reaction by aspirating the substrate solution and rinsing with PBS.

3.7.2. Immunostaining of Surface Antigens

1. Grow the hES cells on the HAFi feeders in 24-well plates (*see* **Note 15**). Aspirate medium and wash once with PBS at the end of the culture period.

2. Fix the cells with 4% paraformaldehyde (PFA) (*see* **Note 16**) at 4°C for 30 min.
3. Add 250 µL/well staining buffer (*see* **Note 17**) supplemented with 0.4% human IgG for 15 min to block nonspecific binding.
4. Add the SSEA-4 antibody (mIgG) (1:100 dilution) or TRA-1-60 (mIgG) (1:20 dilution or TRA-1-81 (mIgG) (1:20 dilution). Incubate at 4°C for 1 h.
5. Aspirate the antibody solution and wash three to five times with the same buffer. Add 300 µL buffer to each well after the last wash.
6. Add secondary antibodies conjugated with different fluorochromes. We have used goat anti-mouse IgG Alexa 546 (for SSEA-4) and goat anti-mouse IgM Alexa 488 (for TRA-1-60 and TRA-1-81) (*see* **Note 18**).
7. Incubate at 4°C for 1 h. Wash three to five times with buffer.
8. Observe under a fluorescence microscope using the appropriate filters.
9. Optional: we also stain cells with Hoechst 33358 (blue emission) to visualize the nucleus; it also gives a better contrast with the surface staining (*see* **Fig. 3**).

3.8. Culture of Cells on Matrigel (see Note 19)

1. Coat six-well plates with Matrigel before seeding the hES cells (*see* **Note 20**). Make 1:30 dilution (in cold ES medium) and coat the plates (1 mL/well for six-well plates). Incubate at room temperature for 1 h, aspirate diluted Matrigel, and wash once with PBS.
2. Suspend the hES cells to be plated in conditioned media (CM) from the feeder cells (mouse or human) (*see* **Note 21**).
3. Plate hES cells at the same split ratio (1:3 to 1:5) used for regular passage.
4. Plate hES cells in a volume of 2.5 mL/well (*see* **Note 22**). Replace CM every day. The hES cells, once confluent (4–5 d), can be used for injecting into mice for teratoma formation.

3.9. Teratoma Formation

The pluripotency of hES cells can be estimated by testing their ability to form teratomas in immunocompromised mouse models like the NOD-SCID or the SCID-beige mouse (*see* **Note 23**).

1. Cells to be used for teratoma formation should be passaged once onto Matrigel before harvesting (*see* **Note 24**).
2. Allow cells to grow to 80–90% confluence. At least 1×10^6 cells or more are necessary for injection to form a teratoma (*see* **Note 25**).
3. Do not remove media from well and scrape cells on the bottom of each well and gently pipet cells up and down to reduce large clumps (*see* **Note 26**).
4. Transfer cells to conical centrifuge tube, but do not pass through a strainer. You may pool wells/plates together in the same tube.
5. Spin cells at 230*g* for 5 min at 4°C.
6. Remove supernatant and resuspend cells in sterile HBSS (*see* **Note 27**).
7. Load resuspended cells into syringes and keep on ice until ready to inject (*see* **Note 28**).

8. Clean injection sites with alcohol. If necessary, remove some fur to expose site (*see* **Note 29**).
9. Examine injection site at least once per week. Teratoma may develop as quickly as 3–4 wk or it may take as long as 3–4 mo.
10. After palpable teratoma has been detected, euthanize animal for excision. Teratomas are usually quite intact and removal of outer layer of skin/fat is sufficient to expose and excise tumors. Tumors may be kept in sterile PBS for a few hours until processing.
11. Weight, size, and morphology can be noted at this time. Tumors should be placed in 4–10% formalin for 24 h before embedding and sectioning and for long-term storage (*see* **Note 30**).

4. Notes

1. Feeder cells adhere to gelatin-coated plates more efficiently. Gelatin will not dissolve in deionized water at room temperature, but will dissolve only on autoclaving. To gelatinize six-well plates, add 2 mL 0.1% gelatin solution per well, keep for 1 h at room temperature in the laminar hood, aspirate, and allow to air dry with lids open. The plates can now be seeded with feeders. They can be wrapped (in plastic wrap) and stored at 4°C for future use. We do not use gelatinized plates that are more than 4 d old.
2. Add 7 µL of β-mercaptoethanol to 10 mL PBS and add this 10 mL/L of hES medium. Make this working solution fresh each time while making the medium.
3. We make a stock of 10 µg/mL bFGF as per the manufacturer's recommendation and aliquot and store stock solution at −20°C. From this add 100 µL/1 L (final concentration 1 ng/mL) for MSC medium and 400 µL/1 L hES medium (final concentration 4 ng/mL).
4. For making the hES medium, add all the requisite volumes including bFGF into the funnel of the Stericup and filter before use.
5. The mitomycin C-treated cells are mitotically inactive and can be used directly. For the untreated cells we mitotically inactivate them by γ-irradiating at 50 Gy.
6. This will minimize the time for the cells to thaw and prevent the prolonged exposure of cells to DMSO (used in the cryopreservation, which is toxic to cells at room temperature). Follow recommended protocol for thawing. Transfer the cells to a 15-mL tube.
7. For a vial containing 5×10^6 cells, usually four six-well plates can be made. Add 2 mL/well of MEF media to the plate. For seeding cells add 0.5 mL of cell suspension per well already containing 2 mL MEF medium. After adding cells to the wells, evenly disperse the cells by moving the plate forward, backwards, and sideways. Do not swirl the plate because this will cause the cells to accumulate and subsequently concentrate more toward the center of the plate.
8. We have compared the growth of these transduced 1087SK cells with the untransduced parental line. Although the untransduced cells undergo senescence

after 31 passages, the hTERT transduced cells continue to proliferate far beyond 42 passages.

9. Add the PBS to the wells slowly by touching the pipet tip to the side walls of the well. Do not allow the PBS to stay on the cells for more than 5 min (work gently, but quickly). Aspirate the PBS and proceed for trypsinization.
10. Check cell dissociation under a microscope after 3–5 min of trypsinization. The cells should be detached. While pipetting, be gentle. The purpose is to break up the colonies into small clusters and not to obtain a single cell suspension. The human ES cells like to grow from small clumps. The cloning efficiency from single cells is extremely poor (<1%).
11. We found that straining through 100-µm strainers efficiently removes the strands of feeder cells that remain after trypsin digestion and minimizes carryover of the feeder cells. We also tried 70-µm strainers but recommend the 100-µm strainers work the best probably because they allow small clumps of hES cells to pass through, but eliminate the strands of feeders that form after trypsinization.
12. Do not use PBS. The phosphate group in PBS will inhibit alkaline phosphatase activity. Dissolve 100 m*M* Tris and 5 m*M* $MgCl_2$ in deionized water. Use this for washing, before adding the substrate.
13. The BCIP/NBT substrate is in form of preweighed tablets. Powder the contents of a single tablet and put in a 15-mL tube. Add 10 mL of deionized water to dissolve the contents by vortexing to give a straw yellow solution. Powdering the tablet helps in quick dissolving of the substrate.
14. The blue-colored reaction develops within 10–20 min. Observe the plate under microscope to check the intensity of the blue color developing. The color developing should be dark (**Fig. 2**), but not saturating. Do not allow the reaction to proceed for very long because the saturation will mask the surface staining and the colonies will appear to have a more diffused appearance. To stop the reaction, aspirate out the substrate solution completely and add PBS to the wells. You can count the numbers of alkaline phosphatase colonies or photograph them. Use bright-field (and not phase contrast) microscopy because this gives the best results.
15. In this case, we generally seed 4.5×10^4 HAFi feeders per well. For hES cells a split ratio of 1:3 to 1:5 is preferable. When colonies develop by d 3–4, proceed with fixing and staining.
16. 4% PFA should be prepared fresh or stored at −20°C. Wear a proper face mask while weighing PFA. Weigh 2 g in a 50-mL flask. Add 40 mL PBS (pH 7.4). Cover the flask and keep it on a stirrer (in a fume hood) with a magnetic needle in the flask. Turn on the heating on the stirrer to facilitate the dissolution (do not boil the solution) of PFA. You may add 0.1% phenol red as pH indicator to the PBS. The solution should be orange red in color (phenol red has a pH range of 6.8–8.4). We add few drops of 0.1 *N* NaOH to further facilitate the dissolution. The solution will turn dark pink in color. After traces of PFA have completely dissolved, add 0.1 *N* HCl to bring the pH to 7.4. The presence of phenol red indicator will help the process. Add 0.1 *N* HCl drop wise until the dark pink color changes back to the

orange red color. In case phenol red is not added, you can adjust the pH back to 7.4 with 0.1 *N* HCl using a pH meter. Make up the volume to 50 mL. We aliquot the PFA and freeze it at −20°C. Do not store at 4°C or room temperature. Use within 15 d to 1 mo.

17. Dissolve 2.5 g bovine serum albumin in 250 mL PBS; add sodium azide (NaN_3) at a final concentration of 0.09%. Make up volume to 500 mL. Store buffer at 4°C.
18. To check for background staining, the primary antibody is excluded. For a more specific control, you can use an isotype control antibody. An isotype control antibody is raised in the same animal as the primary antibody has the same isotype as the primary but is raised against either a nonbiological antigen or an antigen never found on the target cells. This gives you the nonspecific background binding of the antibody to your target cells.
19. Cells can be cultured in the absence of feeder cells on Matrigel (which is an extracellular matrix from mouse sarcoma). This eliminates most of the feeder cells from the previous passage, which we find to be better when hES cells are to be used for teratoma formation. We followed the protocol described by Xu et al. ***(16)***, with necessary modifications.
20. Avoid exposure of Matrigel to room temperature (keep on ice). Matrigel is stored regularly at −20°C. For use, transfer to 4°C to allow complete thawing. Extreme care has to be taken to work with Matrigel because it forms a gel very rapidly at room temperature. Always keep on ice during the coating procedures. To have even coating, we usually cool six-well plates (individually wrapped) in a refrigerator for 10 min before coating with Matrigel. We dilute Matrigel (1:30) in complete hES medium, instead of 1:20 as described in the original article ***(16)***.
21. The CM is collected from the irradiated or mitomycin C-treated feeders. For collecting CM, remove the medium (the original medium in which the feeders were seeded) from the feeder wells. Wash with PBS once and add 2 mL of complete hES medium/well of a six-well plate. Collect the CM the next day and replace wells with fresh medium. The CM can be collected daily for 1 wk. We discard the feeders after collecting CM for 1 wk. We add bFGF at 4 ng/mL to the collected CM, and filter-sterilize using Steriflip before use.
22. Move the plate sideways and back and forth and place in the incubator. Do not swirl the plate because the cells will gather to the center of the well.
23. We used both SCID-beige and NOD-SCID mice for teratoma formation as reported previously ***(1,2)***. We found that SCID-beige strain offers a higher rate and quicker formation of teratomas, although NOD/SCID also gave rise to teratomas.
24. Although good teratoma formation has been seen with cells harvested from both pMEF feeders and Matrigel, the latter is preferred.
25. Cells from a six-well plate per injection are usually sufficient for teratoma formation.
26. Smaller clumps are preferred as compared with a single cell suspension for injection. On the other hand, the clumps should not be very large or they will clog the needle.

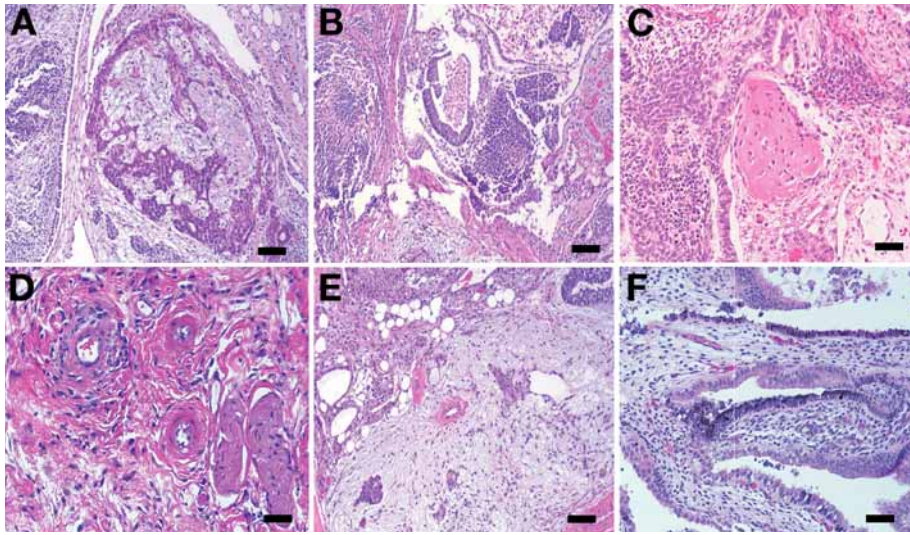

Fig 4. Teratoma obtained in SCID-beige mice from hES cells grown on human adult fibroblasts, immortalized (HAFi) feeders **(A–C)** or primary mouse embryonic fibroblasts feeders **(D–F)**. Representative sections after hematoxylin and eosin staining are shown in **(A)** connective tissue, adipose tissue, endothelium; **(B)** epithelial, gland, endothelial, and neural tissue; **(C)** connective tissue and cartilage; **(D)** smooth muscle and neural tissue; **(E)** adipose, connective, neural, and gland; and **(F)** epithelium with melanin deposition and glandular development. Bar represents 50 μm in original scale. (Please *see* the companion CD for the color version of this figure.)

27. The amount of HBSS is dependent on cell number, number of injections, and injection site. For example: six wells in 250 μL for one intramuscular injection. Usually, 250 μL per injection is good for intramuscular and testicular injection; more may be used for intraperitoneal sites.
28. Because hES cells are very sticky and likely to aggregate and precipitate, it is necessary to load each injection into a separate syringe to prevent unequal distribution of cells through multiple injections.
29. Use accepted techniques for safe injection of an animal.
30. We usually have the hematoxylin and eosin stained slides analyzed by a pathologist for identifying the different tissue types that might be present in the formed teratomas. Representative section slides after hematoxylin and eosin staining are also shown in **Fig. 4** as a reference.

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8

Maintenance of Human Embryonic Stem Cells in Animal Serum- and Feeder Layer-Free Culture Conditions

Michal Amit and Joseph Itskovitz-Eldor

Summary

The availability of human embryonic stem cells (hESCs) reflects their outstanding potential for research areas such as human developmental biology, teratology, and cell-based therapies. To allow their continuous growth as undifferentiated cells, isolation and culturing were traditionally conducted on mouse embryonic fibroblast feeder layers, using medium supplemented with fetal bovine serum. However, these conditions allow possible exposure of the cells to animal pathogens. Because both research and future clinical application require an animal-free and well-defined culture system for hESCs, these conventional conditions would prevent the use of hESCs in human therapy. This chapter describes optional culture conditions based on either animal-free or feeder-free culture methods for hESCs.

Key Words: Embryonic stem cells; foreskin fibroblasts; transforming growth factor-β1; basic fibroblast growth factor; leukemia inhibitory factor; fibronectin.

1. Introduction

When human embryonic stem cells (hESCs) were isolated for the first time by Thomson and colleagues, the culture methods used were similar to the procedures developed earlier for embryonal carcinoma cell lines or mouse ESCs *(1)*, i.e., coculture with mitotically inactivated mouse embryonic fibroblasts (MEFs) using medium supplemented with 20% fetal bovine serum (FBS). However, the potential use of hESCs for clinical purposes such as cell-based therapy requires their isolation and culture in an entirely animal-free environment. This will prevent their exposure to retroviruses or other pathogens potentially present in the mouse feeder layer or FBS. Additionally, for developing therapies based on hESCs and for using the cells as research models, a well-defined and reproducible

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system is required. The use of FBS or mouse feeder layer prevents establishing such a culture system for hESCs.

To promote the development of animal-free and well-defined culture systems for hESCs, one can culture the cells either with or without human feeder layers. Both options require animal serum substitute. The simplest alternative to FBS is human serum *(2)*, though it is not well defined and batch variation cannot be avoided. Another alternative is serum replacement when supplemented with basic fibroblast growth factor (bFGF) *(3)*. The drawback here is that it contains "Albumax," which is a lipid-enriched bovine serum albumin mixed with additional animal products and, therefore, although relatively defined, it is not animal-free.

The first animal-free culture system for hESCs was published by Richards and colleagues based on coculture with human fetal-derived feeder layers or human adult Fallopian tube-epithelial feeder layers and medium supplement with 20% human serum *(2)*. Using this culture system, hESCs can be cultured for prolonged periods while maintaining hESC features, and new hESC lines can be isolated. Foreskin fibroblasts were also found to support both the culture and derivation of hESCs using medium supplemented with either human serum or serum replacement *(4,5)*.

Foreskin fibroblast has several advantages. Unlike fetal fibroblast, which can be grown to reach a certain limited passage, foreskin fibroblast can be grown continuously for at least 6 mo while maintaining the ability to support hESCs as undifferentiated cells. Their long lifespan enables their comprehensive characterization, which is essential for hESCs' future clinical use. These feeders may therefore have an advantage when large-scale growth of hESCs is concerned. Because of their importance, great effort is being invested in the optimization of hESC culture methods and in developing alternative systems based on coculture with human feeder layers *(6)*.

In view of the large-scale and well-defined culture system required for hESC growth for clinical uses, the ideal method for growing these cells would be on matrix using serum and animal-free medium. Xu et al. were the first to demonstrate a culture system in which hESCs were cultured without direct contact with MEFs, using Matrigel matrix and 100% MEF-conditioned medium supplemented with serum replacement and bFGF *(7)*. Using this culture system, hESCs cells may still be exposed to animal pathogens through the MEF-conditioned medium. Later, Amit et al. suggested a feeder-free culture system in which no conditioned medium is used *(8)*, which is based on human fibronectin matrix and medium supplemented with serum replacement, bFGF, and transforming growth factor-β1 ($TGF_{\beta 1}$). The hESCs cultured in these conditions maintained all ESCs characteristics for more than 1 yr. Colonies of hESCs cultured under animal or feeder layer free conditions are illustrated in

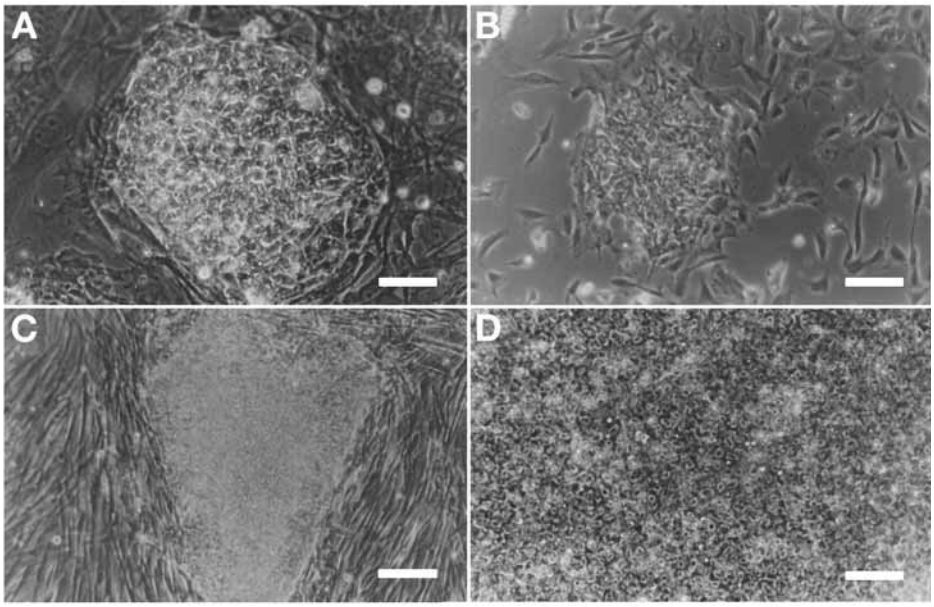

Fig. 1. Human embryonic stem cells colonies of cell line I3 cultured **(A)** with mouse embryonic fibroblasts, **(B)** with human fibronectin and medium supplemented with growth factors, **(C**,**D)** with foreskin fibroblasts. Bar: **A–C** = 50 μ*M*; **D** = 38 μ*M*.

Fig. 1. The existing feeder-free culture systems for hESCs are based on medium supplemented with serum replacement, a component that is not regarded as animal free. Whether these systems are able to support the derivation of hESC lines is yet to be seen.

2. Materials

2.1. Routine Tissue Culture

1. Freezing medium for both hESCs and foreskin fibroblasts: 60% Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA; cat. no. 41965039), 20% dimethylsulfoxide (Sigma-Aldrich Inc., St. Louis, MO; cat. no. D-2650), and 20% human serum (Chemicon International, Temecula, CA; cat. no. S1LITER) (*see* **Notes 1** and **2**).
2. Serum-free freezing medium for both hESCs and foreskin fibroblasts: 50% DMEM, 20% dimethylsulfoxide, 30% serum replacement (knockout serum replacement, SR) (Invitrogen; cat. no. 10828028) (*see* **Note 1**).
3. Splitting medium. DMEM supplemented with 1 mg/mL collagenase type IV (Invitrogen, cat. no. 17104019). For the preparation of 100 mL splitting medium: add 100 mL DMEM into a filter unit, add 100 mg collagen type IV, let set for 2 min for the powder to resolve, and filter (*see* **Note 3**).

4. Freezing box (Nalge Nunc, Rochester, NY; cat. no. 5100-0001).

2.2. Animal-Free Culturing of hESCs

1. 0.1% gelatin (type A, from porcine) (Sigma-Aldrich Inc.; cat. no. G-1890). All culture dishes should be covered with 0.1% gelatin at least 1 h before plating foreskin fibroblasts (*see* **Note 4**).
2. Foreskin fibroblast culture medium: 80% DMEM, 20% human serum, 2 m*M* L-glutamine (Invitrogen Corporation; cat. no. 25030-024) (*see* **Notes 3** and **5**). For the derivation of foreskin fibroblasts, supplement media with the addition of penicillin-streptomycin (Sigma-Aldrich Inc.; cat. no. P-3539) and kanamycin (Amresco, Solon, OH; cat. no. 0408-106).
3. hESC-medium: 80% DMEM or knockout DMEM (ko-DMEM; Invitrogen; cat. no. 10829018), 20% human serum (Sera Care Life Sciences, Inc., Oceanside, CA; cat. no. CC-5520), 1% nonessential amino acids (Invitrogen; cat. no. 11140-035), 1 m*M* L-glutamine, and 0.1 m*M* β-mercaptoethanol (Invitrogen; cat. no. 31350-010) (*see* **Note 3**).
4. hESCs serum-free medium: 85% ko-DMEM, 15% SR, 1% nonessential amino acids, 1 m*M* L-glutamine, 0.1 m*M* β-mercaptoethanol, 4 ng/mL human recombinant bFGF (Invitrogen; cat. no. 13256-029). For the preparation of 500 mL of culture medium: add 75 mL SR, 5 mL nonessential amino acids, 2.5 mL L-glutamine, 1 mL β-mercaptoethanol, 2000 ng bFGF, and 416.5 mL ko-DMEM into a filter unit and filter (*see* **Note 3**).

2.3. Feeder-Free Culturing of hESCs

1. Fibronectin (human): human foreskins fibroblast cellular fibronectin (Sigma-Aldrich Inc.; cat. no. F6277); human plasma fibronectin (Sigma-Aldrich Inc.; cat. no. F2006 or Chemicon; cat. no. FC010-10) (*see* **Note 6**). Dilute 1 mg human fibronectin in 10 mL of sterile water (Sigma-Aldrich Inc.; cat. no. W1503). Coat plates (50 µg/10-cm dish) at room temperature for at least 1 h before plating of hESCs (*see* **Note 7**).
2. Feeder-free culture medium: 85% ko-DMEM, 15% SR, 1% nonessential amino acids, 2 m*M* L-glutamine, 0.1 m*M* β-mercaptoethanol, 4 ng/mL bFGF, 0.12 ng/mL $TGF_{\beta 1}$ (R&D Systems, Minneapolis, MN; cat. no. 240-B). For the preparation of 500 mL of culture medium: add 75 mL SR, 5 mL nonessential amino acids, 2.5 mL L-glutamine, 1 mL β-mercaptoethanol, 2000 ng bFGF, 60 ng $TGF_{\beta 1}$, and 416.5 mL ko-DMEM into a filter unit and filter (*see* **Notes 3** and **8**).

3. Methods

3.1. Routine Culturing

3.1.1. Splitting hESCs

The following protocol is suitable for hESCs cultured either with foreskin fibroblasts or in feeder-free conditions using fibronectin-covered plates.

1. Remove medium from well. Add 0.5 mL (for six-well plates) splitting medium (*see* **Subheading 2.1., item 3**) and incubate for 1 h (most colonies will float).
2. Add 1 mL of culture medium and gently collect cells with a 5-mL pipet (differentiated cells will remain on the plate).
3. Collect cell suspension and place into a conical tube.
4. Centrifuge 3 min at 90*g* at a recommended temperature of 4°C.
5. Resuspend cells in media and plate directly on a ready-to-use culture plate.

3.1.2. Freezing hESCs

The following protocol can be used for hESCs cultured either with foreskin fibroblasts or in feeder-free conditions using fibronectin-covered plates. The recommended freezing ratio is 10 cm^2 per vial (one well in six-well plates).

1. Add splitting medium to plate and incubate for 1 h.
2. Add 1 mL culture medium, gently scrape the cells using a 5-mL pipet and transfer into a conical tube (*see* **Note 9**).
3. Centrifuge 3 min at 90*g* at 4°C.
4. Resuspend cells in a culture medium (*see* **Subheading 2.2., items 3** or **4**).
5. Drop by drop, add an equivalent volume of freezing medium (either **Subheading 2.1., items 1** or **2**) and mix gently (*see* **Note 10**).
6. Pour 0.5 mL into 1-mL cryogenic vials.
7. Freeze overnight at −80°C in a freezing box (*see* **Note 11**).
8. Transfer to liquid nitrogen on the following day (*see* **Note 12**).

3.1.3. Thawing hESCs

This method can be used for hESCs culture with either foreskin fibroblasts or in a feeder-free environment.

1. Remove a vial from the liquid nitrogen.
2. Gently swirl the vial in a 37°C water bath.
3. When a small pellet of frozen cell remains, wash the vial in 70% ethanol.
4. Pipet the content of the vial up and down once to mix.
5. Place the content of the vial into a conical tube and add, drop by drop, 2 mL of culture medium (*see* **Note 10**).
6. Centrifuge 3 min at 90*g* at 4°C.
7. Remove the supernatant and resuspend the cells in 2 mL of medium.
8. Place the cell suspension on one well of a six-well plate (or on a four-well plate) precovered with fibronectin (*see* **Subheading 2.3., item 1**) or foreskin fibroblasts.

3.2. Animal-Free Culturing

3.2.1. Derivation of Foreskin Fibroblast Lines (see ***Note 13***)

1. Place a human foreskin in PBS supplemented with penicillin-streptomycin (*see* **Note 14**).
2. Unfold the foreskin and wash three times with PBS.

3. Cut into small pieces using sharp Iris scissors (approximately eight pieces per foreskin).
4. Transfer the clean pieces into a new Petri dish and mince thoroughly using sharp Iris scissors.
5. Add 6 mL trypsin/ethylenediaminetetraacetic acid (EDTA) and incubate for at least 30 min.
6. Neutralize the trypsin using at least 6 mL of foreskin fibroblasts culture medium (*see* **Subheading 2.2., item 2**). Transfer the foreskin fibroblasts into conical tubes and use foreskin fibroblast culture medium to wash the plate.
7. Divide evenly into T-25 culture flasks at a recommended ratio of three pieces per flask.
8. Add 6 mL foreskin fibroblast culture medium.
9. Grow the foreskin fibroblasts until confluent culture is achieved. Change the medium as needed (do not vacuum the lumps).
10. Freeze the resultant foreskin fibroblasts to create a vial bank of the line (*see* **Subheading 3.4.**).

3.2.2. Splitting Foreskin Fibroblasts

1. Split foreskin fibroblasts every 5–7 d (*see* **Note 15**) by adding 2 mL trypsin/EDTA to cover the entire culture-flask surface.
2. Incubate for 6 min.
3. Tap the side of the flask to loosen the cells and add 4 mL of culture medium (*see* **Subheading 2.2., item 2**) to neutralize the trypsin.
4. Remove the cell suspension into a conical tube and centrifuge for 5 min at 300*g*.
5. Remove the suspension, resuspend in culture medium, and pipet to fracture the pellet.
6. Distribute the cell suspension to a desired number of culture flasks (recommended splitting ratio is 1:3).
7. Add 6 mL of foreskins fibroblast culture medium (*see* **Subheading 2.2., item 2**).

3.2.3. Freezing Foreskin Fibroblasts

1. Remove all lumps as much as possible.
2. Add 2 mL trypsin EDTA and cover the entire culture-flask surface.
3. Incubate for 6 min.
4. Tap the side of the flask to loosen the cells and add 4 mL of culture medium (*see* **Subheading 2.2., item 2**) to neutralize the trypsin.
5. Remove the cell suspension into a conical tube. Let the remaining lumps sink and remove the cell suspension into a clean conical tube.
6. Centrifuge for 5 min at 300*g*.
7. Add culture medium and pipet up and down to break the cell pellet.
8. Drop by drop, add an equivalent volume of freezing medium (*see* **Subheading 2.1., items 1** or **2**) and mix gently (*see* **Note 10**).
9. Place 1 mL into 2-mL cryogenic vials (a recommended freezing ratio of one or two vials per confluent flask).

10. Freeze the vials overnight at −80°C in a Nalgene freezing box (*see* **Note 11**).
11. Transfer the vials into a liquid nitrogen container (*see* **Note 12**).

3.2.4. Thawing Foreskin Fibroblasts

1. Remove the vial from the liquid nitrogen and quickly thaw it in a 37°C water bath.
2. When a small pellet of frozen cells remains, clean the vial using 70% ethanol.
3. Pipet the content of the vial up and down once and transfer the cells into a conical tube.
4. Drop by drop, add 2 mL of culture medium (*see* **Subheading 2.2., item 2,** and **Note 9**).
5. Centrifuge for 5 min at 300*g*.
6. Resuspend the pellet in culture medium.
7. Remove the cell suspension into culture flasks and add 6 mL culture medium.

3.2.5. Preparation of Foreskin Fibroblast-Covered Plates

1. Add 8 µg/mL mitomycin C into a culture flask and incubate for 2 h.
2. Wash four times with PBS.
3. Add 2 mL of trypsin EDTA and cover the entire culture-flask surface.
4. Incubate for 6 min.
5. Tap the side of the flask to loosen the cells and add 4 mL of culture medium (*see* **Subheading 2.2., items 3** or **4**) to neutralize the trypsin.
6. Remove the cell suspension into a conical tube.
7. Centrifuge for 5 min at 300*g*.
8. Add 10 mL of culture medium (*see* **Subheading 2.2., items 3** or **4**) and pipet up and down to break the cell pellet.
9. Count the cells and resuspend them in the desired medium volume.
10. Add the cell suspension into the culture dishes at a plating density of 4×10^5 cells per well in six-well plates (10 cm^2).
11. Let set for at least 3 h before plating the hESCs.

3.2.6. Routine Culture of hESCs With Foreskin Fibroblasts

1. Passage hESCs (*see* **Subheading 3.1.1.**) every 4–6 d directly on foreskin fibroblast-covered plates (*see* **Subheading 3.2.5.**).
2. Change the medium (*see* **Subheading 2.2., items 3** or **4**) on a daily basis.
3. Scrape differentiating colonies every five to seven passages.
4. Freeze the cells and thaw as described in **Subheadings 3.1.2.** and **3.1.3.**, respectively.

3.3. Feeder-Free Culturing

3.3.1. Routine Culture of hESCs in Feeder-Free Conditions

1. Passage hESCs (*see* **Subheading 3.1.1.**) every 4–6 d directly on fibronectin-covered plates (*see* **Subheading 2.3., item 1**).
2. Change the medium (*see* **Subheading 2.3., item 2**) on a daily basis.

3. Scrape differentiating colonies every five to seven passages.
4. Freeze the cells and thaw as described in **Subheadings 3.1.2.** and **3.1.3.**, respectively.

4. Notes

1. The freezing medium should be kept at 4–8°C for no more than 5 d.
2. If the cells are cultured for research purposes only, FBS can be used instead of human serum or serum replacement in order to reduce the costs.
3. All culture media (but those mentioned) should be kept at 4–8°C for no more than 2 wk.
4. Gelatin-coated plates can be prepared in advance and stored in a clean place at room temperature or in a 37°C incubator.
5. If a serum-free culture system is desired, human serum can be replaced with 30% SR.
6. If desirable, bovine fibronectin can be used. In this case it is highly recommended to add 1000 U/mL leukemia inhibitory factor to the culture medium (*see* **Subheading 2.3., item 2**).
7. It is recommended to leave the fibronectin in the culture plate and not to collect it before plating the hESCs. Our experience shows that this improves the plating efficiency.
8. We recommend using human recombinant growth factor as medium supplement.
9. Do not fracture the cells into small clumps.
10. Adding the medium in this stage drop by drop is highly important. If the medium is added all at once, the survival rates decrease dramatically.
11. In our experience, the use of Nalgene special freezing boxes increases cell survivability.
12. It is not recommended to leave the vials at −80°C for less than 24 h or more than a few days.
13. Foreskin fibroblasts are also commercially available (ATCC collection, for example); the commercial lines were found suitable for supporting hESC culture *(5)*.
14. Foreskin donated from adults can also be used. The tissue can be kept at 2–8°C for up to 48 h before deriving the line.
15. The crowding of the culture influences fibroblasts. The first passage after the derivation of foreskin fibroblast is done after about 20 d of culture. Well-established lines are split every 5–7 d. If the growth rate is decreased, uniting two culture flasks into one may recover the culture.

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9

Manipulation of Self-Renewal in Human Embryonic Stem Cells Through a Novel Pharmacological GSK-3 Inhibitor

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Summary

This chapter introduces a new method of maintaining human embryonic stem cells (hESCs) in the undifferentiated state through treatment with a GSK-3 inhibitor, BIO, under a feeder-free condition. Additionally, methods are introduced that determine multidifferentiation potential of hESCs by differentiating into a specific type or random heterogenous cell populations in vitro. These approaches will become a fundamental platform to identify molecular networks regulating cell fate determination in hESCs and mouse embryonic stem cells.

Key Words: GSK-3 inhibitor; human embryonic stem cells; pluripotency; BIO.

1. Introduction

Signaling molecules providing unique spatial and temporal cues to individual cells are thought to regulate organized intercellular cooperation seen in the multicellular organism. Essential roles of specific signaling pathways during embryonic development have been demonstrated in a number of animal model systems *(1–3)*. In mammals, a gene targeting approach through the generation of mutant mice has become a powerful method to understand a specific molecular function or signaling pathway regulating embryogenesis. This approach relies entirely on mouse embryonic stem cells (mESCs) derived from the inner cell mass of the blastocyst that can constitute complete individuals (pluripotency) when injected into the host blastocyst *(4–6)*. Although substantial progress has been made in understanding the role of molecular pathways regulating postimplantation development, much less attention has been paid to

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the elucidation of pluripotency unique in preimplantation embryos, mostly because of the technical difficulty. Because mESCs retain the ability to give rise to all three germ layer derivatives and germ cells in not only in vivo but also in vitro environments *(7–10)*, mESCs can be considered as an in vitro functional equivalent to the inner cell mass of the embryos. It is therefore quite relevant to investigate the genetic mechanisms underlying pluripotency by direct evaluation of mESCs as an in vitro model system. Moreover, the recent derivation of human embryonic stem cells (hESCs) now provides a window into early human embryogenesis *(11,12)*.

The determination of specific signaling pathways that regulate pluripotency during early embryogenesis particularly focusing on hESCs and mESCs has been under intense scrutiny. The leukemia inhibitory factor (LIF)/Stat3 signaling pathway is one of a few known pathways involved in self-renewal of mESCs *(7)*. However, recent studies of mutant mice in which specific components of this pathway are disrupted indicate that the LIF/Stat3 pathway is dispensable for pluripotent state early embryos *(13)*. Moreover, several studies demonstrate that LIF treatment has no or little effect on maintaining self-renewal of hESCs *(11)*. The bone morphogenic protein signaling pathway was recently reported to be sufficient to support self-renewal of mESCs through upregulation of Id genes, although this activity requires the collaboration with the LIF/Stat3 pathway *(14)*. Mutant mice or mESCs lacking both alleles of Grb2 demonstrate impairment of differentiation into the visceral endoderm lineage, whereas blastocyst stage embryos appear to be intact, suggesting an essential role of the extracellular signal-regulated kinase signaling pathway in differentiation but not for maintenance of the pluripotent state *(15)*. These studies together raise the intriguing possibility that a presently unknown signaling pathway is critically regulating self-renewal of pluripotent stem cells.

A group of genes that is specifically enriched in the undifferentiated state of hESCs has recently been determined using the Affymetrix microarray approach *(16)*. Molecular components specific for several signal transduction pathways are included in the enriched gene group. When molecular components of each signaling pathway are searched in the array data, major molecules that constitute the canonical Wnt signaling pathway are all present in the undifferentiated hESCs. Based on this result, evaluating the role of the Wnt signaling pathway in self-renewal of hESCs and mESCs became a primary focus. This lead to the discovery of a dramatic effect of a novel pharmacological GSK-3 inhibitor, 6-bromoindirubin-3′-oxime (BIO) on hESCs and mESCs, that is derived from the Mollusk's Tyrian purple *(17)*. This chemical inhibitor inactivates GSK-3 function at a micromolar level, thereby efficiently stimulating the canonical Wnt pathway while avoiding substantial inhibition of other kinases, such as CDKs. Stimulation of the canonical Wnt signaling pathway using this GSK-3 inhibitor

is sufficient to maintain self-renewal of hESCs in the feeder-free condition *(18)*. This work also suggests the possible application of small chemical compounds to regulate self-renewal of hESCs that would ultimately contribute to tissue replacement therapy.

This chapter introduces a new method of maintaining hESCs in the undifferentiated state through treatment with a GSK-3 inhibitor, BIO, under a feeder-free condition. Additionally, methods are introduced that determine multidifferentiation potential of hESCs by differentiating into a specific type or random heterogenous cell populations in vitro. These approaches will become a fundamental platform to identify molecular networks regulating cell fate determination in hESCs and mESCs.

2. Materials

2.1. Tissue Culture

1. Dulbecco's modified Eagle's medium (DMEM)/F-12 (Gibco, Carlsbad, CA; cat. no. 11320-032).
2. DMEM with D-glucose and L-glutamine, and pyridoxine hydrochloride, but no sodium pyruvate nor sodium bicarbonate (Gibco; cat. no. 12100-046).
3. α-minimum essential medium (αMEM) (Gibco; cat. no. 11900-024).
4. Knockout-DMEM medium (Gibco; cat. no. 10892-018).
5. Knockout serum replacement (Gibco; cat. no. 10828-028).
6. Fetal bovine serum (FBS) (HyClone, Logan, UT; cat. no. SH30070.03).
7. 2.0 m*M* L-glutamine (Gibco; cat. no. 25030-081).
8. 0.1 m*M* nonessential amino acids (Gibco; cat. no. 11140-050).
9. Human recombinant basic fibroblast growth factor (Gibco; cat. no. 13256-029).
10. 0.05% trypsin-ethylenediaminetetraacetic acid (Gibco; cat. no. 25300-054).
11. Dispase, lyophilized (Gibco; cat. no. 17105-041).
12. Cell dissociation buffer (Sigma, St. Louis, MO; cat. no. G5914).
13. 55 m*M* (1000X) 2-mercaptoethanol (Gibco; cat. no. 21985-023).
14. Penicillin/streptomycin (Gibco; cat. no. 15070-063).
15. Dulbecco's phosphate-buffered saline (1X) without calcium/magnesium (Gibco; cat. no. 14190-14).
16. Gelatin, Type A (Sigma; cat. no. G-1890).
17. Matrigel (BD Biosciences, San Jose, CA; cat. no. 354234).
18. NYL Filter Unit, 500-mL (Nalgene, Rochester, NY; cat. no. 151-4020).
19. 0.2-μm syringe filter (Gelman, East Hills, NY; cat. no. 4612).
20. Falcon multiwell six-well tissue culture-treated plate (BD Biosciences, San Jose, CA; cat. no. 353046).
21. 10 × 20-mm cell culture dish (Corning, Corning, NY; cat. no. 430167).
22. Falcon 14-mL polypropylene round-bottom tube (BD Biosciences; cat. no. 352059).
23. Falcon 50-mL polypropylene conical tube (BD Biosciences; cat. no. 352098).

2.1.1. Cell Lines

1. We use CJ7 and E14 mESCs lines, both of which were derived from the 129 strain of mice. Because these lines are generally used for gene targeting, they should be available from the transgenic facility in each institute.
2. Mouse stromal cells for stromal-derived induction activity (SDIA): PA6 cells (MC3T3-G2/PA6, RCB1127) can be obtained from Riken Cell Bank (Tokyo, Japan; cellbank@rtc.riken.go.jp).

2.1.2. Media

1. Mouse embryonic stem cell medium: we grow mESCs on MEFs-feeder cells or 0.1% gelatin-coated plates in knockout DMEM supplemented with 15% FBS, 100 m*M* MEM nonessential amino acids, 0.55 m*M* 2-mercaptoethanol, 1 m*M* L-glutamine, and 100 U/mL of penicillin/streptomycin. When mESCs are grown on 0.1% gelatin-coated plates, a final concentration of l400 U/mL LIF is added to the culture medium. For 1 L: mix 820 mL knockout DMEM, 150 mL FBS, 10 mL nonessential amino acids, 100 μL 2-mercaptoethanol, 10 mL L-glutamine, 10 mL penicillin/streptomycin, and 1.4 mL LIF (if required).
2. Medium for PA6 stromal cells: αMEM supplemented with 10% FBS and 50 U/mL of penicillin/streptomycin is used for the cultivation of PA6 cells. For 1 L: mix 895 mL αMEM, 100 mL FBS, and 5 mL penicillin/streptomycin.
3. SDIA-differentiation medium: knockout-DMEM supplemented with 10% KSR, 1 m*M* L-glutamine, 1% nonessential amino acids, and 0.1 m*M* 2-mercaptoethanol is used for differentiation of hESCs on PA6 feeder cells. For 1 L: mix 880 mL knockout DMEM, 100 mL KSR, 10 mL L-glutamine, 10 mL nonessential amino acids, and 100 μL 2-mercaptoethanol.

2.1.3. Establishment of mESC Reporter Lines

1. Lipofectamine 2000 (1.5-mL; Invitrogen, Carlsbad, CA; cat. no. 11668-019).
2. OptiMEM (500-mL; Invitrogen; cat. no. 31985-070).
3. G418 (50-mg/mL; Sigma; cat. no. G8168).

2.1.4. Treatment of hESCs or mESCs With GSK-3 Inhibitors

1. 6-bromoindirubin-3′-oxime (BIO) (1 mg; Calbiochem, San Diego, CA; cat. no. 361551).
2. 1-methyl-6-bromoindirubin-3′-oxime (MeBIO, a kinase inactive analog of BIO) (1 mg, Calbiochem; cat. no. 361551).

2.2. Immunocytochemistry

1. 4% paraformaldehyde (Poly Scientific, Bay Shore, NY; cat. no. s2303-32oz).
2. Wash buffer: phosphate-buffered saline (PBS) (Gibco; cat. no. 14040-133) with 0.1% bovine serum albumin (BSA) (Sigma; cat. no. B4287). For 1 L: mix 1 L PBS and 1.0 g BSA.
3. Primary antibody against Oct-4 (BD Biosciences; cat. no. 611202).

4. Primary antibody against Tuj-1 (BAbCO, Berkeley, CA; cat. no. MMS-435P).
5. Primary antibody against cytokeratin (Chemicon, San Diego, CA; cat. no. MAB1611).
6. Primary antibody against glial fibrillary acidic protein (Dako, Carpenteria, CA; cat. no. H0083).
7. Primary antibody against smooth muscle actin (Dako; cat. no. M 0851).
8. Primary antibody against α-fetoprotein (Cell Sciences, Canton, MA; cat. no. PS 076).
9. Primary antibody against Troma-1 (Developmental Studies Hybridoma Bank, Iowa City, IA).
10. Goat anti-mouse immunoglobulin G conjugated to Cy3 (Zymed Laboratories, South San Francisco, CA; cat. no. 115-166-062).
11. Goat anti-rabbit immunoglobulin G conjugated to Cy2 (Zymed Laboratories; cat. no. 111-226-045).

2.3. Luciferase Reporter Assay

1. Dual-luciferase reporter assay system (Promega, Madison, WI; cat. no. E1920).
2. Lipofectamine 2000 (Invitrogen; cat. no. 11668-027).
3. Luminometer (Berthold Technologies, Bad Wildbad, Germany; model no. Lumat LB9507).

3. Methods

3.1. Self-Renewal Monitoring Systems for hESCs and mESCs

To dissect the molecular mechanism underlying pluripotency in hESCs and mESCs, it is quite valuable to establish the in vitro system in which the self-renewing state is monitored and manipulated by exogenous factors. This is known as the hESCs feeder-free culture method, in which self-renewal of hESCs can be easily manipulated and their undifferentiated state can be briefly monitored by evaluating their morphological changes. Using this system, self-renewal of hESCs is sustained by treatment with a novel pharmacological GSK-3 inhibitor through activation of Wnt signaling. Additionally, a protocol will be introduced that establishes a mESCs reporter line used to monitor the undifferentiated state in a real-time manner.

3.1.1. Passive Differentiation of Self-Renewing hESCs Under the Feeder-Free Condition

There are numerous ways to differentiate hESCs into specific types or heterogeneous populations of cells such as retinoic acid treatment and embryoid body formation. One of the most passive and gentle methods to differentiate hESCs involves conditioned medium (CM) simply switched to non-CM (hESCs medium not exposed to MEFs) under the feeder-free condition *(**19**)*. Because this method does not require extra passaging to grow hESCs under the nonadherent condition

(for embryoid body formation) or treatment of exogenous factors (e.g., retinoic acid), it is suitable to directly evaluate differentially regulated genes between undifferentiated and differentiated hESCs without considering any environmental effects or exogenous signaling mediated transcriptional modulations. Moreover, as this system makes possible an evaluation of the undifferentiated state of hESCs through monitoring morphological changes on differentiation, factors that prevent hESCs from differentiation can be easily screened in a large-scale manner. This chapter describes the general protocol of the feeder-free culture method and gene chip analysis of human embryonic stem cells.

3.1.2. Treatment of hESCs With GSK-3 Inhibitors

Several signaling pathways, based on microarray analysis, indicate possible involvement in the support of pluripotency in hESCs *(16)*. A focus on the Wnt signaling pathway has been retained in this chapter given that all major signal transducers in this pathway are present in the self-renewing hESCs. There are several different ways to positively manipulate the canonical Wnt signaling pathway. Overexpression of positive regulators (e.g., Wnt ligands, Dishevelled, β-catenin) by plasmid transfection would be one of the most convenient methods. It may not however be the best way to activate the Wnt pathway in hESCs because of their relatively low transfection efficiency. Alternatively, the Wnt signaling pathway can be stimulated through inhibition of GSK-3 that negatively regulates β-catenin, a major signal transducer in the Wnt pathway. BIO, a novel GSK-3 inhibitor found in Tyrian purple, was recently discovered through a screening of a number of kinase inhibitors. BIO efficiently inactivates GSK-3 function at a micromolar level while its affinity to other kinases remains minimum (**Table 1**) *(20)*. BIO activates Wnt signaling in hESCs in the feeder-free system as described previously. hESCs treated with BIO have been found to self-renew even in the absence of CM *(18)*.

To use BIO for supporting self-renewal of hESCs under the feeder-free condition:

1. Plate hESCs on Matrigel-coated 6- or 12-well plates in CM. Approximately 20 (for a 12-well plate) or 40 (for a 6-well plate) hESCs colonies per each well are enough to evaluate hESCs differentiation (*see* **Note 1**).
2. Incubate them in a humidified CO_2 incubator more than 12 h to allow them to adhere to Matrigel substrate.
3. 24 h after plating, CM is switched to non-CM containing GSK-3 inhibitors at appropriate concentrations (*see* **Note 2**). Medium containing BIO should be replaced every other day.

The effect of BIO can be briefly monitored by the morphological change of hESCs. Usually 1–2 d after BIO treatment, hESCs show slightly flattened morphology as compared with hESCs in CM, although they are less flattened

Table 1
Selectivity of Indirubin Derivatives

	GSK-3α/β	CDK1/CyclinB	CDK5/p25
Indirubin	1.000	10.000	10.000
6-bromoindirubin-3′-oxime (BIO)	0.005	0.320	0.083
1-methyl-6-bromoindirubin-3′-oxime (MeBIO)	44.000	55.000	>100.000
			IC_{50} (μ*M*)

than hESCs grown in non-CM. About 3–4 d after BIO treatment, hESC colonies clearly demonstrate compact and tight morphology similar to that observed in undifferentiated hESCs. During this time, majority of hESCs colonies grown in non-CM show prominently flattened and large cell shape typical for differentiated hESCs (*see* **Note 3**). The differentiated state of hESCs can be further determined by immunocytochemistry using Oct-3/4-specific antibody, or reverse transcription polymerase chain reaction with primers for pluripotent state-specific genes (Oct-3/4, Rex-1, Nanog).

We usually grow hESCs in non-CM containing BIO for 5 d or up to 7 d (**Fig. 1**). Beyond this period, hESCs treated with BIO start showing the sign of differentiation as hESCs grown in CM cultured for a long period. In some cases, a small population of hESCs colonies demonstrates fatigue or apoptotic features probably from BIO-mediated nonspecific CDKs inhibition, although using the lowest effective concentration of BIO can minimize it. BIO-treated hESCs can be passaged for several times under the feeder-free condition using the regular passaging procedure described in the chapter, Gene Chip Analysis of Human Embryonic Stem Cells (*see* **Note 4**).

3.1.3. Establishment of mESC Reporter Lines

Although we primarily focus on hESCs as a model system to investigate the molecular mechanism of pluripotency, we also work on mESCs to understand the genetic networks conserved or distinctive between the two species. In addition, it should be noted that maintenance of mESCs is less labor intensive than that of hESCs, and the genetic manipulation is relatively easier in mESCs. More importantly, because pluripotency can be only tested in the in vivo system through generation of a complete individual, mESCs are the best model system for this rigorous experiment while hESCs are not applicable. We here describe a protocol to establish a mESCs reporter line that expresses a marker gene (GFP) driven by a pluripotent-specific gene (Rex-1) promoter/enhancer (***18,21***). Using the mESCs reporter line is one of the powerful approaches to monitor the undifferentiated state of mESCs in a real-time manner.

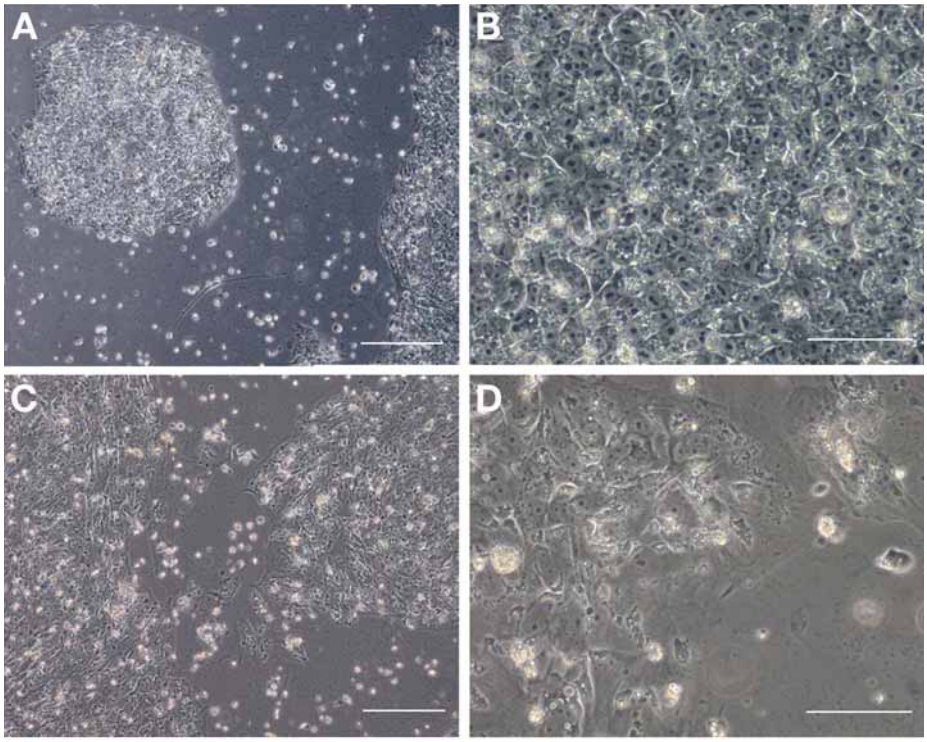

Fig. 1. Human embryonic stem cells (hESCs) (H1 line) are treated with 2 μ*M* 6-bromoindirubin-3′-oxime (BIO) or 1-methyl-6-bromoindirubin-3′-oxime (MeBIO) in nonconditioned medium for 5 d under the feeder-free condition. hESCs treated with BIO maintain compact cell morphology typically seen in the undifferentiated cells **(A**,**B)**, whereas MeBIO-treated hESCs show flattened morphology similar to that of differentiated hESCs grown in nonconditioned medium **(C**,**D)**. Scale bars: **(A**,**C)** 100 μ*M*; **(B**,**D)** 50 μ*M*. (Please *see* the companion CD for the color version of this figure.)

1. Grow mESCs on either MEFs or gelatin-coated dishes until reaching subconfluency.
2. Transfect the plasmid containing the drug resistant gene (e.g., neo, hygro, puro) by using a commercially available transfection method according to the manufacturer's protocol (e.g., Lipofectamine, Fugene, Exgen) (*see* **Note 5**). If the reporter plasmid does not contain any drug selection markers, cotransfect the reporter plasmid with a plasmid containing a drug resistant gene.
3. One day after transfection, harvest the cells by trypsinization, and replate on gelatin-coated six-well plate at subcloning density (*see* **Note 6**).
4. One day after replating, start adding the selection drug at a concentration optimal for selecting cells (*see* **Note 7**).
5. When drug-resistant mESCs form distinct colonies, each colony can be evaluated by fluorescent microscope to choose colonies consisting of uniformly GFP-positive mESCs.

6. Pick up GFP-positive colonies using P20 Pipetman. Separate the colony from surrounding MEFs by carefully scratching colony-MEFs border with the P20 Pipetman under the inverted microscope. Then, gently push the bottom of the colony with the P20 to remove the colony from the surface. Aspirate the removed colony using the P20 and transfer it into 1 well of a 96-well plate filled with 70 µL trypsin (*see* **Note 8**).
7. After picking up 10–20 GFP-positive colonies, place the 96-well plate in a humidified 37°C CO_2 incubator for 5 min.
8. Gently dissociate the colony by pipetting up and down using a P200, and add 100 µL of mESC medium to neutralize the trypsin activity.
9. Transfer the entire contents in each well onto one well of gelatin- or drug-resistant MEF-coated six-well plates filled with 2 mL mESCs medium, and gently rock the plate back and forth, and side to side to evenly distribute the mESCs.
10. When the plated mESCs become distinct colonies, colonies are harvested and further expanded to make enough frozen stocks (*see* **Note 9**).

3.2. Functional Evaluation of the Multidifferentiation Potential of hESCs

Because pluripotency is a functional definition, it is important to test the differentiation potential of self-renewing hESCs by either in vivo or in vitro experiment. The following methods can induce a cell type (neuron)-specific or random heterogeneous differentiation from undifferentiated hESCs as in vitro evaluation system. Immunocytochemistry, Western analysis, or reverse transcription polymerase chain reaction using tissue specific molecular markers should determine the differentiated tissues generated by these methods.

3.2.1. Induction of Neurons by the Stromal-Derived Induction Activity Method

Recent studies report that mESCs or nonhuman primate ES cells can be specifically differentiated into neurons by growing them on PA6 stromal feeder cells *(**22**,**23**)*. This method was applied for induction of neurogenesis from hESCs, efficiently generating neuronal cell lineage after 3 wk of culture.

1. Plate PA6 cells on gelatin-coated 12-well plates to form a confluent monolayer.
2. One day after plating PA6 cells, plate hESCs colonies (100–200 cells/clump, approx 5 clumps/well of a 12-well plate) on each well in hESCs medium (*see* **Note 10**).
3. Twenty-four hours after plating hESCs, replace the medium with stromal-derived induction activity (SDIA)-differentiation medium. Initially replace the medium with fresh SDIA-differentiation medium every other day. When hESCs colonies become larger, they should be fed with fresh medium every day (*see* **Note 11**).
4. Approximately 3 wk after starting culture, hESCs generate substantial number of neurons on PA6 feeder cells. Because there is no obvious morphological sign of neurogenesis detectable under the phase contrast microscope, immunocytochemistry

using the neuron-specific antibody (e.g., Tuj-1) is required to visualize neurogenesis derived form hESCs.

3.2.2. Embryoid Body-Mediated Random Differentiation of hESCs

Embryoid body (EB) formation is a classical method to induce differentiation of mESCs and hESCs. Because this method leads to random cell differentiation, including three germ layer-derived somatic tissues and germ cells, it is suitable to examine multidifferentiation function of pluripotent cell types. After EB formation, we grow them under the adherence condition to further differentiate into mature cell types.

1. Harvest hESCs using Dispase treatment, and plate hESCs at approx 2×10^3 clumps/cm^2 (100–200 cells/clump) on nontissue culture treated dishes to induce EB formation.
2. Grow EBs in hESCs medium (non-CM) for 7 d.
3. After 7 d of EB differentiation, plate EBs on collagen-coated 12-well plates in MEFs medium (DMEM supplemented with 10% FBS) to allow them to adhere to the surface and further differentiate.
4. After another 7 d of culture, fix them in 4% paraformaldehyde, and evaluate by immunocytochemical analysis using tissue specific antibodies (*see* **Note 12**). Alternatively, cells are harvested, and subjected to Western blot or reverse transcription polymerase chain reaction analysis.

3.3. Molecular Analysis of Differentiated Cells

When hESCs or mESCs are manipulated to produce differentiated cells, tissue-specific molecular markers should be examined to confirm the lineage of generated cells. Immunocytochemistry is a convenient, yet powerful, approach to determine the differentiated cell type because the specific molecular signal and cell morphology can be simultaneously analyzed at high resolution. To further molecularly dissect the role of a signaling pathway in the cell fate determination, the luciferase assay can be used as a routine technique. This method quantifies the activity of the reporter construct driven by the tissue specific promoter (e.g., Rex-1 or Oct-3/4 for the undifferentiated hESCs or mESCs ***[13]***, Gata4 or Gata6 for early endodermal differentiation ***[24]***, Tα-1 for neuronal differentiation ***[25]***) under the conditions in which the activity of the specific signaling pathway is altered.

3.3.1. Immunocytochemistry

We usually use fluorescence-conjugated secondary antibodies to locate the signal recognized by the primary antibody. Other methods including immunoperoxidase can be also used to visualize the signal when the autofluorescence level of the sample is too high.

1. Aspirate the culture medium from hESCs or mESCs.
2. Fix cells in 4% paraformaldehyde for 15 min.
3. Wash cells with PBS/0.1% BSA for 5 min, three times.
4. If the antigen to be detected is cytoplasmic (e.g., intermediate filaments) or in the nucleus (e.g., transcription factors), permeabilize cells with 0.3% Triton X-100 in PBS/0.1% BSA containing 10% serum for 45 min (*see* **Note 13**). If the antigen is on the cell surface, skip this step because this treatment will severely disrupt surface antigens.
5. Add an appropriate concentration of a primary antibody dissolved in PBS/0.1% BSA/10% serum onto each well, and incubate for 1–3 h at room temperature or overnight at 4°C.
6. Wash cells with PBS/0.1% BSA for 5 min, three times.
7. Add an appropriate concentration of a fluorescence-conjugated secondary antibody dissolved in PBS/0.1% BSA/10% serum onto each well, and incubate for 45 min at room temperature (*see* **Note 14**).
8. Wash cells with PBS/0.1% BSA for 5 min, three times.
9. Observe the sample under fluorescent microscope.

3.3.2. Luciferase Reporter Assay

A dual luciferase reporter assay system is used because the experimental reporter activity (firefly luciferase) can be easily standardized by the internal control Renilla reporter activity (Renilla luciferase) in a single sample in the same tube.

1. Plate cells (mESCs; 5000 cells/cm^2 or hESCs; approx 50 cells/clump, 100 clumps/cm^2) on 24-well plates.
2. Transfect the firefly or Renilla reporter plasmid (mESCs; 100 ng or 10 ng per well, respectively, or hESCs; 500 ng or 20 ng per well, respectively) and specific effector constructs (100–500 ng) in triplicate by using Lipofectamine 2000 according to the manufacturer's protocol.
3. Forty-eight hours after transfection, aspirate the medium and add 100 µL of 1X passive lysis buffer to each well.
4. Gently shake the plate on an orbital shaker for 15 min.
5. Harvest the cell lysate by using a scraper and transfer it into 1.5-mL Eppendorf tubes.
6. Mix 10 µL of the lysate with 100 µL of Stop & Glo Buffer in a glass tube.
7. Measure the luciferase activity using a Lumat luminometer.
8. Standardize the reporter activity by the Renilla reporter activity.

4. Notes

1. Too many colonies in the same well would attach together during the culture period and tend to delay the differentiation process.
2. The optimal concentration of the GSK-3 inhibitor (BIO) should be predetermined for each hESCs line by testing different concentrations of BIO, generally ranging

from 1 to 5 μ*M*. In our experience, hESCs growing in shorter doubling time require relatively lower concentration of BIO. Although BIO is exceptionally highly specific to GSK-3, other kinases, including CDKs, can be also affected by BIO treatment. It is therefore critical to find out a minimal concentration of BIO that can maintain each hESCs line in the undifferentiated state to avoid any significant effect on their viability or growth rate.

3. Some hESCs at the margin of the colony may start migrating and spreading out from the colony. Generally, hESCs in smaller colonies exhibit morphological change faster and clearer than hESCs in larger colonies, which sometimes tend to remain undifferentiated even after 5 d of differentiation period.
4. We find that after two to three passages, BIO-treated hESCs tend to reduce their growth rate and self-renewal function. We are establishing the passaging protocol that maximizes BIO-mediated self-renewal in hESCs through combining different types of chemical compound to reduce cytotoxicity of BIO. Although it is quite challenging to replace biological Wnt activity simply with the synthetic chemical compound without affecting any cell functions, we believe that developing this method is one of the first steps to provide self-renewing hESCs toward therapeutic applications.
5. For instance, in case of Lipofectamine, 5 μg of the reporter plasmid or 10 μL of Lipofectamine is dissolved in 250 μL of OptiMEM in separate Falcon 2095 tubes and incubated for 5 min. Then, mix these contents together and incubate another 20 min. Add the mixture onto mESCs in one well of a six-well plate and gently swirl the culture vessel to mix the content with the culture medium.
6. If the MESCs tend to differenciate under the drug selection conditon, MESCs should be plated on mutant mice-derived MEFs resistant to the drug used for the selection.
7. It is highly recommended to predetermine the optimal concentration of the drug to completely kill the parental nontransfected cells by 5–7 d after treatment. Timing or robustness of cell death after drug treatment is highly variable depending on types and concentrations of drugs to be used. Generally, neomycin is used at concentrations ranging of 80–200 μg/mL. Cells start dying as early as 2–4 d after treatment. In case of Puromycin, we use at 0.5–2 μg/mL. Cell death is visible as early as 1 d after treatment.
8. It is recommended to use a laminar flow hood equipped with a dissecting microscope to avoid any airborne contamination.
9. During further passaging, mESCs in several colonies sometimes start losing GFP expression even in the undifferentiated state under the drug selection. When it occurs, repeat the cloning process shown above to maintain uniformly GFP-positive mESC colonies.
10. Too many colonies in the same well may obscure the border between each colony because hESCs grow during a long period (3 wk to complete neurogenesis in hESCs).
11. After around 2 wk of the culture period, PA6 cells tend to shrink and curl up from the culture vessel. To prevent coming off of the entire hESCs colonies and feeder cells, it is important to replace the medium as gentle as possible. Pour the medium

slowly while attaching the tip of the pipet to the vessel wall. Never flash the medium directly onto the cell surface.

12. Here is the example of tissue-specific antibodies. Cytokeratin (epidermis, ectoderm origin), smooth muscle actin (mesoderm origin), Tuj-1 (neuron, ectoderm origin), α-fetoprotein (liver, endoderm origin), glial fibrillary acidic protein (GFAP, astrocyte, ectoderm origin), and so on.
13. The species of the serum should be matched to the species by which the secondary antibody is raised.
14. After adding the fluorescence-conjugated secondary antibody, the culture plates should always be kept in dark to avoid photobleaching of positive signals.

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10

Derivation of Human Feeders for Prolonged Support of Human Embryonic Stem Cells

Chui-Yee Fong and Ariff Bongso

Summary

Human embryonic stem (hES) cell lines are derived and propagated using animal-based feeder cells and xenoproteins. Reliance on a xenosupport system introduces many disadvantages with respect to exploiting the therapeutic potential of hES cells because of the risk of transmission of pathogens from the animal feeders to hES cells. Recently, human feeders (commercial and in-house derived) in the presence of human-based culture ingredients have been successfully used to derive and propagate xeno-free hES cell lines. In-house-derived human feeders, however, have the advantage over commercial feeders of not being previously exposed to xeno-proteins. This chapter describes the collection of human tissue biopsies and the establishment of human feeder monolayers for the derivation and support of hES cell lines in the presence of human-based culture ingredients.

Key Words: Establishment of human feeders; human embryonic stem cells; support; xeno-free.

1. Introduction

A major drawback in the derivation and propagation of human embryonic stem (hES) cells on mouse embryonic fibroblasts (MEFs) is the risk of transmitting pathogens; in particular, small viruses from the animal feeder cells to the hES cells because of direct contact. Reliance on a xeno-support system introduces considerable disadvantages with respect to exploiting the therapeutic potential of hES cells. Human feeders have been successfully used to grow human inner cell masses and isolate hES cells as far back as 1994 *(1,2)*. It was recently shown that both commercially available and in-house-derived human feeders support the derivation and propagation of hES cell lines *(3,4)*. Additionally, the animal-based xenoproteins in the culture media nourishing the hES cells and feeders can be

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substituted with human-based ingredients, making it a completely xeno-free humanized in vitro system. Unfortunately, the commercial feeders have the disadvantage that they have been grown in the presence of calf sera and other animal proteins and hence exposed to xenoproteins. Feeder-free in vitro systems using noncellular matrices (e.g., Matrigel, collagen) with conditioned media or hES media substituted with specific growth factors support hES cell growth for only six to seven passages and are xenosupport systems because many of the matrices are of animal origin (*3*,*5*). Additionally it was recently suggested that feeder-free conditions could induce chromosome anomalies (recurrent gain of chromosomes 12 and 17q) similar to embryonal carcinoma karyotypes at late passages (*6*). Thus until such time as a feeder-free system that is xeno-free, and has no risk of inducing embryonal carcinoma-like genotypes is available, in-house-derived human feeders appear to be the best choice. Of the in-house-derived human feeders, human fetal muscle, human fetal skin, and human adult skin appear to be the best support cells for hES cells (*3*,*4*). Although ranked third, human adult skin offers the advantage of obtaining biopsies by a noninvasive, simple procedure for feeder derivation and propagation. Additionally, a skin biopsy from the same patient donating in vitro fertilization embryos for hES cell derivation will be the perfect autologous disease-free in vitro system because the patient would have already been screened for HIV, hepatitis B, and other communicable diseases before enrolling for in vitro fertilization.

2. Materials

2.1. Establishment of Human Feeder Cell Lines

1. Individually wrapped sterile universal containers for collection and transport of biopsies (Sarstedt, Ingle Farm, Australia; cat. no. 75.9922-721).
2. Earle's or Hank's balanced salt solution (100 mL, Sigma Chemical Co, St. Louis, MO; cat. no. H9269) supplemented with 1 mL penicillin-streptomycin solution (Invitrogen, Carlsbad, CA; cat. no. 15070-063).
3. hES medium: 50% Dulbecco's modified Eagle's medium (Invitrogen; cat. no. 11960-044), 50% human serum (HS) (Irvine Scientific, Santa Ana; cat. no. 5009), 2 m*M* L-glutamine (Invitrogen; cat. no. 15070-063), 1% nonessential amino acids (Invitrogen; cat. no. 11140-050), 0.1 m*M* 2-mercaptoethanol (Invitrogen; cat. no. 21985-023), 50 µL/mL penicillin/50 µg/mL streptomycin solution (Invitrogen; cat. no. 15070-063 (*see* **Note 1**).
4. 0.05% trypsin-0.53 *M* ethylenediaminetetraacetic acid (EDTA) (100 mL, Invitrogen; cat. no. 25300-062).
5. Plastic cell culture flasks (BD BioSciences, Bedford, MA; cat. no. 353018) or one-well culture dishes (BD Falcon; cat. no. 353653).
6. Two pairs of sterile watchmaker forceps (Lawton, Fridingen, Germany; cat. no. 09-0959).
7. One pair of pointed, curved scissors (Lawton; cat. no. 05-0311).

8. 1-mL, 5-mL sterile serological pipets (Becton Dickinson [BD], Franklin Lakes, NJ; cat. no. 7543, 7521).
9. 5-mL, 10-mL sterile plastic tubes (BD; cat. no. 357553, 357551).
10. Sterile cell scraper (Nunclon, Roskilde, Denmark; cat. no. 179693).

2.2. Freezing of Human Feeder Cells

1. Cell culture freezing medium with 10% dimethylsulfoxide as cryoprotectant (Invitrogen; cat. no. 11101-011).
2. hES medium (*see* **Subheading 2.1., item 3**).
3. 1-mL cryotubes (Nunclon; cat. no. 347783).
4. 1-mL cryoflex (Nunclon; cat. no. 343958).
5. 5-mL, 10-mL sterile plastic tubes (BD Falcon, Bedford, MA; cat. no. 2003 and 2001).
6. Programmable freezing machine (Planer, Stanbury UK; Series III).

2.3. Thawing of Human Feeder Cells

1. hES medium (*see* **Subheading 2.1., item 3**).
2. 5-mL, 10-mL sterile plastic tubes (Falcon; cat. no. 2003 and 2001).
3. Plastic cell culture flasks (BD Falcon; cat. no. 353018).

3. Methods

3.1. Establishment of Human Feeder Cell Lines

1. Aseptically collect either single or multiple 4 mm^2 adult skin biopsies from the abdominal regions of adult donors or similar sized fetal muscle biopsies from the thigh regions of 14-wk-old normal human abortuses.
2. Transfer biopsies to a sterile Petri dish containing hES medium.
3. For adult skin, using sterile watchmaker forceps and pointed scissors, separate the dermis from epidermis and cut the epidermis into very tiny explants (1 mm^3). For fetal muscle, using sterile pointed scissors, mince the tissue into tiny pieces or clusters of cells.
4. Transfer the epidermal explants or fetal muscle tissue pieces to fresh hES medium in another sterile Petri dish. Rinse explants in this medium several times.
5. Transfer the explants to small cell culture flasks or one-well culture dishes containing a layer of hES medium enough only to completely immerse and bathe the explants (*see* **Note 2**). Incubate flasks/dishes at 37°C in 5% CO_2 in air and do not disturb/shake the cultures for at least 3 d (*see* **Note 3**).
6. Examine cultures after 3 d under phase contrast-inverted optics for cellular outgrowths and to see if medium has become straw colored. Whether there are outgrowths or not, add an equal amount of fresh hES medium to the existing medium and incubate further (*see* **Note 4**). If there is further growth of outgrowths from explants after 2 d of incubation, keep replacing medium with fresh hES medium until confluency or substantial growth from the explants are obtained. Epidermal outgrowths from adult skin start their primary growth as epithelioid islands

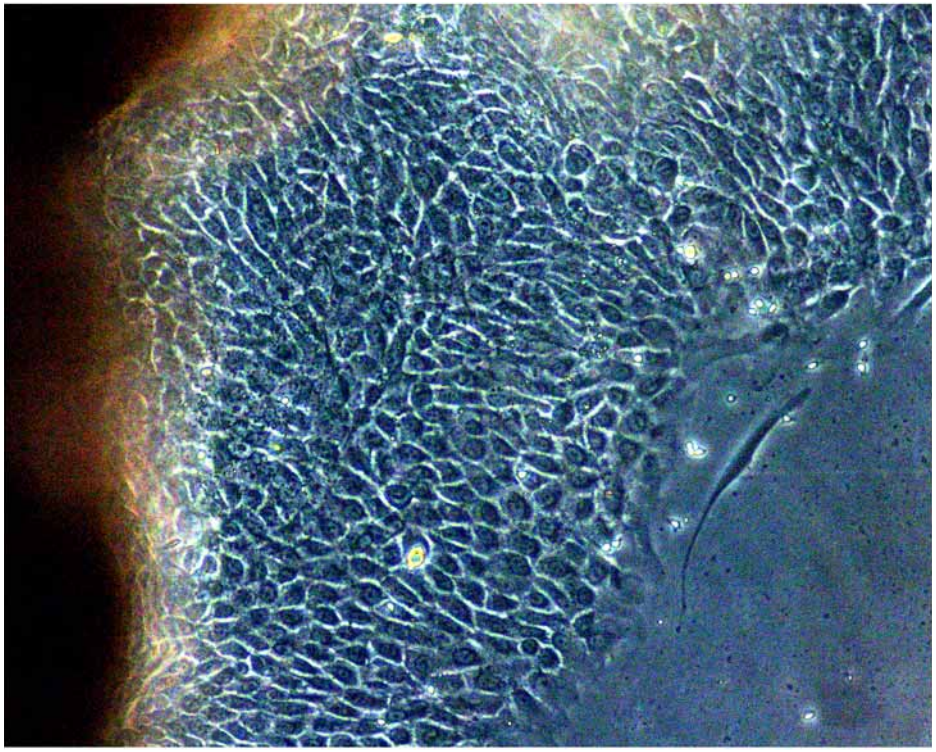

Fig. 1. Light micrograph of an island of epithelioid outgrowth from a primary explant of human adult epidermal skin (×200). (Please *see* the companion CD for the color version of this figure.)

(**Fig. 1**). After the first passage, the epithelial cells transform into fibroblasts (**Fig. 2**). Fetal muscle explants commence their primary growth as fibroblasts and remains as fibroblasts thereafter in subsequent passages.

7. For passaging, aspirate medium and discard. Add 2 mL 0.05% trypsin-0.53 m*M* EDTA. Incubate for a few minutes, checking each time whether the monolayer has detached from plastic (*see* **Note 5**). After explants and individual cells have detached, transfer entire contents to sterile 5- or 10-mL plastic tubes and centrifuge at 300*g* for 5–10 min (*see* **Note 6**).
8. Discard supernatant. Add 2 mL hES medium to the pellet. Resuspend and seed cell suspension into new cell culture flasks/one-well dishes containing hES medium. Incubate and do not disturb for 2 d. For passaged cells, faster growth with earlier confluency is expected. The cells can now be frozen for later use as support for hES cells (*see* **Note 7**).

3.2. Freezing of Human Feeder Cells (see ***Note 8***)

1. Monolayers are washed and trypsinized (similar to passaging fresh cells described in **Subheading 3.1., step 7**) to allow detachment from plastic.

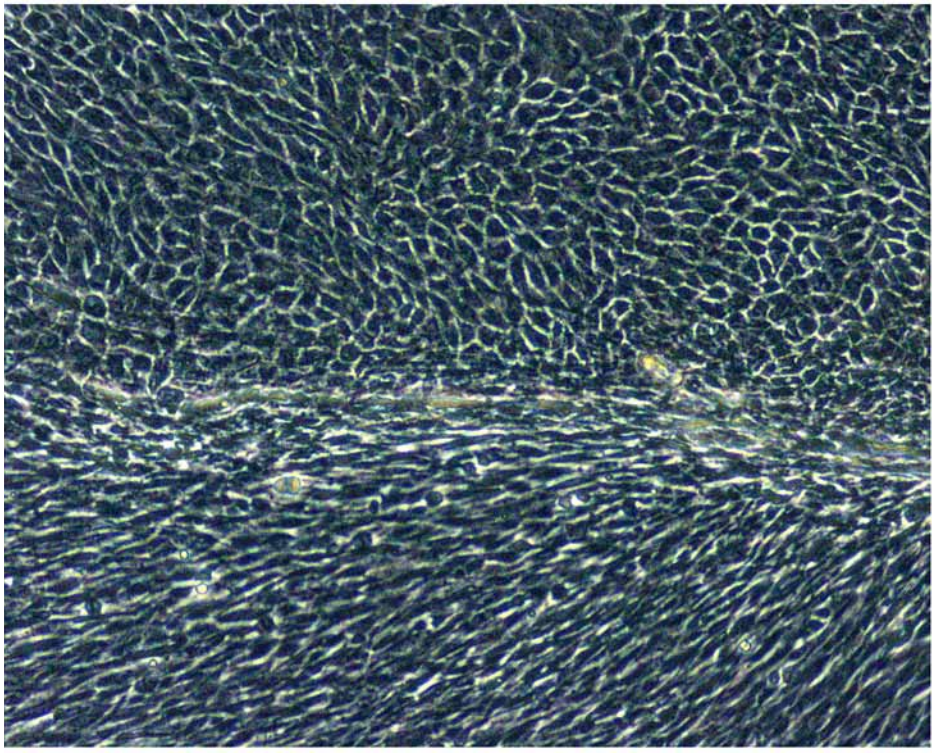

Fig. 2. Light micrograph of epithelioid outgrowths transforming into fibroblasts in early passages of human adult epidermal skin cultures (×200). (Please *see* the companion CD for the color version of this figure.)

2. The trypsinized cells are transferred to 5-mL tubes and washed twice with hES medium by centrifugation to remove all traces of trypsin.
3. The washed cell pellet is resuspended in 2 mL of freezing medium and approx 2–3 million cells are seeded into each cryotube, which is labeled with passage number, cell type, and date of freezing.
4. The cryotubes are frozen using the slow-programmed freezing machine. Cooling is from room temperature to −30°C at 1°C per minute. At −30°C, the cryotubes are plunged directly into liquid nitrogen.

3.3. Thawing of Human Feeder Cells

1. The cryotubes are warmed at 37°C using a water bath and the cells are transferred to a sterile 10-mL Falcon tube.
2. About 9 mL of culture medium is added slowly to the cells drop by drop to prevent osmotic shock to the cells and the cell suspension is centrifuged at 300*g* for 5 min.
3. The supernatant is removed and the pellet is resuspended in 1 mL hES medium.

4. The cell suspension is then seeded into two to three cell culture flasks, each containing 2–3 mL of hES medium, and the flasks are incubated at 37°C in 5% CO_2 in air. Growth to confluency is monitored.

4. Notes

1. If a xeno-free in vitro system is not mandatory, Chang's medium (Irvine Scientific; cat. no. C106) supplemented with 200 m*M* L-glutamine, streptomycin (100 µg/mL), and Fungizone (0.1 mL/mg) (Invitrogen; cat. no. 15290-018) is a better substitute for hES medium + 50% HS. Chang's medium is an enriched super-complex commercial medium that contains salts, energy substrates, newborn calf serum, amino acids, hormones, and vitamins. Good cell growth and shorter times to monolayer confluency are produced with Chang's medium *(7,8)*.
2. If a cell culture flask is used, loosen cap.
3. The cultures are not disturbed for 3 d to allow the explants to attach to the plastic and produce outgrowths. This is usually the point of failure if one is not careful. The medium is adequate for 3 d and does not need changing.
4. Do not change old medium, because the attached explants will be disturbed. It is common to see some explants floating without attachment. After 2 d of incubation, if there is no growth, there is a likely possibility of contamination.
5. Detachment can be hastened by mechanical agitation.
6. If it is necessary to avoid trypsin, the cells can be easily detached mechanically with a sterile cell scraper. The detached sheets of cells need to be broken into cell clusters or individual cells by vigorous resuspension using a sterile Pasteur pipet.
7. For primary cultures and early passages, use hES medium supplemented with 50% HS; for subsequent passages, use hES medium supplemented with 20% HS.
8. Maintenance of live cells in the incubator (37°C) can be very labor intensive and costly. Thus cells are frozen in liquid nitrogen for future use without the need for frequent passaging.

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11

Transplantation of Human Embryonic Stem Cells to the Chick Embryo

Ronald S. Goldstein

Summary

The traditional methods of studying the differentiation of human embryonic stem cells (hESCs) are to differentiate them in vitro or in immune-deficient mice as teratomas. The chick embryo is a well-studied and accessible experimental system that has been shown to permit the development of mammalian cells, including murine embryonic stem cells. We therefore performed experiments transplanting colonies of hESCs into organogenesis-stage chick embryos, hoping this might provide a novel system for studying the developmental programs and decisions of these important cells. hESCs, constitutively expressing green fluorescent protein or labeled with the dye CFDA, were used to allow the following the hESC in living embryos. As a first step, we chose to transplant hESCs into the trunk of chick embryos, both into and instead of developing somites. Our first results showed that hESCs survive, migrate, and integrate into the tissues of the chick embryo. Some of the hESCs differentiate and the type of embryonic microenvironment that the implanted cells were exposed to modified their differentiation. Therefore, this hESC-chick embryo system has potential for complementing studies in rodents and in vitro, and uniquely, to shed light on early processes in the development of human cells in the embryonic context.

Key Words: Human embryonic stem cells; xenograft; human embryogenesis; chick embryo; somites; in ovo microsurgery.

1. Introduction

The most clinically important, and imagination stimulating application of human embryonic stem cells (hESCs), is of course the generation of normal differentiated cells for replacement of those lost in disease or injury. However, there are several additional important ways in which hESCs can be used productively. Use of these cells has the potential to reveal important details about early human embryonic development, because early human embryos, for

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both practical and ethical reasons, are not accessible to researchers. Arguments in the scientific and general community now seem to preclude the production of early chimeras using hESCs, whether the host blastocyst is human or mammalian (mouse) *(1)*. By contrast, transplantation of hESCs into adult animals is routine today and does not appear to raise any ethical problems. However, the adult tissue environment likely lacks many of the factors required to direct differentiation of the hESCs, and the complex and compact three-dimensional structure can impede migration and integration of hESCs. This is clearly shown by the development of teratomas from hESCs when injected in adult mice *(2,3)*.

The chick embryo has been studied intensively at the anatomical, cell biological, and molecular levels, and the precise timing and placement of many of the inductions and morphogenetic events underlying its development are known. In addition, experimental manipulations of the embryo are quite uncomplicated because of the easy accessibility of the embryo by simply opening a "window" in the shell. A number of studies have shown that mammalian cells survive and differentiate in response to environmental signals of avian embryos. For example, when chick neural tube is replaced with that of mouse embryos, mouse neural structures develop, including a spinal cord, a neural crest, and their derivatives the peripheral ganglia *(4)*. Mouse embryonic stem cells and their derivatives have also been transplanted successfully to the chick embryo. In one elegant study, motoneuron precursors derived from mouse embryonic stem cells were transplanted to the neural tube of the chick, and mouse motoneurons not only differentiated, but sent their axons out chick nerves, and innervated peripheral muscles *(5)*.

We therefore have transplanted hESC colonies into the developing tissues of early chick embryos in an attempt to provide hESC with an environment of developing tissues, while avoiding the ethical problem of generating embryos with extensive chimerism *(6)*. We found that placement of the hESC in direct contact with the host neural tube consistently resulted in the generation of neural tube-like structures ("neural rosettes") that grew with the same orientation as the host central nervous system, with their axis parallel to that of the chick. In contrast, interspersing the hESC within somites led to integration of individual human cells into chick structures such as peripheral ganglia and vertebral antagen. The present chapter details the techniques that were involved in these experiments, with the primary focus on the surgical techniques themselves (*see* **Note 1**).

2. Materials

2.1. Culture

1. 3.5-cm culture tissue culture plates (Nunc, Roskilde, Denmark; cat. no. 153066).
2. Gelatin (Merck, Whitehouse Station, NJ; cat. no. 1.04078.1000). Prepare a 0.1% solution in dH_2O, autoclave at 121°C for 30 min. The sterile stock is stored in the refrigerator.

3. Vybrant CFDA SE Cell Tracer Kit (Molecular Probes, Eugene, OR; cat. no. V12883). Make a stock solution by dissolving the contents of vial of powder in 90 μL supplied dimethylsulfoxide.

2.2. Surgery

1. Fertile chick eggs (*see* **Note 2**).
2. Surgical instruments (*see* **Note 3**): fine scissors (Fine Science Tools, North Vancouver, Canada; cat. no. 91460-11); Dumont no. 5 forceps (Fine Science Tools; cat. no. 91150-20); pin holder (Fine Science Tools; cat. no. 26016-12) × 2; sterilization case with rubber insert (Fine Science Tools; cat. no. 20311-21), Minuten pins (Fine Science Tools; cat. no. 26002-10).
3. Microscalpels are made in one of two ways:
   a. For very fine dissection, including opening of the ectoderm, they are made by electrolysis of tungsten wire (A-M systems, Carlsborg, WA; cat. no. 7185) (*see* **Note 4**). One lead of a 12-V alternating current transformer is connected to a bath of saturated NaOH in a plastic Petri dish by a paper clip, and the other lead is attached to a pin holder (Fine Science Tools; cat. no. 26016-12) with the tungsten wire inserted. The pin holder is held in any simple micromanipulator and the tip of the wire lowered just into the solution while monitoring with an old binocular microscope (*see* **Note 5**). The current is then applied, and the wire dipped and removed from the solution, and the tip examined with the binocular microscope until the appropriate shape and sharpness is obtained. Varying the angle of the wire can produce many different shapes of scalpels *(7)*, but we find that straight is fine for the operation described here.
   b. For removal of somites and manipulation of tissue during the implantation, pins (Fine Science Tools; cat. no. 26000-40-45) are sharpened on a stone (Fine Science Tools; cat. no. 29008-22) using a drop of paraffin oil. The scalpel blade should look like a butter knife when finished. That is, it should be thin and not very sharp at the end. Obtaining a thin blade requires holding the pin almost parallel to the surface of the stone and rubbing it back and forth laterally, switching sides periodically. For sharpening, the pin should be inserted into the holder most of the way, only leaving 2–3 mm outside for sharpening. Otherwise, it just bends when it is rubbed against the stone. When the sharpening is finished, the pin is pulled out with forceps, leaving approx 5 mm inside the holder. The longer the pin, the more visibility there is during the operation, because the pin holder can block the field of view if it is too close.
4. Cellotape (*see* **Note 6**).
5. Egg incubator (GQF Manufacturing, Savannah, GA; cat. no. 1550).
6. Stereo microscope with bifurcated fiber optic light guides for surgery, and epifluorescence for viewing graft in vivo.
7. Cold plate that can maintain 12°C (homemade from a Pelletier device, digital thermostat, and black-anodized aluminum plate. Plexiglas cover retains cooling).
8. Dulbecco's phosphate-buffered saline (PBS) with divalent cations (Biological Industries, Bet HaEmek, Israel; cat. no. 020-020-1).

9. Pancreatin (Invitrogen, Carlsbad, CA; cat. no. 610-8728-AE).
10. Penicillin-streptomycin-amphotericin (Biological Industries; cat. no. 03-033-1).
11. India ink.
12. Gel-loading tips (i.e., USP, San Leandro, CA; cat. no. TGL-1000).
13. Embryo dish (Electron Microscopy Sciences, Hatfield, PA; cat. no. 70543-30).
14. PBS+ (Dulbecco's PBS with divalent cations) (Biological Industries, cat. no. 02-020-1A).

2.3. Fixation, Embedding, and Location of Grafted Cells

1. 4% paraformaldehyde (*see* **Note 7**). For 200 mL: add 8 g paraformaldehyde granules (Electron Microscopy Sciences; cat. no. 19208) to 100 mL dH_2O. Heat to 60°C while stirring in a fume hood. Add 1 *M* NaOH one drop at a time until clear and cool to ambient temp. Bring volume to 200 mL with 2X PBS without divalent cations (double the quantity of salts in **Subheading 2.4.1.** in the same volume).
2. Paraplast+ (Sigma-Aldrich, St. Louis, MO; cat. no. P-3683).
3. Sylgard elastomer (World Precision Instruments, Sarasota, FA; cat. no. SYLG184).

2.4. Immunocytochemistry

1. PBS without divalent cations (PBS–). For 500 mL: dissolve 80 g NaCl, 2 g KCl, 11.5 g Na_2HPO_4 (anhydrous), 2 g KH_2PO_4, 1 g Thimerosal (Sigma-Aldrich, cat. no. T-5125) in 450 mL dH2O. Bring volume to 500 mL when dissolved. This makes a 20X stock that is diluted before use.
2. Microwave oven (*see* **Note 8**).
3. Plastic staining dishes for deparaffinization, hematoxylin and eosin staining, and dehydration (Tissue-Tek, Torrance, CA; cat. no. 4451).
4. Glass staining dishes for immunocytochemistry (6X) (Electron Microscopy Sciences; cat. no. 70312-20).
5. Polypropylene staining dish for microwave antigen retrieval (Electron Microscopy Sciences; cat. no. 70321-10).
6. Staining rack (Electron Microscopy Sciences; cat. no. 70321-20).
7. 1X citrate buffer (diluted from 10X solution). For 500 mL 10X solution: add 14.5 g trisodium citrate dihydrate (Carlo Elba, Milan, Italy; cat. no. 479487) to 400 mL ddH_2O. Bring to pH 6.0 with 1 *N* HCl.
8. Blocker 1% bovine serum albumin (USB, Cleveland, OH; cat. no. 70195), 0.5% Triton X-100 (USB; cat. no. 22686) in PBS+. Make up 100 mL and freeze in sterile 15-mL tubes.
9. Anti-mammalian neurofilament-M (Developmental Studies Hybridoma Bank, Iowa City, IA; cat. no. 2H3).
10. Anti-βIII tubulin MAb (Promega, Madison, WI; cat. no. G712A).
11. Anti-human Desmin, (DAKO, Glostrup, Denmark; cat. no. M0760).
12. Anti-islet-1 (Developmental Studies Hybridoma Bank; cat. no. 40.2D6).

13. Anti-green fluorescent protein (GFP) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; cat. no. sc-8334).
14. Anti-human mitochondria (Chemicon, Temecula, CA; cat. no. MAB 1273).
15. Anti-HNK (supernatant from growing ATTC, Manassas, VA; hybridoma no. TIB-200, or Sigma; cat. no. C0678).
16. Alexa Fluor 488 goat anti-mouse IgG (H+L) conjugate (Molecular Probes; cat. no. A-11001).
17. Alexa Fluor 594 goat anti-rabbit IgG (H+L) conjugate (Molecular Probes; cat. no. A-11012).
18. Slides (Superfrost Plus) (Menzel-Glaser, Braunschweig, Germany; cat. no. 041300).
19. Glass cover slips 24 × 50 mm, 24 × 60 mm.
20. Mounting medium: 75% glycerol (Sigma; cat. no. G-8773) 25% PBS–, add 1% n-propyl gallate (Sigma; cat. no. P-3130) for antibleaching.
21. 0.1 mg/mL Hoechst 33258 (bisbenzimide) (dilute 1:1000 from stock solution). For 10 mL stock solution: add 10 mg Hoechst 33258 (Sigma; cat. no. B-2883) to 100 mL ddH_2O. Working solution can be reused and kept in light-protected container at 4°C until staining is no longer strong.
22. Nail polish (*see* **Note 9**).
23. Polylysine for coating slides (Sigma; cat. no. P8920).

2.5. Histology

1. Harris hematoxylin (Sigma; cat. no. HHS-16).
2. Eosin yellowish (Gurr, London, UK; cat. no. 22232).
3. Cold Schiff: dissolve 10 g basic fuchsin (MCB, Norwood, OH; cat. no. B300/BX135) in 300 mL 1 *N* HCl (24 mL HCl conc. + 276 mL dH_2O). Dissolve 10 g sodium metabisulfite (Sigma; cat. no. S9000) in 1700 mL dH_2O. Mix the solutions. Add activated charcoal (BDH DARCO G60, Poole, England; cat. no. 33187) and mix. Let stand for 24 h at room temperature. Filter with no.1 Whatman filter paper; solution should be colorless. Store at 4°C in a well-closed bottle.
4. Sulfurous rinse solution: prepare a stock of 10% w/v sodium metabisulfite. Working solution: 10 mL stock + 10 mL 1 *N* HCl + 180 mL dH_2O.
5. Fast green FCF (Sigma; cat. no. F7258).
6. Entellan (Merck; cat. no. 1.07961.0500).
7. Ethanols (70%, 95% denatured diluted with dH_2O, 100% anhydrous analytical reagent).
8. Xylene.
9. Toluene.

2.6. Digital Photography

1. Frame grabber (Scion, Frederick, MD; model no. LG-3).
2. Black and white video camera (Cohu, Poway, CA; model no. 4192-5000).
3. Nikon Coolpix camera (Nikon, Tokyo, Japan; model no. 990).
4. ImageJ (http://rsb.info.nih.gov/ij).

3. Methods

3.1. Culture of ES Cells

3.1.1. Growth of Human ES Cells

The growth of hESC was described *(6)* and (in Benvenisty et al., this volume). In some experiments, hESC that constitutively expressed enhanced GFP (eGFP) *(8)* were used for grafting.

3.1.2. Partial Removal of Fibroblasts and Initiation of Differentiation

Before grafting, hESC were cultured for 1–3 d on gelatin-coated plates (cover bottom of plate in sterile hood at least 1 h at room temperature, then rinse with dH_2O and medium) without fibroblast feeder lines to obtain a purer population of hESC for grafting and to begin the process of hESC differentiation.

3.1.3. Vital Staining of hESC

In some experiments, cells were vitally stained with CFDA.

1. Warm 1.5 mL PBS+ to 37°C.
2. Add 3 µL CFDA stock solution (*see* **Note 10**) and mix.
3. Replace the medium in the 35-mm culture plate containing the hESC colonies with the dye, and incubate 15 min in a CO_2 incubator.
4. Rinse with warm PBS+.
5. Change to 1.5 mL Dulbecco's minimum essential medium and incubate at least 30 s in the CO_2 incubator.
6. When ready for use, change to PBS+, and collect colonies as in **Subheading 3.2.6.**

3.2. Microsurgery for Transplants

3.2.1. Incubation and Preparation of Embryos

1. Incubate fertile chicken eggs *without turning* in a horizontal position from 40 to 45 h to obtain embryos of 10–20 somite pairs.
2. Mark the approximate position of the embryo by drawing a small pencil mark on the topmost position on the shell. Care should be exercised from this point on so that the eggs should not rotate in any direction.
3. At least 20 min before beginning surgery, lower the embryo away from the shell by removing 1–1.5 mL albumin. This is accomplished by wiping the point of the egg with a tissue moistened with 70% ethanol and making a small hole in the point by tapping sharply with pointed fine surgical scissors.

4. Insert a 21-G needle attached to a 2-mL syringe into the hole, and withdraw the albumin very slowly. Rapid removal of the albumin makes large bubbles above the embryo, making subsequent surgery nearly impossible.

3.2.2. Visualizing the Embryo

Although some use vital dyes to stain chick embryos before surgery, in our experience the best visualization is obtained when black ink is injected in the sub-blastodermal space. A small amount of India ink diluted 1:3 in PBS+ is injected sub-blastodermally using a hand-drawn pipet (*see* **Notes 11** and **12**).

3.2.3. Exposure of the Embryo for Surgery

In the experiments described here, hESC is transplanted to the area of the somites **(Fig. 1A,B)** (*see* **Note 13**).

1. Make the slit in the shell membrane above the three or four most recently formed somites with a microscalpel. Intrinsic tension makes the membrane separate and reveal the underlying ectoderm of the embryo.
2. Immediately moisten the shell membrane with PBS+ after opening the egg to prevent damage to the embryo.
3. When there is a thin layer of PBS+ over the membrane, pierced it gently with the microscalpel held at about 45°, and then lift up the scalpel with a sharp movement; this results in a small tear of the membrane.

3.2.4. Preparation of Embryos for Grafts of hESC Into Somites

1. Cut three slits in the ectoderm to access the somites, one between the somites to be damaged and the neural tube, one between the last formed somite and the segmental plate, and one between the most rostral somite to be damaged and the somite rostrad to it.
2. Cut the ectoderm by piercing with a sharp movement at one end of the cut and lift the scalpel upward.
3. After making the three cuts, insert the scalpel between the somites to be damaged and the flap of ectoderm formed by the cuts, lifting up the flap and exposing the somites.
4. Gently, make several slices in the somites using the scalpel, taking care not to cut so deep that the endoderm is damaged. If the endoderm is cut (it is only 10-µm thick at this stage), the ink that was injected for contrast (**Subheading 3.2.2.**) comes seeping up through the cut, and the embryo is likely not to survive the surgery (*see* **Note 14**).

3.2.5. Preparation of Embryos for Grafts of hESC in Place of Somites

1. Remove the shell membrane as in **Subheading 3.2.3.** and make a longitudinal slit in the ectoderm between the somites to be removed and the neural tube as in the previous section.

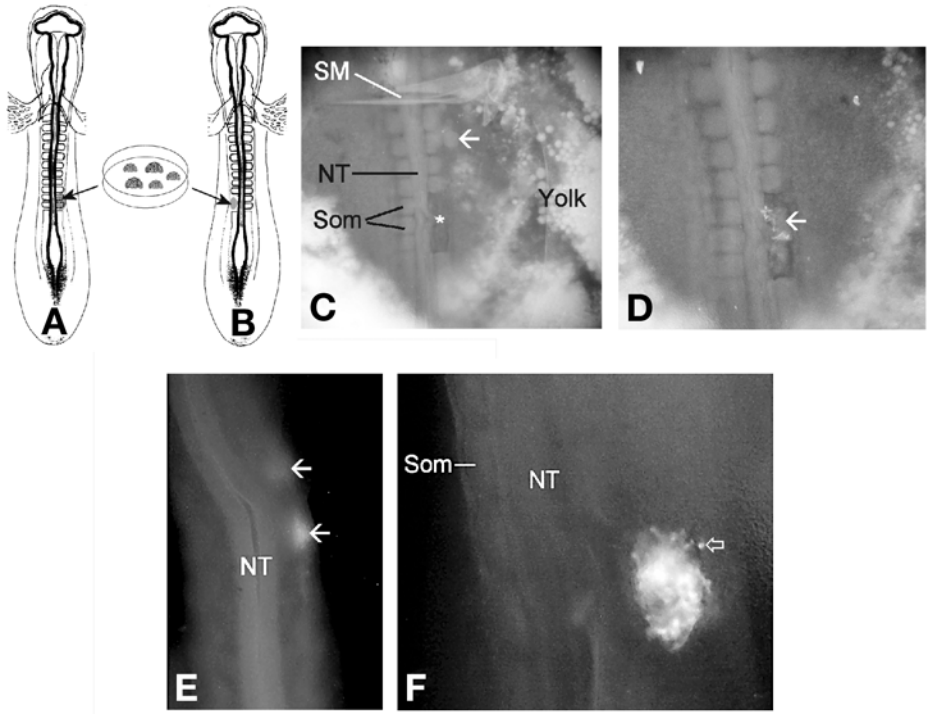

Fig. 1. Diagrams of grafts of human embryonic stem cell (hESC) into (**A**) and instead of (**B**) epithelial somites of chick embryos of about 36 h incubation. (**C**,**D**) A portion of the trunk of a live embryo being grafted. An asterisk is in the middle of a gap made by the removal of three intact epithelial somites in **C**. The white arrow indicates one of the removed somites. **D** shows the same embryo, with the gap filled in with hESC cells (arrow). The bright white material below the arrow in **D** is a small piece of plastic removed from the Petri dish along with the hESC colony. (**E**,**F**) Images of live embryos that had been implanted with GFP-expressing hESC 24 h previously. In **E**, two areas of fluorescent cells are visible (arrows), the upper one is less bright because the cells are less superficial. In **F**, individual cells, such as the one indicated by the open arrow, can be seen migrating away from the graft. SM, shell membrane; NT, neural tube; Som, somites.

2. Drip a 10-µL drop of pancreatin on this region to loosen the somites from their surrounding tissues. The tip of the pipet should not touch the embryo, the drop should "fall" from a short distance above. When the drop is placed correctly, a small pool of liquid forms.
3. Incubate for 5–10 min, with the aperture in the egg shell covered (but not sealed) with a large glass cover slip to prevent desiccation of the embryo (*see* **Note 15**). The somites are ready for removal when a gentle nudge from the microscalpel moves them out of place (**Fig. 1C**) (*see* **Note 16**).
4. Neutralize the enzyme with a drop (from a Pasteur pipet) of 5% heat-inactivated serum in PBS+ (*see* **Note 17**).

3.2.6. Transfer and Implantation of hESC to the Chick Embryo

1. Remove the Petri dish containing the hESC from the CO_2 incubator and replace the culture medium with PBS+ at room temperature (*see* **Note 18**).
2. Cut the colonies into small pieces under the dissecting microscope and scrape off the bottom of the culture dish.
3. Collect the colony pieces and transfer, several at a time, into an embryo dish containing PBS+ that is kept on a cooling plate maintained at 12–15°C (*see* **Note 19**). One can prepare the colonies before beginning to operate on the eggs or while waiting for the pancreatin to act. In our experience, when maintained at 12–15°C, the hESC remain viable for at least 6 h.
4. Immediately before transfer of the hESC to the egg, place a drop of PBS+ in the graft area, again making a "pool" of liquid between the embryo and the shell membrane.
5. Now, as quickly as is practical, select a few pieces of hESC colony from the embryo dish and aspirate them into a gel-loading tip in a volume of 5 µL.
6. Transfer the tissue by placing the tip into the "pool" of PBS in the egg and gently expel liquid until the hESC leaves the tip (*see* **Note 20**).
7. Set down the pipetor and, using the microscalpel (preferably a dull one), coax the tissue into the damaged somite or space left by the removal of the somites (**Fig. 1D**). In the case of somite replacement, it is advisable to try to fill the gap as well as possible with hESC, because of the tremendous regenerative ability of somites *(**10**)*. This regeneration results in the chick cells competing with the foreign (hESC) cells and preventing their expansion.
8. After the hESC tissue is in place, the ectodermal flap is smoothed over the area of the graft to act as a "blanket" holding the transplanted cells in place.
9. Seal the egg with Cellotape, label it with the exact details of the experiment using a graphite pencil, and return the egg carefully to the incubator.

3.2.7. Follow-Up of Grafts

1. The morning after the operation, open the eggs aseptically, and view with a binocular microscope equipped with epifluorescence for visualizing GFP (**Fig. 1E,F**) or CFDA. The implanted hESC are still relatively superficial at this point, and the embryos can be photographed under both fluorescence and bright-field illumination.
2. Gently drip four to five drops of PBS+ containing 10X concentrated antibiotic solution onto the embryo to prevent dehydration and combat infection (*see* **Note 21**).

3.3. Fixation and Embedding

3.3.1. Fixation

1. After the second incubation period, remove embryos from the egg.
2. Transfer the embryos to a Petri dish with a 5-mm layer of Sylgard elastomer, and pin them out with Minuten pins in PBS+ at room temperature (*see* **Note 22**). The GFP

fluorescence, although visible after paraformaldehyde fixation, is stronger before fixation, so this is a good time to photograph the embryo for future localization of transplanted cells (*see* **Subheading 3.3.3.**). One can pin out and fix several embryos at once by drawing a "map" of their positions in the dish before fixation.
3. After photography/observation, the PBS+ is aspirated carefully with a Pasteur pipet and 4% paraformaldehyde added.
4. Carefully unpin the embryos (which become fairly stiff after about 20 min fixation at room temperature) and place them into fresh fixative in scintillation or similar glass vials. If the surgery is not near the head, it is worthwhile to decapitate the embryo to improve the subsequent dehydration and embedding. Fixation can be performed for 2–3 h at room temperature or overnight at 4°C.

3.3.2. Embedding and Sectioning

1. Rinse fixed embryos 2×5 min in dH_2O and dehydrate via ethanol, two changes each 70, 95, and 100% analytical reagent. The times are dependent on the size of the embryo, each step for 4-d embryos is 15 min; for 9-d embryos, the times should be doubled.
2. The embryos are then cleared in two changes of xylene of 15–30 min according to the size of the embryo.
3. Perform three changes of paraffin, and finally embed the embryo longitudinally, with the neck positioned in such a way that it will be cut in cross-section.

3.3.3. Serial Sectioning and Searching for Graft

One of the major challenges in this entire experimental procedure is finding the relatively few grafted ES and their progeny within the "background" of an entire embryo. The cells can be positively identified without ambiguity in sections in one of three ways: (1) immunostaining for human specific markers such as human mitochondrial marker; (2) using cells marked either a genetically (i.e., expressing a marker such as GFP) or with a vital dye such as CFDA; or (3) using mammalian or human specific antibodies to differentiation markers (such as mammalian neurofilament protein).

1. Prepare long ribbons of serial sections are prepared and lay them out in long, flat boxes (*see* **Note 23**).
2. Cut the ribbons into strips about two-thirds the length of a microscope slide; using an old, blunt scalpel blade; a paper or cardboard "ruler" of the right length is helpful for estimating.
3. Mount the ribbons onto slides in a staggered manner. That is, if the width of the ribbon is such that it is possible to mount four ribbons per slide, every fourth ribbon is mounted on slide 1, the next of the series on slide 2, and so on. This allows sampling of large extents of the embryo in order to find the graft. Initial staining with anti-GFP or anti-human markers is performed on one slide from each set and the stained sections scanned until the graft is found (*see* **Note 24**).

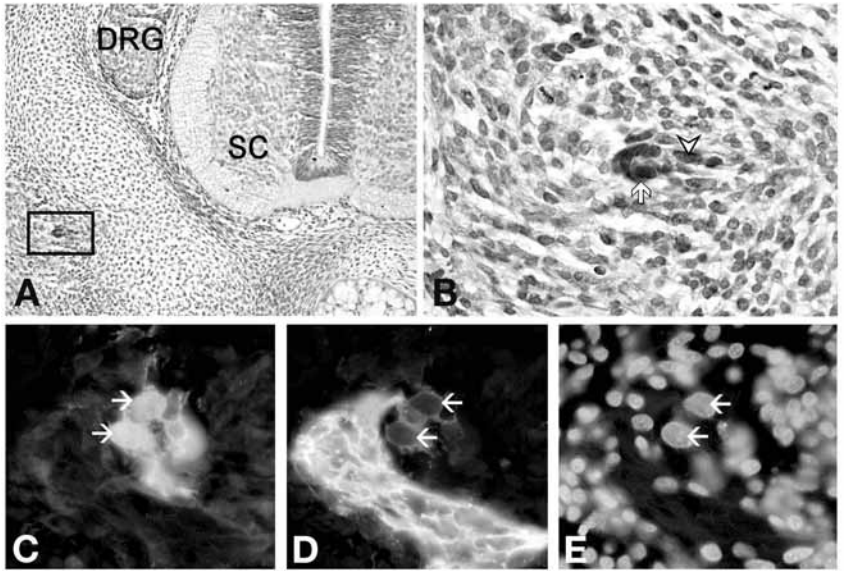

Fig. 2. Feulgen staining of a section through a grafted embryo is shown (**A**,**B**). A small cluster of human cells (in the black rectangle) is easily observed even at low magnification using Feulgen nuclear staining. At higher magnification in **B**, the open arrow indicates a cluster of a few human cells, with their larger, more intensely staining nuclei. The arrowhead points to a single human cells, which can be identified in the background of the smaller chick nuclei. **(C–E)** Fluorescent micrographs of a field stained with; antibodies to GFP, neuron-specific tubulin, and Hoechst nuclear stain, respectively. The arrows in each panel point to a pair of human neurons. In **F**, their large nuclei can easily be distinguished from those of the surrounding chick cells. The large fluorescent structure in **D** is a peripheral nerve of the chick that expresses higher levels of neuron-specific tubulin than the newly differentiated human neurons. DRG, dorsal root ganglion; SC, spinal

3.3.4. Feulgen Staining

The nuclei of mammalian cells, like those of quail cells, are much larger than those of the chick embryo (i.e., **ref. *4***). The graft can therefore often be easily identified by Feulgen staining (**Fig. 2A**,**B**), which gives a sharper and more intense nuclear staining than routine hematoxyline and eosin. In addition, Feulgen combined with fast green counterstaining often gives better general histology than hematoxylin and eosin.

1. Rehydrate to dH_2O.
2. Incubate in 5 *N* HCl 30 min (40 mL HCl/60 mL dH_2O).
3. Incubate in tap water 15 min.
4. Rinse in dH_2O rinse 5 min.
5. Incubate for 90 min in cold Schiff reagent.

6. Incubate in sulfurous wash 1 min, then 4 min.
7. Wash in gently running tap water 15 min.
8. Wash in dH_2O for 5 min.
9. Counterstain lightly with fast green (1% aqueous solution) for 5 min.
10. Dip in 70% ethanol.
11. Dehydrate and mount with Entellan.

3.3.5. Immunocytochemistry

For absolute identification of the ES cells, it is necessary to perform antibody staining.

1. Bake slides in paraffin oven (56°C) for 30 min (*see* **Note 25**).
2. Deparaffinize by 2–15 min incubations in toluene, followed by 5 min each in 100, 95, and 70% ethanol. Wash for 10 min in dH_2O.
3. Incubate in a microwave for antigen retrieval for 15 min in 10 m*M* sodium citrate buffer in a polypropylene container. Cover the slides with 200 mL buffer and start the microwave at full heat. Watch the container until the solution reaches boiling, then lower the power to 30–50%. If the level of liquid falls below the top of the slides, stop the microwave procedure midway and add more buffer. When finished, remove from oven and allow the container to cool to room temperature.
4. Wash for 15 min in PBS.
5. Block for 15 min.
6. Drain blocker (do not rinse) and then add the primary antiserum diluted in blocker (*see* **Note 26**).
7. Incubate overnight or 48 h at 4°C in a humid chamber (*see* **Note 27**).
8. Warm for 1 h, then remove cover slips by placing the slides slowly into the glass staining dish carrier containing 200 mL PBS. After about 2 min, slowly raise the slide carrier with the metal handle and the cover slips will fall off by themselves. Rinse another 3 min, and then transfer to second PBS rinse bath for 5 min.
9. Drain slides, and add secondary antibody(ies); incubate for 1–2 h in a humid chamber.
10. Remove cover slips as in **step 8**, and rinse in PBS– 2×5 min.
11. Counterstain with Hoechst 1 μg/mL 5–10 min.
12. Cover slip in anti-fade medium and seal cover slips with nail polish.
13. Let nail polish dry in a covered box for 10–15 min and view with a fluorescence microscope (*see* **Note 28**).

Figure 2C–E shows two human neurons adjacent to a nerve of the chick. The human cells are double-stained for GFP and neuron-specific tubulin, whereas the chick nerve is only stained with the neural marker.

3.3.6. Photomicrography

For photography of fluorescence, we have been using a combination of a Cohu analog video camera and a Scion scientific frame grabber card (*see* **Note 29**). Bright-field images are collected using a Scion CFW1310 color Firewire camera or a Nikon Coolpix 990 digital camera.

4. Notes

1. An additional method of introducing hESC cells to embryos is injection of a suspension of cells ***(11)***, as has been done with mouse neural crest precursors ***(12)***.
2. We obtained freshly laid fertile eggs from a local farm (Moshav Sitria). The eggs were kept at 12°C for up to 10 d before incubation without noticeable ill effects.
3. Instruments should be cleaned promptly after each use and rinsed with dH_2O and 70% ethanol to remove water. Dry sterilization was performed at 180°C for 90 min. With careful cleaning and dry sterilization, instruments last for many years.
4. We used a Teflon-coated wire we had in the lab. The Teflon must be stripped off first by scraping with scissors. Noncoated wire can also be purchased.
5. The NaOH splatters when the current is applied, so eye protection and a lab coat should be worn, and a microscope that is near the end of its life used.
6. There are many brands of Cellotape on the market; they vary greatly in their ability to remain adherent to egg shell in high-humidity and 37°C. It is worthwhile buying a few types and testing to see which sticks best after 3–4 d in the incubator.
7. Any paraformaldehyde can be used. We buy the crystals to reduce exposure to this toxic substance; the powder is easily spread around the lab. Even using a chemical hood, the granules (even if they spill) cannot be carried by wind currents. Many labs use formalin and dilute 1:10 with PBS; we have not tried this.
8. After having one oven rust, we bought one with an enamel interior.
9. Others have claimed that certain brands interfere with fluorescence; we buy bargain-counter samples and have not had a problem.
10. We found that although the manufacturer recommends using the stock dye only immediately after reconstitution with dimethylsulfoxide that we obtained substantially identical results with 1-wk-old stock solution.
11. Although the ES cells have been tested for hepatitis and AIDS, for extra precaution, the ink was injected using a graduated microsyringe (GS-1200 [VX-07844-00] Gilmont Micrometer Syringes, Barnant, Barrington, IL) connected to the pipet via a 19-G steel needle (whose tip had been cut off with electrician's pliers) and mineral oil-filled PE tube.
12. A tuberculin syringe can also be used for injecting ink. However, in our experience, making too large a hole with the ink injection pipet is one of the primary causes of loss of operated embryos. The surface tension of the external membranes is very strong and can cause a continual tearing if the hole is not small enough to repair itself within a day or so.
13. Of course, transplantation of the HES can be made virtually anywhere in the chick embryo; many years of study have elucidated the precise positions and timing of development of its various tissues and organs.
14. If embryos older than about 20 somite pairs are used, excessive damage of the somites with the scalpel can cause bleeding, an occurrence that also reduces the chance of survival after surgery.
15. If the enzyme is permitted to act too long, the neural tube begins to look like an "accordion," and survival chances are small.
16. It is advisable to have one microscalpel with a dull tip for removing the somites to minimize the possibility of damage to the endoderm (and embryo).

17. Use whichever type is cheapest and most easily available in the laboratory.
18. All of the preceding steps can be performed in a laminar flow biological hood with positive air flow. However, it is not advised to use such a hood for work with human biological material. Therefore, the blower should be shut down before the hES is brought to the hood. Alternatively, a biohazard hood can be used, with holes cut for the oculars of the binocular dissecting microscope.
19. To prevent the sticking of the tissue to the pipet tip, aspirate/collect some medium without tissue into the tip, keep the plunger of the pipetor fixed, and then up the tissue in the remaining volume by allowing the plunger to spring up until it stops. It is advisable to cut many more pieces than will actually be transplanted, because there is invariably loss of tissue both in transit to the embryos and within the embryos themselves.
20. This is a critical step; if the expelling is done too rapidly, the tissue "swims away" and is lost within the embryo.
21. Survival of the embryos is between 50 and 100% 1 d after surgery; at 5 d, 20–50%.
22. A slotted spoon is a valuable aid in removing the delicate younger embryos without damage.
23. For example, such as those in which 8×10 photographic paper is sold.
24. For routine histology (e.g., hematoxylin and eosin, Feulgen), conventional microscope slides coated with polylysine solution are fine. For immunostaining, and most particularly, for microwave antigen retrieval, Superfrost Plus slides are absolutely required. Otherwise, the sections will fall off the slides during the microwaving procedure.
25. The use of Superfrost plus slides is absolutely critical; otherwise, sections fall off in the microwave.
26. Antibody dilutions vary from batch to batch; we use the manufacturer's suggestion as a lower guide, and perform tests on each vial received.
27. We use 75 µL/slide, and carefully cover slip with 24×50 cover glass to keep the antibody in place. Two antibodies from different species (mouse/rabbit) or two monoclonal subtypes (e.g., immunoglobulin M) can be mixed and applied simultaneously.
28. The slides are stored at 4°C in regular plastic slide boxes, Alexa, Cy2, and Texas-Red fluorescence are stable for more than 6 mo.
29. This system has the advantage of being extremely sensitive and displaying live (30 frames/s) video on a conventional composite (television) monitor for group viewing when not using the computer. We built a small potentiometer-based "gain" control for the camera, which simplifies exposure control. Images are captured, one wavelength (filter set) at a time using ImageJ. We always use the averaging feature of the Scion's capture module because it reduces background noise dramatically and is quite fast (takes only 1–2 s) with a Pentium IV computer. The individual images are then merged into a single red-green-blue image, or a montage is made using the "stacks" commands in ImageJ. Images are usually sharpened a bit using unsharp mask filtering, and text is added using commercial graphics editors such as Paint-Shop Pro.

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12

Derivation and Characterization of Neuronal Precursors and Dopaminergic Neurons From Human Embryonic Stem Cells In Vitro

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Summary

Dopaminergic neurons of human origin have many potential research applications such as in vitro studies on biochemical pathways related to neuronal disorders, and potentially direct cell replacement for therapeutic use. Dopaminergic neurons with apparently normal properties can be produced from embryonic stem cells from mice and sub-human primates by the simple procedure of coculturing with the PA6 stromal cell line. Recently, we have demonstrated that this coculture system can induce dopaminergic differentiation in human embryonic stem cells, and the human embryonic stem cell-derived dopaminergic cells exhibit biochemical and functional properties of mature dopaminergic neurons.

Key Words: Embryonic stem cells; neural differentiation; dopaminergic; PA6 stromal cells.

1. Introduction

Pluripotent human embryonic stem (hES) cells, isolated from the inner cell mass of preimplantation embryos, are able to self-renew and to generate any cell type of the developing embryo *(1)*. hES cells are therefore potentially useful for both in vitro developmental studies and for in vivo cell replacement therapies. The possible therapeutic use of hES cells has attracted great attention since the first derivation of hES cells in 1998 (e.g., to generate dopaminergic neurons for the treatment of the neurodegenerative disorder Parkinson's disease). Before hES cells can be applied for treating degenerative disorders, however, reliable methods to induce differentiation of hES cells to specific mature cell types must be developed.

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There are several methods to efficiently generate tyrosine hydroxylase (TH)-positive dopaminergic neurons from mouse embryonic stem (mES) cells. The most common method relies on a multistep protocol involving in embryoid body (EB) formation followed by exposure to several growth factors and selection for nestin-positive cells *(2,3)*. Overexpression of nuclear receptor related-1 transcription factor (Nurr1) increases the proportion of TH-positive cells by more than 10-fold by this method *(3)*.

Another protocol that produces dopaminergic neurons at high frequency involves coculturing ES cells with the mouse PA6 stromal cell line *(4)*. In the absence of exogenous bone morphogenic protein 4 (BMP4), mES cells efficiently differentiate into neural precursors and neurons. Greater than 90% of cells become neural cell adhesion molecule (NCAM)-positive when cultured on PA6 cells for 1 wk. After 2 wk of coculture with PA6 under such conditions, mES cells differentiate into midbrain dopaminergic neurons at a high frequency in which 30% of neurons derived from mES cells are dopaminergic and produce significant amounts of dopamine *(4–6)*. Coculture with PA6 cells can also be employed to generate dopaminergic neurons from sub-human primate ES cells *(7)* and hES cells *(8,9)*. Although the molecular mechanism underlying neural induction by PA6 cells is not clear, the efficiency of generating TH-positive neurons by this simple method is comparable to that of the more complex method by overexpressing Nurr1 combined with multiple growth factor treatment.

In this chapter, we describe methods for generating neural progenitors via EB formation and for generating and characterizing dopaminergic neurons from hES cells by coculturing with PA6 cells.

2. Materials

2.1. Tissue Culture

2.1.1. Supplies

1. Dulbecco's modified Eagle's medium/Ham's (DMEM) (Invitrogen, Carlsbad, CA; cat. no. 1195073).
2. DMEM/F12 (Invitrogen; cat. no. 11330032).
3. Glasgow minimum essential media (GMEM) (Invitrogen; cat. no. 11710035).
4. Minimum essential medium alpha-medium (Invitrogen; cat. no. 32571036).
5. 1X phosphate-buffered saline (PBS) (Invitrogen; cat. no. 14190144).
6. Fetal bovine serum (FBS) (Hyclone, Logan, UT; cat. no. SH30071.03).
7. Knockout serum replacement (KSR) (Invitrogen; cat. no. 10828028).
8. Penicillin-streptomycin (100X) (Invitrogen; cat. no. 15140122).
9. Nonessential amino acids (Invitrogen; cat. no. 11140050).
10. L-glutamine (Invitrogen; cat. no. 25030081).
11. β-mercaptoethanol (Specialty Media, Phillipsburg, NJ; cat. no. ES-007-E).
12. Sodium pyruvate (Sigma, St. Louis, MO; cat. no. S8636).

13. Basic fibroblast growth factor (bFGF) (Sigma; cat. no. F0291).
14. Trypsin-ethylenediaminetetraacetic acid (EDTA): 0.05% trypsin, 0.53 m*M* EDTA (Invitrogen; cat. no. 25300054).
15. Cell dissociation buffer (CDB) (Invitrogen; cat. no. 13150016).
16. Dimethylsulfoxide (Sigma; cat. no. D8418).
17. Mitomycin C (Sigma; cat. no. M-0503).
18. 0.1% gelatin (Specialty Media; cat. no. ES-006-B).
19. Collagen type I-coated Biocoat 25-mm culture dish (Becton Dickinson, Franklin Lakes, NJ; cat. no. 356456).
20. Papain dissociation system (Worthington Biochemical Corp., Lakewood, NJ; cat. no. LK003150).
21. B27 Supplement (Invitrogen; cat. no. 17504-044).
22. Collagenase IV (Invitrogen; cat. no. 17104-019) (*see* **Note 1**).
23. Fibronectin, human (Invitrogen; cat. no. 33016-015).
24. Human brain-derived neurotrophic factor (R&D Systems, Minneapolis, MN; cat. no. 248-BD).
25. Human epidermal growth factor, recombinant (R&D Systems; cat. no. 236-EG).
26. Human neurotrophin 3 (R&D Systems; cat. no. 267-N3).
27. Laminin (Sigma; cat. no. L2020).
28. N2 Supplement (Invitrogen; cat. no. 17502-048).
29. Neurobasal media (Invitrogen; cat. no. 17504).
30. Poly-L-lysine 30–70 kDa (Sigma; cat. no. P9404).

2.1.2. Media

1. Medium for undifferentiated hES cells: hES cell line BG01 is maintained in DMEM/F12, supplemented with 15% FBS, 5% KSR, 2 m*M* nonessential amino acids, 2 m*M* L-glutamine, 50 µg/mL Penn-Strep, 0.1 m*M* β-mercaptoethanol and 4 ng/mL of bFGF. For 100 mL: 75 mL DMEM/F12, 15 mL FBS, 5 mL KSR, 2 mL L-glutamine, 1 mL nonessential amino acids, 0.5 mL penicillin-streptomycin, 1 mL β-mercaptoethanol, and 16 µL bFGF (25 mg/mL).
2. Medium for mouse embryonic fibroblast (MEF) cells: MEF feeder cells are maintained in DMEM, supplemented with 10% FBS and 50 µg/mL penicillin-streptomycin. For 100 mL: 90 mL DMEM, 10 mL FBS, and 0.5 mL penicillin-streptomycin.
3. Media for PA6 cells: the mouse stromal cell line PA6 is maintained in minimum essential medium, supplemented with 10% FBS and 50 µg/mL penicillin-streptomycin. For 100 mL: 90 mL minimum essential medium, 10 mL FBS, and 0.5 mL penicillin-streptomycin.
4. Medium for differentiation of hES cells on PA6: hES cells are differentiated on a monolayer of PA6 in GMEM, supplemented with 10% KSR, 2 m*M* L-glutamine, 1 m*M* sodium pyruvate, and 0.1 m*M* β-mercaptoethanol. For 100 mL: 87 mL GMEM, 10 mL KSR, 1 mL L-glutamine, 1 mL β-mercaptoethanol, and 1 mL sodium pyruvate.
5. Medium for EB differentiation: hES cell-derived EBs are maintained in DMEM supplemented with 20% FBS, 1% nonessential amino acids, 1 m*M* L-glutamine and

0.1 m*M* β-mercaptoethanol. For 100 mL: 77 mL DMEM, 20 mL FBS, 5 mL KSR, 1 mL L-glutamine, 1 mL nonessential amino acids, and 1 mL β-mercaptoethanol.

6. Media for neural progenitor proliferation: neural progenitor proliferation media comprises DMEM/F12 with B27 and N2 supplements, 10 ng/mL human epidermal growth factor, and 10 ng/mL bFGF. For 100 mL: 88 mL DMEM/F12, 1 mL B27, 1 mL N2, and 100 µL of each human epidermal growth factor (10 µg/mL) and bFGF (10 µg/mL).
7. Media for neural progenitor differentiation: Neural progenitor differentiation media is comprised of Neurobasal media supplemented with B27, 10 ng/mL human neurotrophin 3, and 10 ng/mL human brain-derived neurotrophic factor. For 100 mL: 89 mL Neurobasal, 1 mL B27, and 100 µL of each human neurotrophin 3 (10 µg/mL) and human brain-derived neurotrophic factor (10 µg/mL).

2.1.3. Cells

1 hES cells: the hES cell line BG01 was obtained from BresaGen Inc., Athens, GA; cat. no. BG01.
2. MEF feeder cells: MEF feeder cells from strain SVB are obtained from Specialty Media; cat. no. PMEF-N.
3. PA6 cells: PA6 cells are purchased from the Riken Bioresource Center Cell Bank, Koyadai, Japan; cat. no. RCB1127.

2.2. Immunocytochemistry

2.2.1. Primary Antibodies

1. Goat anti-Oct4, polyclonal, immunoglobulin (Ig)G (Santa Cruz, Santa Cruz , CA; cat. no. sc-8628).
2. Mouse anti-Nestin, monoclonal, IgG (BD Bioscience; cat. no. 611659).
3. Mouse anti-NCAM, monoclonal, IgG (Sigma; cat. no. C9672).
4. Mouse anti-β tubulin, monoclonal, IgG (Sigma; cat. no. T8660).
5. Mouse anti-tyrosine hydroxylase, monoclonal, IgG (Chemicon, Temecula, CA; cat. no. MAB318).
6. Rabbit anti-tyrosine hydroxylase, polyclonal, IgG (Chemicon; cat. no. AB151).
7. Mouse anti-bromodeoxyuridine, monoclonal, IgG (BD Pharmingen, San Diego, CA; cat. no. 555627).

2.2.2. Secondary Antibodies

1. Alexa Fluor 488 goat anti-mouse (Molecular Probes, Eugene, OR; cat. no. A-11001).
2. Alexa Fluor 488 goat anti-rabbit (Molecular Probes; cat. no. A-11008).
3. Alexa Fluor 594 goat anti-rabbit (Molecular Probes; cat. no. A-11005).
4. Alexa Fluor 594 goat anti-mouse (Molecular Probes; cat. no. A-11012).
5. Alexa Fluor 594 rabbit anti-goat (Molecular Probes; cat. no. A-11080).
6. Alexa Fluor 486 goat anti-rat (Molecular Probes; cat. no. A-11006).
7. Alexa Fluor 594 goat anti-rat (Molecular Probes; cat. no. A-11007).

2.2.3. Other Reagents

1. Blocking buffer: 5% blocking serum in 0.1% PBS/Triton-X. For 100 mL: 95 mL 1X PBS, 5 mL serum, and 0.1 mL Triton-X.
2. Fixatives: 4% paraformaldehyde. For 100 mL: 100 mL 1X PBS and 4 g paraformaldehyde.
3. Slowfade Light Antifade kit with DAPI (Molecular Probes; cat. no. S-24636).

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

1. RNA STAT-60 (Tel-Test Inc., Friendswood, TX; cat. no. CS-110).
2. Chloroform (Sigma; cat. no. C-2432).
3. Isopropanol (Fisher, Hanover Park, IL; cat. no. A416-500).
4. Ethanol.
5. DEPC-treated water.
6. Reverse transcription kit (Ambion, Austin TX; cat. no. 1710).
7. PCR master kit (Roche Molecular Biochemicals; cat. 1-636-103).
8. PCR primers (**Table 1**).

3. Methods

3.1. Coating Plates

3.1.1. Poly-L-lysine-Coated plates

1. Incubate six-well plates in 13.3 µg/mL poly-L-lysine (30–70 kDa) dissolved in sterile distilled water (1 mL/well) for 1 h.
2. Wash plates once with sterile water and air-dry before use.

3.1.2. Fibronectin-Coated Plates

1. Incubate six-well plates in 20 µg/mL fibronectin in sterile water for at least 4 h at room temperature.

3.1.3. Laminin-Coated Plates

1. Incubate six-well plates in 20 µg/mL laminin dissolved in PBS for at least 4 h at room temperature.

3.2. Maintenance of MEF

1. Thaw a frozen vial of MEF cells quickly at 37°C (*see* **Note 2**).
2. Transfer the cells into a 15-mL centrifuge tube containing 9 mL of MEF medium and centrifuge at 270*g* for 5 min.
3. Resuspend the cell pellet gently in 10 mL MEF medium and plate the cells into 10-cm dishes precoated with gelatin. Incubate cells at 37°C, 5% CO_2. The cells should form a confluent monolayer after 3–4 d.
4. Remove the medium and wash once with PBS.

Table 1
Primer Sequences

Gene name	Genbank accession no.	Primer sequence
TH	NM_000360	5′- GGTTCCCAAGAAAAGTGTCAG-3′ 5′- GGTGTAGACCTCCTTCCAG-3′
AADC	NM_000790	5′- GGGACCACAACATGCTGCTC-3′ 5′- CCACTCCATTCAGAAGGTGCC -3′
Nurr1	X75918 NM_006186 Hs. 82120	5′- CGGACAGCAGTCCTCCATTAAGGT -3′ 5′-CTGAAATCGGCAGTACTGACAGCG-3′
trkA	M23102 NM_002529 Hs.406293	5′-CCATCGTGAAGAGTGGTCTC-3′ 5′-GGTGACATTGGCCAGGGTCA-3′
trkB	S76473 NM_006180 Hs.422220	5′-AGGGCAACCCGCCCACGGAA-3′ 5′-TTGGTGGCCTCCAGCGGCAG-3′
trkC	S76475 NM_002530 Hs.26776	5′-ACGCCAGGCCAAGGGTGAGCT-3′ 5′-TTCATGACCACCAGCCACCAC-3′
GTPCH I	NM_000161	5′-CAGGAGACCATCTCAGATGTC-3′ 5′-TTCTTCTCCCTTCCCAGGCC-3′
PITX3	NM_005029	5′-ACTAGGCCCTACACAC-3′ 5′-TTTTTTTGACAGTCCGC-3′
LMX1B	NM_002316	5′-AACTGTACTGCAAACAAGACTACC-3′ 5′-TTCATGTCCCCATCTTCATCCTC-3′
PCBD	NM_000281	5′-GTGATGCCATCTTCAAGCAG-3′ 5′-CAAATTAGTGTAACAGAGCCCC-3′
QDPR	NM_000320	5′-AAGGAAGGACGGAACTCAC-3′ 5′-TCCCCAATACCAACAAATCAAC-3′
DAT	L24178 NM_001044 Hs.406	5′-AGCAGAACGGAGTGCAGCT-3′ 5′-GTATGCTCTGATGCCGTCT-3′
VMAT2	L23205 NM_003054 Hs.50458	5′-CTTTGGAGTTGGTTTTGC-3′ 5′-GCAGTTGTGATCCATGAG-3′

(continued)

Gene name	Genbank accession no.	Primer sequence
DBH	NM_000787	5′-GTGCTACATTAAGGAGCTTCCAAAG-3′ 5′-GGCCTCATTGCCCTTGGT-3′
CHAT	NM_020549 Hs.302002	5′- ATGGGGCTGAGGACAGCGAAG-3′ 5′- AAGTGTCGCATGCACTGCAGG-3′
VAChT	U10554 NM_003055 Hs.302002	5′- ACGTGGATGAAGCATACG-3′ 5′- CTGAGACATGGCGCACGT-3′
GAD67	NM_000817	5′-ATTCTTGAAGCCAAACAG-3′ 5′-TAGCTTTTCCCGTCGTTG-3′
Glutaminase (*KGA*)	AF097494 NM_014905 Hs.128410	5′-GGTCTCCTCCTCTGGATAAGATGG-3′ 5′-CCCGTTGTCAGAATCTCCTTGAGG-3′
Glutaminase (*GAC*)	AF158555 Hs.128410	5′-GATGTCCTCATTTGACTCAGGTGAC-3′
TPH1	NM_004179 Hs.356479	5′-ATGATTGAAGACAATAAGGAG-3′ 5′-AGTTTCCATACCATCTTCCTTC-3′
TPH2	NM_173353	5′-CTCTCCAAACTCTATCCCACTC-3′ 5′-GCATTCCTGTAAGCAAGTTGTC-3′
p75	NM_002507	5′-AGCCAACCAGACCGTGTGT-3′ 5′-TTGCAGCTGTTCCACCTCTT-3′
c-RET		5′-AGGAGGCTGAGTGGGCTACGT-3′ 5′-GGACCTCAGATGTGCTGT-3′
GFRA1	NM_005264	5′-AGGGAAATGATCTGCTGGAGGA-3′ 5′-CTCTGGCTGGCAGTTGGTAAAA- 3′
GFRA2	NM_001495	5′-AGGCCCTGCGCCAGTTCTTCGA-3′ 5′-ACGTTCACGTCCGTGCCGTTGC-3′
GFRA3	NM_001496	5′-CTGCACCTCTAGCATAAGCACC-3′ 5′-GGCCTTCTCGAAGAAAGTGAGC-3′
PTCH	NM_000264	5′-TCCCAAGCAAATGTACGAGCA-3′ 5′-TGAGTGGAGTTCTGTGCGACAC-3′
Smo	NM_005631	5′-TATTCACTCCCGCACCAAC-3′ 5′-AGCCAGACATCCAGAACTC-3′

5. Add 3 mL trypsin-EDTA and incubate the dish at 37°C, 5% CO_2, until the cells come off the dish (approx 5 min). Add 7 mL MEF medium and break any cell aggregates by gently pipetting. Plate the cells into five 10-cm dishes (approx 5×10^5 cells/dish).
6. Passage the cells again on confluence. MEF cells are maintained only to the seventh passage.

3.3. Preparation of MEF Feeder Layers

1. Remove the medium from the confluent MEF dish and add 10 mL MEF medium containing 10 μg/mL mitomycin C (*see* **Note 3**). Swirl dish to ensure an even distribution of medium.
2. Incubate cells at 37°C, 5% CO_2, for 2–2.5 h.
3. Wash the monolayer of cells twice with 10 mL PBS. Change the medium to hES medium before feeding hES cells (*see* **Note 4**).

3.4. Thawing hES Cells

1. Transfer a cryovial of hES cells quickly into a 37°C water bath (*see* **Note 5**). Transfer vial content into a 15-mL tube containing 10 mL of prewarmed hES medium immediately after the cells are thawed.
2. Count cells and assess viability.
3. Centrifuge at 270*g* for 5 min, resuspend the cell pellet gently in an appropriate volume of hES medium, and plate at 5×10^5 cells per 60-mm dish.

3.5. Maintenance of Undifferentiated hES Cells

1. Passage the cells when confluent (*see* **Note 6**). For a 60-mm dish, gently aspirate the medium, add 4 mL PBS and incubate for 4 min at room temperature.
2. Remove the PBS and add 2 mL CDB. Gently pipet the cells up and down until most of the hES cells detach from the MEF feeders (*see* **Note 7**).
3. Transfer the hES cells to a 15 mL centrifuge tube containing 9 mL of hES medium and centrifuge at 270*g* (or 1000 rpm) for 5 min (*see* **Note 8**).
4. Gently resuspend the cell pellet in hES medium. Count cells and plate 5×10^5 cells per 60-mm dish on the MEF layer.
5. Leave the plated hES cells untouched for 48 h, then change the medium daily.

3.6. Freezing hES Cells

1. Wash undifferentiated hES cells in the exponential phase of growth with PBS and collect the cells by CDB (*see* **Subheading 3.5., step 2**).
2. Centrifuge at 270*g* for 5 min and resuspend the cells in 0.5 mL of solution containing 50% hES medium and 50% FBS at a density of $1–2 \times 10^6$ cells/mL.
3. Add 0.5 mL of solution containing 80% hES medium and 20% dimethylsulfoxide.
4. Transfer the hES cells to a polypropylene cryovial and place the vial in an isopropanol freezing chamber in a –70°C freezer overnight (*see* **Note 9**). Transfer the cryovial to liquid nitrogen.

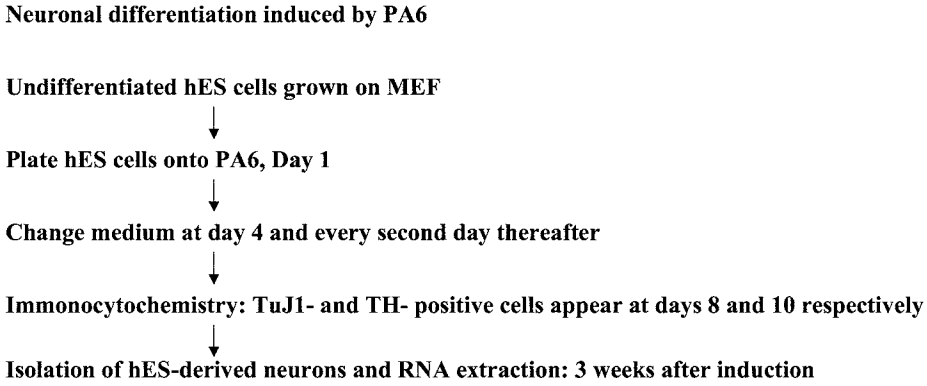

Fig.1. Neuronal differentiation of human embryonic stem cells induced by the mouse stromal cell line PA6. The process of generating dopaminergic neurons is illustrated.

3.7. Maintenance of PA6 Cells

1. Remove PA6 medium and wash once with PBS (*see* **Note 10**).
2. For a T-25 flask, add 2 mL trypsin-EDTA and incubate at 37°C until the cells come off the dish (approx 5 min). Add 4 mL PA6 medium and break any cell aggregates by gently pipetting. Plate the cells into five T-25 flasks (approx 3×10^5 cells/flask).
3. Passage cells again on confluence (3 d).

3.8. Preparation of PA6 Feeder Layers

1. Plate PA6 cell suspensions into 35-mm collagen type I-coated dishes; make sure that a confluent monolayer is produced (*see* **Note 11**).
2. Allow PA6 cells to attach overnight.

3.9. Neuronal Differentiation of hES Cells on PA6

1. Wash undifferentiated hES cells with PBS and collect the cells by CDB (*see* **Subheading 3.5., step 2**).
2. Transfer the hES cells to a 15-mL centrifuge tube containing 9 mL of hES differentiation medium and centrifuge at 270*g* for 5 min.
3. Resuspend the cells in hES differentiation medium, count the cells, and dilute them to a density of 2×10^3 clumps/mL (*see* **Fig. 1** and **Note 12**).
4. Rinse PA6 feeder cells with hES differentiation medium.
5. Plate 30 µL of hES cells into each 35-mm dish and incubate cells at 37°C, 5% CO_2.
6. Change the medium on d 4 and every second day thereafter.
7. Fix and stain the cells with appropriate antibodies as needed. Nestin is expressed during the entire period of differentiation, and about 90% of colonies contain NCAM and TuJ1-positive cells after 2 wk of differentiation (*see* **Fig. 2**). TH-positive colonies first appear in culture between d 8 and 10 (*see* **Fig. 3**). After 3 wk of differentiation, approx 80–90% of the hES colonies will contain TH-positive neurons, and a high percentage of the cells in most of the colonies express TH (*see* **Note 13**).

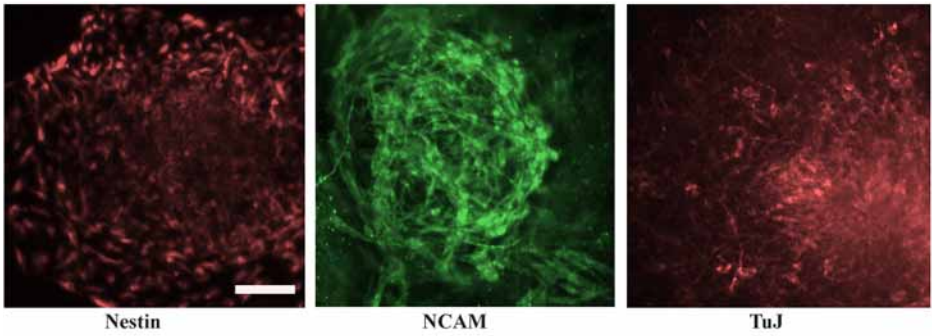

Fig. 2. Differentiation into neuronal precursors. After 2 wk on a layer of PA6 cells, most colonies are positive for nestin, neural cell adhesion molecule and TuJ1. The scale bar is 20 μm. (Please *see* the companion CD for the color version of this figure.)

3.10. Isolation of Differentiated Colonies From PA6 Feeder Layer

1. Reconstitute papain, ovomucoid inhibitor, and DNase according to the manufacturer's directions (*see* **Note 14**).
2. Rinse differentiated hES cells with PBS containing Ca^{2+} and Mg^{2+} (*see* **Note 15**). Remove PBS and add 0.5 mL of the reconstituted papain solution for a 35-mm dish and incubate at 37°C for 2 min.
3. Gently rock the dish until the colonies are detached from the feeder layer.
4. Transfer the colonies into a 15-mL tube and spin the cells down briefly at 270*g*. Resuspend the cells in appropriate medium.

3.11. Neuronal Differentiation of hES Cells Via EB Formation

3.11.1. EB Formation

1. Aspirate the medium from the hES cells and add 1 mL collagenase IV (200 U/mL) to each well if using six-well plates (otherwise, adjust volumes accordingly) and incubate at 37°C for 5–10 min.
2. Aspirate the collagenase IV and add 1–2 mL of differentiation medium to each well.
3. Scrape the cells off the surface with a cell scraper and transfer them to low attachment plates (*see* **Note 16**).
4. Add differentiation medium to each well for a final volume of 4 mL/well (*see* **Note 17**).
5. Change medium every 2–3 d by transferring the EBs into a 15-mL tube and letting the aggregates settle for about 5 min. Aspirate the supernatant, add fresh differentiation media, and transfer the EBs back to the low attachment plates.

3.11.2. Generation of hES Cell-Derived Neural Progenitors

1. Maintain EBs in suspension culture for 4 d.
2. Harvest EBs by transferring them into a 15-mL tube and letting the aggregates settle for about 5 min.

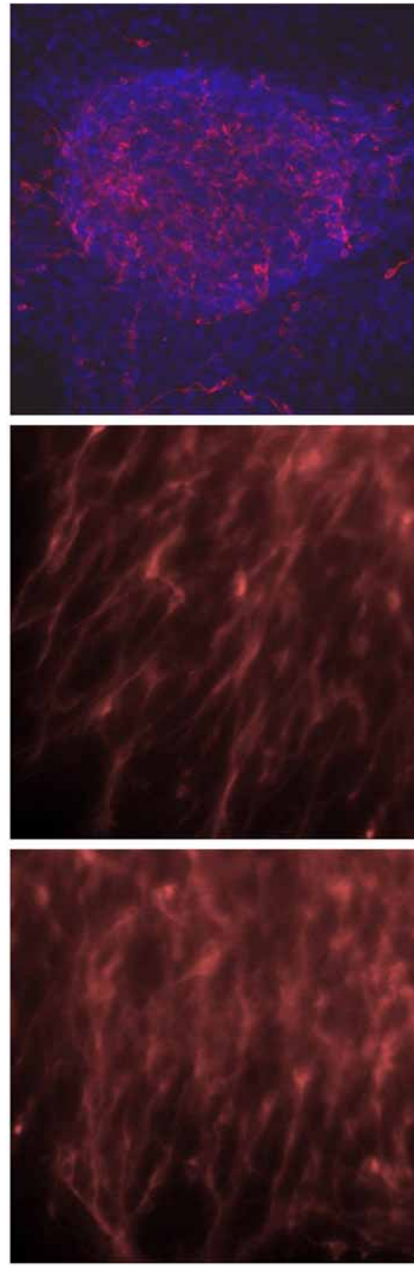

Fig. 3. Differentiation of human embryonic stem cells into dopaminergic neurons. Immunostaining of PA6-induced neurons with anti-tyrosine hydroxylase (TH) after 18 d of coculture with PA6 cells. A colony containing TH-positive cells (top) and some single TH-positive cells (middle and bottom) are shown in the figure. (Please *see* the companion CD for the color version of this figure.)

3. Aspirate media and resuspend EBs in neural progenitor proliferation media.
4. Plate EBs onto poly-L-lysine/fibronectin-coated plates (*see* **Note 18**). The EBs will adhere to the plate, and cells will migrate out of the EBs over the next few days.
5. Change the media every other day.

3.11.3. Differentiation of hES Cell-Derived Neural Progenitors

1. Harvest the plated EBs after 3–7 d in proliferation conditions (when cultures are about 80% confluent) by incubating in trypsin for 2–3 min at 37°C. Watch the cells carefully and remove the trypsin when the cells round up and begin to release from the plate.
2. Remove the cells from the dish with a cell scraper or a pipet.
3. Resuspend the cells in neural progenitor differentiation media.
4. Plate on poly-L-lysine/laminin coated plates (*see* **Note 19**).
5. Change the media three times per week.

3.12. Immunocytochemistry

3.12.1. Single Staining

1. Aspirate medium from dish and wash three times with PBS.
2. Add 4% paraformaldehyde to cover for 10 min.
3. Wash the cells three times with PBS.
4. Add 5% blocking serum for 20 min.
5. Prepare primary antibodies in 8% goat serum in 0.1% PBS/Triton-X (*see* **Note 20**).
6. Aspirate blocking reagent, then add primary antibody solution overnight at 4°C.
7. Aspirate the primary antibody solution and wash three times with PBS.
8. Prepare secondary antibodies in 8% goat serum in 0.1% PBS/Triton-X.
9. Add secondary antibody solution for 1 h in the dark at room temperature.
10. Wash three times with PBS.
11. Add equilibration buffer from Antifade Kit (enough to cover).
12. Aspirate the buffer and add three to four drops of antifade reagent in glycerol buffer with DAPI.
13. Add two to three drops antifade reagent in PBS and keep in dark at 4°C until viewing.
14. Observe the staining under a fluorescence microscope with appropriate filter combinations.

3.12.2. Double Staining

Double or triple labeling of different antigens in single cells is in many cases needed to localize the antigens. The following is an example of a double labeling for BrdU and TH.

1. Add 10 μ*M* of BrdU into the culture at 37°C, 5% CO_2, for 24 h.
2. Fix the cells with 4% paraformaldehyde for 15 min at room temperature and wash three times with PBS.
3. Add 95% methanol for 30 min at –20°C and wash three times with PBS.
4. Permeabilize the cells with 2 *M* HCl for 10 min at room temperature followed by washing three times with PBS.

5. Neutralize residual HCl with 0.1 *M* sodium borate for 10 min at room temperature.
6. Rinse once with PBS and incubate the cells with 10% goat serum in PBS with 0.1% Triton X-100 for 20 min at room temperature.
7. Incubate with anti-BrdU antibody and anti-TH for 1 h at 37°C (*see* **Note 21**).
8. Wash three times with PBS and incubate with appropriate secondary antibodies for 30 min at room temperature in the dark.
9. Wash three times with PBS and counter stain with DAPI before viewing under fluorescence microscope.

3.13. RT-PCR

3.13.1. RNA Extraction

1. Collect undifferentiated cells by CDB (*see* **Subheading 3.5., step 5**) or differentiated cells using the Papain dissociation system (*see* **Subheading 3.10.**).
2. Transfer cells to a 15-mL tube and centrifuge at 270*g* for 5 min.
3. Remove supernatant and resuspend pellet in 1 mL RNA-STAT 60 (for 10^6–10^7 cells). Pipet up and down to lyse cells. Transfer the lysates into a 1.5-mL tube and incubate 5 min on ice.
4. Add 200 µL of chloroform and gently mix by inversion six to eight times. Incubate 3 min on ice.
5. Centrifuge at 13,000*g* for 15 min.
6. Transfer aqueous phase to a new 1.5-mL tube.
7. Add 500 µL of isopropanol, vortex, and incubate 10 min on ice.
8. Centrifuge at 13,000*g* for 15 min.
9. Wash twice with 70% EtOH. Air-dry the pellet and resuspend in 50 µL DEPC-treated water.

3.13.2. Reverse Transcription

1. Mix 1–5 µg RNA (bring up to 20 µL by H_2O), 20 µL dNTP, 10 µL decamers, and 30 µL H_2O.
2. Denature at 72°C for 3 min.
3. Chill on ice; quick spin.
4. Add 10 µL 10X buffer, 5 µL RNase inhibitor, and 5µL RT.
5. Incubate at 42°C for 1 h.

3.13.3. PCR

1. Prepare the PCR mixture: 1–10 µL template DNA, 4 µL dNTP (2.5 m*M* each), 5 µL 10X Taq buffer, 4 µL 5′ primer, 4 µL 3′ primer, 1 µL Taq polymerase, and 22–31 µL H_2O.
2. Run PCR cycles as desired; for example:
   a. Hot start at 94°C for 2–5 min.
   b. Thirty-five cycles of the following: 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s.
   c. Extend at 72°C for 10 min and hold at 4°C.

4. Notes

1. In our hands, 200 U/mL is usually 1 mg/mL.
2. All materials and reagents for cell culture must be sterile. All media and solutions must be prewarmed to 37°C. Medium with growth factors should be used within 1 wk.
3. Mitomycin C is highly toxic. We use premeasured mitomycin C solution in glass vials.
4. A very high MEF density (2×10^6 cells per 60-mm dish) is required for BG01 line. Lower MEF densities result in differentiation of BG01 cells.
5. hES cells are very sensitive to pH and temperature changes. One must work quickly but gently when culturing hES cells. After the cells are thawed, they need to be tended *every day* and there is *no shortcut* for this routine care. hES medium more than 1 wk old should not be used. We make fresh medium every week.
6. For a 60-mm dish, the yield is typically $5–10 \times 10^6$ cells when seeded with 5×10^5 cells 5 d previously. The cells are passaged every 5 d.
7. It is important to minimize the time during which hES cells are exposed to CDB. We recommend that hES cells not be exposed to CDB more than 2 min.
8. If large debris from the feeder cells is present, allow the feeders to settle out and remove the supernatant.
9. We use the NALGENE Cryo 1°C freezing container to freeze cells.
10. PA6 cells should be carefully maintained in culture and frequently passaged (every 3 d).
11. We normally use one T-25 flask cells for two 35-mm dishes.
12. hES colonies are formed from small clumps of hES cells (approx 10 cells per clump). The cell numbers increase more than 100-fold within 2 wk of differentiation.
13. Immunocytochemistry is routinely used to examine the percentage of tyrosine hydroxylase positive (TH) colonies. We expect about 85% of the colonies to express TH after 3 wk of differentiation.
14. We routinely use the Papain dissociation system to isolate hES cells from the PA6 feeder layer for RNA purification or other purposes.
15. PBS containing Ca^{2+} and Mg^{2+} should be used for isolating hES colonies from PA6 cells using papain, because activity is weakened in the absence of Ca^{2+}.
16. We find that a 1:1 or 1:2 split ratio works well, depending on the confluency of the hES cells.
17. EBs should be apparent the day after harvest and will grow in size and complexity over time in culture.
18. We find that a 1:1 ratio works well, depending on the density of EBs.
19. We find that a 1:1 ratio works well, depending on cell density.
20. To avoid frequent freezing and thawing of antibodies, primary antibodies should be frozen in aliquots.
21. Make sure the host species for the two primary antibodies are different.

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13

In Vitro Differentiation of Neural Precursors From Human Embryonic Stem Cells

Xue-Jun Li and Su-Chun Zhang

Summary

We describe a procedure for efficient and reproducible differentiation of neuroectodermal cells from human embryonic stem (ES) cells using an adherent colony culture. ES cell colonies are detached intact from the fibroblast feeder layer using dispase or collagenase. The ES cell aggregates, after 4–6 d in suspension culture, are adhered to the culture surface and form colonies of monolayer in a chemically defined medium. Under this culture condition, columnar neuroectodermal cells appear in the center of each colony and organize into neural tube-like rosettes after 14 d of differentiation culture. These neuroectodermal cells in the rosettes can be effectively isolated through differential enzymatic and adhesion treatment and the neural population accounts for at least 70% of the total differentiated progenies. Thus, our system not only provides a source of synchronized neuroectodermal cells, but also offers a paradigm to dissect mechanisms of neural induction and cell lineage specification during early human development.

Key Words: Neural induction; embryonic stem cells; neural precursor; differentiation; cell replacement.

1. Introduction

Embryonic stem (ES) cells are in vitro counterparts of the inner cell mass of a preimplantation embryo at the blastocyst stage and can be expanded in an artificial culture environment for a prolonged period with a stable genetic background *(1,2)*. They have the ability to generate almost all cell types of the body including neural cells and, thus, offer an in vitro model for tracing early cell lineages in mammals. ES cells established from human embryos *(3)* are also a source for potential future cell therapy. A first step toward the use of ES cells is a directed differentiation to a specific cell lineage such as neuroectodermal lineage.

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Developmental principles learned from animal studies form the basis of designing protocols for directed neural differentiation. Studies in low vertebrate animals such as amphibian, zebrafish, and chick indicate that activation of fibroblast growth factor (FGF) signaling or inhibition of bone morphogenetic protein signaling is necessary for specification of the neuroectoderm from the embryonic ectoderm *(4–6)* (for review, *see* **ref.** *7*). The neuroectoderm folds to form the neural tube, during which it is patterned into regionally specialized domains along rostrocaudal and ventral–dorsal axes. Progenitor cells in each region differentiate to postmitotic neurons with unique transmitter and positional identities. In humans, the neuroectoderm is specified in the third week of gestation and the neural tube is formed by the end of the third week of gestation *(8)*.

The most commonly used approach for neural differentiation from mouse ES cells is the spontaneous aggregation of ES cells into the so-called embryoid bodies and treatment of these ES cell aggregates with retinoic acid to promote neural differentiation *(9)* or with other morphogens such as FGF2 to preferentially promote neuroepithelial proliferation *(10)*. Recently, protocols are developed to guide mouse ES cells toward neuroectoderm *(11)* and then to specialized neurons such as dopaminergic and motor neurons based on developmental principles *(12,13)* (for review, *see* **ref.** *14*).

We have designed a chemically defined colony culture system to induce human ES cells toward a neural fate based on the developmental principles. This neural differentiation protocol takes into consideration the similarities and differences between human and mouse ES cells. We also expect that this in vitro system will mimic in vivo neural development in humans and that it will allow us to dissect mechanisms of early human neural development. Because human ES cells survive better as clusters as opposed to disaggregated individual cells, the neural differentiation process is initiated by detachment of ES cell colonies from the fibroblast feeder layer by enzymes such as dispase or collagenase and grown as aggregates in suspension *(15,16)*. Most differentiation protocols involve treatment of these ES cell aggregates with morphogens or growth factors in suspension for neural differentiation. However, such suspension culture system has significant drawbacks. ES cell aggregates grown in suspension culture for an extended period often form cysts, resulting in stochastic rather than directed differentiation. An unusually high concentration of morphogens or growth factors is required for the factors to reach cells inside the aggregates *(15–18)*. Even so, cells on the surface and those inside the aggregates will have a varied degree of exposure to morphogens, thus, creating a wide range of cell lineages or cells at various developmental stages. The cluster nature also makes it difficult to visualize the continual change in cell morphology in response to treatments. For these reasons, we plate the ES aggregates to a plastic culture surface in a serum-free neural medium at a low density so that the aggregates

form individual colonies (*19*). The colony culture allows semiquantitation analysis and the monolayer nature also permits continual assessment of changes in cell morphology. Cells in the colony center transform into small columnar cells, whereas those in the periphery gradually become flattened. The small columnar cells organize into neural tube-like rosette formations by 7–10 d after plating the aggregates. The rosette-forming columnar cells express transcription factors such as Pax6 and Sox1, confirming the neural identity. Thus the neuroectodermal differentiation process resembles normal human neural development in terms of timing and morphology.

2. Materials

2.1. Supplies

1. T25 flasks, the polystyrene flasks with polyethylene filter cap (Fisher Scientific, Pittsburgh, PA; cat. no. 12-565-57; or Nunc, Roskilde, Denmark; cat. no. 136196).
2. T75 flasks, the polystyrene flasks with polyethylene filter cap (Fisher Scientific; cat. no. 12-565-31; or Nunc; cat. no. 178891).
3. Polystyrene plates, 6-well and 24-well (Fisher Scientific; cat. no. 12-565-73 and 12-565-75; or Nunc; cat. no. 140675 and 143982).
4. Polystyrene conical tube, 15- and 50-mL (Fisher Scientific; cat. no. 05-527-90 and 14-432-23; or BD Biosciences, Bedford, MA; cat. no. 352095 and 352073).
5. Serological pipets 5-, 15-, and 30-mL (Fisher Scientific; cat. no. 13-678-11D, 13-678-11E and 13-678-11).
6. 9-in. Pasteur pipets (Fisher Scientific; cat. no. 13-678-8B and 13-678-20D).

2.2. Stock Solutions

1. L-glutamine solution (200 m*M*) (Sigma, St. Louis, MO; cat. no. G7513). Make aliquots of 2.5 mL and store at –20°C.
2. MEM nonessential amino acids solution (Gibco-BRL, Rockville, MD; cat. no. 11140-050).
3. Kockout serum replacer (Gibco-BRL; cat. no. 10828-028). Make aliquots of 50 mL and store at –20°C.
4. Dulbecco's modified Eagle's medium (DMEM): nutrient mixture F-12 1:1 (DMEM/F12) (Gibco-BRL; cat. no. 11330-032).
5. β-mercaptoethanol (14.3 *M*) (Sigma; cat. no. M7522).
6. Recombinant human FGF basic (R&D Systems, Minneapolis, MN; cat. no. 233-FB) is dissolved in sterilized PBS with 0.1% bovine serum albumin (Sigma; cat. no. A-7906) at a final concentration of 10 µg/mL. Aliquot 0.5 mL into sterilize tubes and store at –80°C.
7. Dispase solution (1 mg/mL): dissolve 10 mg dispase (Gibco-BRL; cat. no. 17105-041) in 10 mL F12/DMEM in a water bath for 15 min (*see* **Note 1**) and filter-sterilize the dispase solution with a 50-mL Steri-flip (Fisher Scientific; cat. no. SCGP00525).

8. Heparin (1 mg/mL); dissolve 10 mg heparin (Sigma; cat. no. H3149) in 10 mL DMEM medium. Aliquot 0.5 mL into sterilized tubes and store at –80°C.
9. DMEM (Gibco-BRL; cat. no. 11965-092).
10. F-12 nutrient mixture (HAM) (Gibco-BRL; cat. no. 11765-054).
11. N2 supplement (Gibco-BRL; cat. no. 17502-048).
12. Fibronectin from human plasma (Sigma; cat. no. F2006).
13. Fetal bovine serum (Gibco-BRL; cat. no. 16000-044).
14. Laminin from human placenta (Sigma; cat. no. L6274).
15. Poly-2-hydroxyethylmethacrylate (Poly-HEME) (Sigma; cat. no. P3932).

2.3. Media

1. ES cell growth medium. In a 500-mL filter unit (0.22-µm sterilizing low protein-binding membrane) (Corning Incorporated, Corning, NY; cat. no. 430513), combine 392.5 mL DMEM-F12, 100 mL knockout serum replacer, 5 mL MEM nonessential amino acids solution, 2.5 mL of 200 m*M* L-glutamine solution (final concentration of 1 m*M*), and 3.5 µL 14.3 *M* β-mercaptoethanol (final concentration of 0.1 m*M*). Filter and store at 4°C for up to 7–10 d.
2. Neural induction medium (DMEM/F12/N2). Sterilely combine: 163 mL F-12, 326 mL DMEM, 5 mL N2 supplement, 5 mL MEM nonessential amino acids solution, and 1 mL of 1 mg/mL Heparin (*see* **Subheading 2.2., item 8**). Media be stored at 4°C for up to 2 wk.

3. Methods

3.1. Aggregation of ES Cells (Formation of Embryonic Bodies)

1. Aspirate medium from each well of the six-well plate containing the ES cells (*see* **Fig. 1A**).
2. Add to each well 1 mL of fresh ES cell growth medium (s*ee* **Subheading 2.3., item 1**) with dispase (*see* **Subheading 2.2., item 7**) at a final concentration of 0.5–1 mg/mL.
3. Incubate in a CO_2 incubator for 5 min; observe the cells every 2 min under a phase-contrast microscope until the edges of ES cell colonies begin to curve up (*see* **Fig. 1B**). It usually takes about 5–10 min to reach this point.
4. Aspirate the dispase-containing medium from the six-well plate and add fresh, warm ES cell medium to each well (1.5 mL/well).
5. Swirl the six-well plate to dislodge the ES cell colonies from the mouse fibroblast feeder layer.
6. Use a 5-mL pipet to gently blow the ES cell colonies off the bottom of the well; if some ES cell colonies remain attached, pool all the cells to a 15-mL tube (*see* **Note 2**).
7. Centrifuge at 200*g* for 5 min at room temperature.
8. Aspirate the medium off the cell pellet.
9. Resuspend the ES cell colonies in 10–12 mL ES cell medium and culture them in a T25 flask (*see* **Note 3**).

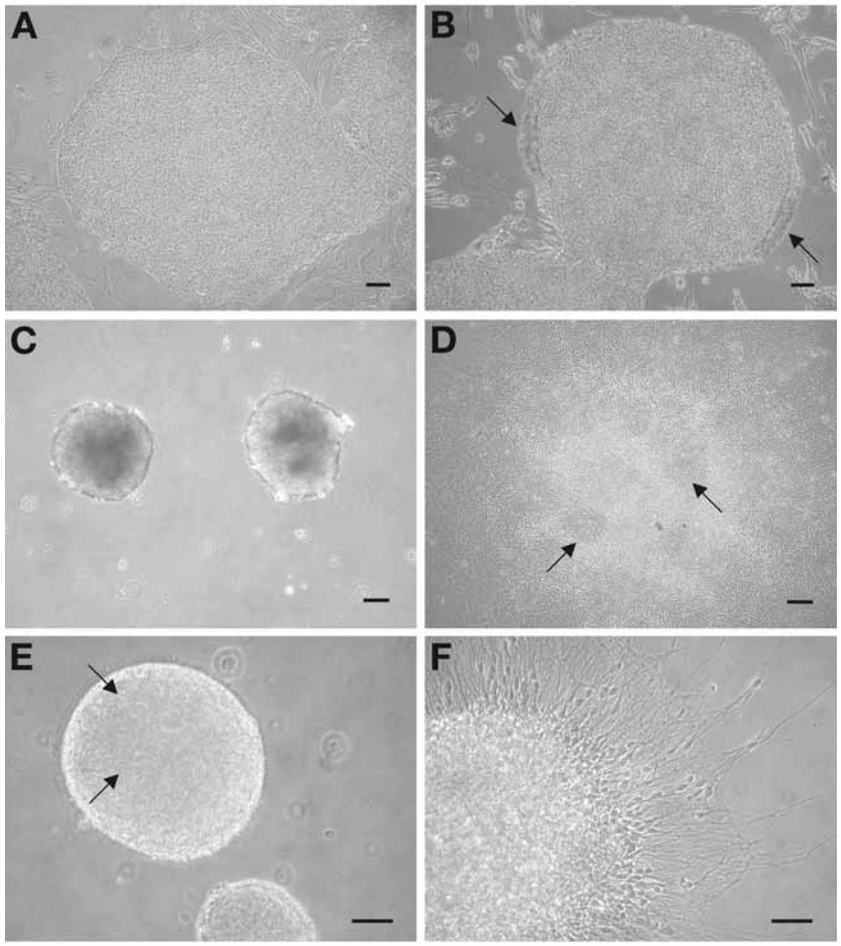

Fig. 1. Differentiation of neuroectodermal cells from human embryonic stem (ES) cells. **(A)** Phase contrast image showing an ES cell colony grown on the mouse embryonic fibroblasts feeder layer. **(B)** The edge of the ES cell colony, after treatment with dispase for 5 min, began to curve up (arrows). **(C)** ES cell aggregates suspended in the ES cell growth medium for 4 d. **(D)** ES cell aggregates, after 10 d of culture in the neural induction medium, developed multiple rosettes (arrows) in the colony center. **(E)** Rosettes cells, separated from peripheral flat cells and cultured in neural induction medium with FGF2 (10 ng/mL), formed spheres, which have rosettes inside (arrows). **(F)** After differentiating the neuroectodermal cells on the polyornithine and laminin substrate for 1 wk, numerous neurites extended out of the sphere. Bar = 50 μm.

10. The ES cell aggregates (*see* **Note 4, Fig. 1C**) should be fed every day and cultured for 4 d. If there are mouse embryonic fibroblasts attaching to the flask, you may get rid of them simply by transferring the floating ES cell aggregates to a new flask (*see* **Note 5**). To feed the ES cell aggregates, set the flask down at a titled angle so that the ES cell aggregates settle in one corner of the flask. Aspirate off about half of the medium and add fresh ES cell medium to replace the amount of aspirated.

3.2. Neuroepithelial Differentiation (Formation of Neural Tube-Like Rosettes)

1. After culturing for 4 d, the ES cell aggregates are ready to be differentiated.
2. Collect the ES cell aggregates and transfer them to a 15-mL centrifuge tube.
3. Centrifuge at 50*g* for 1–2 min at room temperature.
4. Wash with 5 mL neural induction medium (*see* **Subheading 2.3., item 2**).
5. Centrifuge at 50*g* for 1–2 min at room temperature.
6. Aspirate off medium and resuspend ES cell clusters in 8–10 mL neural induction medium supplemented with 10 ng/mL FGF2 (final concentration, *see* **Subheading 2.2., item 6**) and transfer to a T25 flask.
7. After 2 d in the neural induction medium, the ES cell aggregates attach to the plastic substrate (*see* **Note 6**). Once attached, feed the culture with neural induction medium with 10 ng/mL FGF2 every other day.
8. Observe the culture daily (*see* **Note 7**). There is a temporal change of cell morphology in each colony. At 1–2 d after attachment, cells in the colony center become elongated compared with those in the periphery. In another 2–3 d, these elongated cells in the colony center become compacted and start to form rosettes. With further culturing in the same medium for another 4–5 d, the area and thickness of rosettes increase and almost every colony contains multiple neural tube-like rosettes often with lumens (*see* **Note 8, Fig. 1D**).

3.3. Isolation of Neuroepithelial Cells in the Neural Tube-Like Rosettes (see Note 9)

1. Rinse the rosette culture with PBS once and add fresh neural induction medium with dispase (*see* **Subheading 2.2, item 7**) at a final concentration of 0.2–0.5 mg/mL.
2. Incubate the culture for 15–30 min in a CO_2 incubator until the clump of rosettes retract and begin to come off the plate. At this stage, most of the peripheral flat cells remain attached (*see* **Note 10**).
3. Gently swirl the flask to release rosettes clumps. Let the flask stand and collect the rosette clumps into a 15-mL centrifuge tube. Triturate the clumps with a 5- or 10-mL serological pipet up and down twice, but not to break up the clumps.
4. Centrifuge at 50*g* for 2 min at room temperature.
5. Wash cells with 5 mL of neural induction medium and centrifuge again at 50*g* for 2 min at room temperature.
6. Aspirate the medium, resuspend the cells in 10–15 mL of neural induction medium +10 ng/mL FGF2, and transfer the cells to a T25 flask. Place in an

incubator for about 2 h to allow the non-neural cells to differentially attach to the flask.

7. Transfer the floating cells (mostly aggregates of neural rosette cells) to a new flask. The rosette aggregates will roll up to form round clusters after about 1 d (*see* **Note 11, Fig. 1E**).
8. Feed the neuroectodermal aggregates every other day with neural induction medium +10 ng/mL FGF2 (*see* **Notes 12** and **13**).

4. Notes

1. Dispase should be incubated in a 37°C water bath for 10–15 min to dissolve completely before filter. Make fresh dispase solution before use.
2. Under most circumstances, swirling the plate will dislodge most ES cell colonies. Use gentle force to blow the remaining attached ES cell colonies to avoid the detachment of fibroblasts. After removal of ES cell colonies, the plate will be left with a layer of mouse fibroblasts in which there are empty "holes" where ES cell colonies used to sit.
3. The amount of the medium depends on the number of ES cell colonies. In general, 10–12 mL of medium is appropriate for ES cells from two wells of a six-well plate.
4. The aggregates of ES cells described here is to initiate a differentiation process. The aggregates cultured for 4 d do not display structures of the three germ layers and form cysts inside; hence, they do not resemble an embryo. These aggregates, after plating onto the mouse fibroblast feeder layer, can regrow as ES cells. We therefore recommend the name of "ES cell aggregates" instead of embryoid bodies, a more widely used name.
5. ES cell aggregates usually do not attach under this culture condition. They may attach if there are a lot of contaminating fibroblasts, which attach and reform a "feeder." In that case, transfer the aggregates right after the fibroblasts attach to the flask. This can be repeated to remove the contaminating fibroblasts.
6. ES cell aggregates, after switching from ES cell growth medium to FGF-containing neural induction medium for 2 d, become brighter and healthier looking. They are now ready to attach to the plastic substrate for neural differentiation. If the aggregates do not attach, they may be plated onto the culture vessels that are precoated with fibronectin (20 µg/mL) or laminin (20 µg/mL). Alternatively, addition of 10% fetal bovine serum into the culture overnight will promote the attachment of the aggregates. It should be noted that fetal bovine serum has an adverse role in neural induction and thus it should be avoided or used as short time as possible.
7. This protocol mimics in vivo neuroectodermal development in terms of timing and morphology. It takes about 12–14 d for the human ES cells to differentiate into columnar neuroectodermal cells that organize into neural tube-like rosettes. Considering ES cells are equivalent to a d 5–6 embryo, development of the neuroectoderm in vitro takes about 18–20 d, the time window when the neural tube forms in a human embryo (***8***,***20***).

8. There is a temporal change of cell morphology in each colony. Such temporal pattern may change if the ES cells are already partially differentiated or if the ES cell aggregates do not attach according to the schedule described.
9. This method works well when neuroepithelial cells in the neural tube-like rosettes accumulate in the colony center whereas the peripheral cells remain a monolayer of flat cells, creating a clear boundary between rosettes and the peripheral cells. It happens usually around 10 d after plating the ES cell aggregates. Other techniques may be developed if a set of neuroectodermal cell-specific surface markers are available.
10. Because the differential response of rosette cells vs surrounding cells to dispase is very narrow, it is extremely important to watch the culture closely to obtain best separation.
11. These rosette clusters can be maintained or expanded in the suspension culture for several weeks (*see* **Fig. 1E**). These neuroectodermal cell clusters are different from the neurospheres that are formed from the fetal or adult tissues. The ES cell-generated neuroectodermal spheres contain rosettes inside. The rosettes gradually disappear after several weeks of expansion, which then resemble brain-derived neurospheres. The morphogens to be used depend on the objective of the study.
12. The human ES cell-derived neuroectodermal spheres do not normally attach to general plastic culture surface. If they attach easily, it usually suggests contamination of non-neural lineage cells. The neuroectodermal spheres can be detached by gently tapping the flask. Alternatively, they can be transferred to a bacterial-grade Petri dish, or poly-HEME coated flask to prevent cell attachment.
13. Neuroepithelial cells isolated using this protocol should consist of at least 95% of the cells that are stained positively for nestin and musashi-1, the commonly used neuroepithelial markers. These ES-generated neuroepithelial cells can be expanded as neurospheres or can be differentiated into neurons and glia cells under appropriate culture conditions (*see* **Fig. 1F**).

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14

Derivation and Characterization of Hematopoietic Cells From Human Embryonic Stem Cells

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Summary

In vitro, the aggregation of pluripotent human embryonic stem cells (hESC) into cell clusters termed embryoid bodies (EB) allows for the spontaneous differentiation of hESC into progeny representing endoderm, mesoderm, and ectoderm lineages. During human EB (hEB) differentiation, stochastic emergence of hematopoietic cells can be enhanced by a combination of hematopoietic cytokines and the ventral mesoderm inducer bone morphogenetic protein (BMP)-4. Dependent on the presence of hematopoietic cytokines and BMP-4, vascular endothelial growth factor (VEGF-A_{165}) selectively promotes erythropoietic development toward the primitive lineage. The effects of VEGF-A_{165} can be augmented by erythropoietin (EPO). Hematopoietic cells are derived from a rare subpopulation of hemogenic precursors during hEB development. These hemogenic precursors lack CD45, but express PECAM-1, Flk-1, and VE-cadherin (hereinafter $CD45^{neg}$PFV) and are solely responsible for hematopoietic cell fate. Human ESC-derived hematopoietic cells have similar colony and cellular morphologies to those derived from committed adult hematopoietic tissues, and also show repopulating capacity in immune deficient mice after intrabone marrow transplantation. In this chapter, we describe methods that have been successfully applied in our laboratory, including (1) generation of hematopoietic cells by EB formation; (2) augmentation of hematopoiesis by use of hematopoietic cytokines and BMP-4; (3) promotion of erythropoietic development by addition of VEGF-A_{165} and EPO; (4) isolation of $CD45^{neg}$PFV hemogenic precursors and generation of hematopoietic cells from these precursors; and (5) characterization of hESC-derived hematopoietic cells in vitro and in vivo.

Key Words: hESC; hematopoiesis; BMP-4; VEGF; precursor; transplantation; NOD/SCID mice.

1. Introduction

Human embryonic stem cells (hESC) are pluripotent cells and can grow infinitively in feeder-free media *(1)*. In vitro, the aggregation of hESC into cell

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clusters termed embryoid bodies (EB) allows for the spontaneous differentiation of hESC into progeny representing endoderm, mesoderm, and ectoderm lineages *(2,3)*. During human EB (hEB) differentiation, stochastic emergence of hematopoietic cells can be enhanced by a combination of hematopoietic cytokines and the ventral mesoderm inducer bone morphogenetic protein (BMP)-4 *(4,5)*. Functional CD45+ hematopoietic cells capable of in vitro colony forming activity emerge after 10 d of hEB development and the role of hematopoietic cytokines is restricted during these first 10 d *(5)*. Under treatment with hematopoietic cytokines and BMP-4, up to 90% of cells dissociated from 22 d EB express pan-leukocyte marker CD45 (*see* **Fig. 1**) *(5)*. In addition, dependent on the presence of hematopoietic cytokines and BMP-4, vascular endothelial growth factor (VEGF-A_{165}) selectively promotes erythropoietic development toward the primitive lineage. The effects of VEGF-A_{165} can be augmented by erythropoietin (EPO) *(6)*, suggesting that hematopoietic differentiation from hESC can be influenced.

Recently, we have demonstrated that hematopoietic cells are derived from a rare subpopulation of hemogenic precursors during hEB development *(7)*. These hemogenic precursors lack CD45, but express PECAM-1, Flk-1, and VE-cadherin (hereinafter $CD45^{neg}$PFV) and are solely responsible for hematopoietic cell fate *(7)*. $CD45^{neg}$PFV precursors can be fractioned from d 10 hEB development by a FACSVantage (*see* **Fig. 2**). Up to 98.5% of CD45+ cells are generated from $CD45^{neg}$PFV precursors, but not the remaining d 10 hEB cells after culture in a hematopoietic conducive condition for 7 d, with approx 8% of these $CD45^{neg}$ cells coexpressing CD34 (*see* **Fig. 3**) *(7)*.

hESC-derived hematopoietic cells show similar colony and cellular morphologies to those derived from committed adult hematopoietic tissues *(5–7)*. They possess characteristic progenitors of all myeloid lineages, including macrophage, granulocyte, erythroid, and multipotent hematopoietic progenitors containing granulocytic, erythroid, macrophage, and megakaryocytic lineages. Further, similar to adult peripheral blood, cord blood, and bone marrow, hESC-derived hematopoietic progenitor capacity is enriched in the CD34+ subfraction, suggesting that, in addition to producing mature hematopoietic cells, these precursors possess the ability to sustain production of primitive blood cells with appropriate function and phenotype *(7)*.

Despite these in vitro phenotypic and functional assays, clinical promise of hESC-derived hematopoietic cells can only be functionally defined by sustained multilineage in vivo reconstitution on transplantation. Experimentally, the non-obese diabetic-severe combined immunodeficient (NOD/SCID) xenotransplant assay has provided a powerful tool to functionally define candidate human hematopoietic stem cells, defined as SCID-repopulating cells *(8)*. However, recent evidence from our laboratory indicates that intra-

venous transplantation of hESC-derived hematopoietic cells causes mortality in recipient immune deficient mice because of emboli formed from rapid cellular aggregation in response to mouse serum *(9)*. We suggest that intrabone marrow transplantation (IBMT) that bypasses the recipient mouse circulation may be necessary to observe hematopoietic engraftment from hESC-derived hematopoietic cells *(9)*.

In this chapter, we will describe methods that have been successfully applied in our laboratory, including: (1) generation of hematopoietic cells by EB formation; (2) augmentation of hematopoiesis by use of hematopoietic cytokines and BMP-4; (3) promotion of erythropoietic development by addition of VEGF-A_{165}; (4) isolation of $CD45^{neg}$PFV hemogenic precursors and generation of hematopoietic cells from these precursors; and (5) characterization of hESC-derived hematopoietic cells in vitro and in vivo.

2. Materials

2.1. Derivation of Hematopoietic Cells From hEB

1. Dulbecco's modified Eagle's medium (DMEM) (500 mL; Gibco, Burlington, Canada; cat. no. 11965-092).
2. Knockout DMEM (ko-DMEM) (500 mL; Gibco; cat. no. 10829-018).
3. D-phosphate buffered saline (PBS), Ca^{2+}, and Mg^{2+} free (500 mL; Gibco; cat. no. 14190-144).
4. Knockout serum replacement (ko-SR) (500 mL; Gibco; cat. no. 10828-028).
5. 200 m*M* L-glutamine (100 mL; Gibco; cat. no. 15039-027).
6. Nonessential amino acid solution, 10 m*M*, 100X (100 mL; Gibco; cat. no. 11140-050).
7. Sterile filter system, Corning 0.22-µm cellulose acetate filter, low binding protein (150 mL and 500 mL; Fisher, Ottawa, Canada; cat. no. 09-761-119 and 09-761-5).
8. Collagenase IV (1 g; Gibco; cat. no. 17104-019). To prepare stock solution: calculate the total amount of International Units (IU) in each individual bottle, dilute with ko-DMEM to make 10,000 IU/mL, aliquot under 2 mL, and store at –30°C. To prepare working solution: combine 2 mL of stock solution with 98 mL of ko-DMEM. The working solution is then filtered through a 0.22-µm membrane and stored at –30°C. Avoid repeated freeze-thaw cycles. Thawed working solution can be stored at 4°C up to 1 wk.
9. Ultra free-MC, 0.22-µm filter, sterile, low binding protein (Millipore, Nepean, Canada; cat. no. UFC30GVOS).
10. Bovine serum albumin (BSA), 30% solution (50 mL; Sigma, Oakville, Canada; cat. no. A9576).
11. Human basic fibroblast growth factor (bFGF), recombinant (10 µg; Gibco, cat. no. 13256-029). To prepare working solution (10 µg/mL): dissolve each vial of bFGF in 1 mL D-PBS containing 0.1% BSA (add 30 µL of 30% BSA into 10 mL D-PBS). Prefilter Ultra free-MC 0.22-µm filter with 3 mL D-PBS containing 10% BSA (combine 1 mL of 30% BSA with 2 mL D-PBS), then filter bFGF working

solution. Aliquot 200 µL of filtered bFGF into sterile Eppendorf tubes and store at –30°C. Avoid repeated freeze-thaw cycles. Thawed bFGF can be kept at 4°C up to 1 mo.

12. Fetal bovine serum (FBS), heat inactivated and nonheat inactivated (500 mL; Hyclone, Logan, UT; cat. no. 30071-03).
13. Six-well plates, flat bottom (VWR, Mississauga, Canada; cat. no. CA62406-161).
14. Matrigel (10-mL; BD, Mississauga, Canada; cat. no. 353234). To prepare stock solution: thaw Matrigel slowly to avoid gel formation (overnight at 4°C). Add 10 mL of cold ko-DMEM (4°C). Put Matrigel bottle and 20 tubes (15-mL polypropylene conical tubes) on ice. Pipet 20 times to mix Matrigel and ko-DMEM medium. Make 1-mL/tube aliquots. Store at –30°C. To prepare working solution: thaw one tube of stock Matrigel overnight at 4°C or in cold water at 4°C for 2 h. Add 5 mL of cold ko-DMEM, pipet 10 times. Then, add 9 mL of cold ko-DMEM and pipet 10 times. Add 1 mL of working solution to one well of a six-well plate. Wrap the plate with plastic film. Keep the plate overnight at 4°C. Coated plates can be kept at 4°C no longer than 7 d.
15. Six-well plates, ultra low attachment (Fisher; cat. no. CS003471).
16. 14.3 *M* β-mercaptoethanol (100 mL; Sigma; cat. no. M7522). To prepare 1.43 *M* β-mercaptoethanol: combine 2 mL of 14.3 *M* β-mercaptoethanol with 18 mL of D-PBS and store at –30°C in 0.25-mL aliquots. Keep thawed 1.43 *M* β-mercaptoethanol at 4°C up to 1 mo. To prepare 55 m*M* (β-mercaptoethanol, add 10 µL of 1.43 *M* β-mercaptoethanol to 240 µL Iscove's modified Dulbecco's medium (IMDM) and store at 4°C up to 2 wk.
17. Cell dissociation buffer, enzyme-free PBS-based (100 mL; Gibco; cat. no. 13151-014).
18. 40-µm nylon cell strainer (BD; cat. no. 352340).
19. IMDM (500 mL; Gibco; cat. no.12200-028).
20. Mouse embryonic fibroblast conditioned medium: the mouse embryonic fibroblast conditioned medium (MEF-CM) is produced by daily collection of feeding medium from irradiated (40 Gy) mouse MEF cells within a 7–10 d period. The medium consists of 80% ko-DMEM supplemented with 20% ko-SR, 1% nonessential amino acids, 1 m*M* L-glutamine, 0.1 m*M* β-mercaptoethanol, and 4 ng/mL bFGF. To prepare 500 mL of medium: combine 100 mL ko-SR, 5 mL nonessential amino acids, 2.5 mL L-glutamine, 35 µL 1.47 *M* β-mercaptoethanol, and 392.5 mL ko-DMEM. The medium is then filtered through a 0.22-µm sterile cellulose acetate membrane and kept at 4°C up to 14 d. Immediately before use, add 200 µL of bFGF (working solution, 10 ng/µL) to 500 mL of medium. Feed MEF cells and collect medium daily up to 10 d. Pool all collected MEF-CM, filter them through a 0.22-µm sterile cellulose acetate membrane. Store aliquots in –80°C up to 6 mo. Store thawed MEF-CM at 4°C no more than 7 d (*see* **Note 1**).
21. Medium for hEB (EB Medium): human EBs are cultured in ko-DMEM supplemented with 20% of nonheat-inactivated FBS, 1% nonessential

amino acids, 1 m*M* L-glutamine, 0.1 m*M* β-mercaptoethanol. To prepare 100 mL of medium: combine 20 mL of nonheat-inactivated FBS, 1 mL nonessential amino acids, 0.5 mL L-glutamine, and 7 µL β-mercaptoethanol (1.43 *M*). Filter through a 0.22-µm sterile cellulose acetate membrane. Store at 4°C no longer than 14 d.

2.2. Promotion of hEB Development Into Hematopoietic Fate by Combinations of Hematopoietic Cytokines and BMP-4

1. Stem cell factor (SCF), recombinant, methylated, human (2500 µg; Amgen Inc., Thousand Oaks, CA; donated). To prepare working solution (1500 µg/mL): add 1.67 mL of D-PBS containing 2% heat-inactivated FBS to the bottle, pipet, and aliquot 50 µL/tube. Store at –80°C. Keep thawed SCF at 4°C no longer than 1 mo. Avoid repeated freeze-thaw cycles.
2. Interleukin (IL)-3, recombinant, human (50 µg; R&D; Minneapolis, MN; cat. no. 203-IL). To prepare working solution (25 µg/mL): add 2 mL of D-PBS containing 2% heat-inactivated FBS to the bottle, pipet, and aliquot 100 µL/tube. Store at –30°C no longer than 3 mo. Keep thawed IL-3 at 4°C no longer than 1 mo. Avoid repeated freeze-thaw cycles.
3. IL-6, recombinant, human (50 µg; R&D; cat. no. 206-IL). To prepare working solution (50 µg/mL): add 1 mL of D-PBS containing 2% heat-inactivated FBS to the bottle, pipe, and aliquot 50 µL/tube. Store at –30°C no longer than 3 mo. Keep thawed IL-6 at 4°C no longer than 1 mo. Avoid repeated freeze-thaw cycles.
4. Flt-3 Ligand (Flt-3L), recombinant human (250 µg; R&D; cat. no. 308-FK/CF). To prepare working solution (250 µg/mL): add 1 mL of D-PBS containing 2% heat-inactivated FBS to the bottle, pipet, and aliquot 100 µL/tube. Store at –30°C no longer than 3 mo. Keep thawed Flt-3L at 4°C no longer than 1 mo. Avoid repeated freeze-thaw cycles.
5. Granulocyte-colony stimulating factor (G-CSF) (300 µg/1 mL; Amgen Inc.; cat. no. 3105100). Aliquot 100 µL/tube and store at –80°C.
6. BMP-4, recombinant, human (300 µg; R&D; cat. no. 314-BP). To prepare stock solution (1000 µg/mL): add 300 µL of D-PBS containing 2% heat-inactivated FBS to the bottle, pipet, and aliquot 50 µL/tube. Store at –80°C. To prepare working solution (50 µg/mL): add 950 µL of D-PBS containing 2% heat-inactivated FBS to 50 µL of stock solution. Aliquot 50 µL/tube. Store working solutions at –30°C no longer than 3 mo. Keep thawed BMP-4 at 4°C no longer than 1 mo. Avoid repeated freeze-thaw cycles.

2.3. Augmentation of hEB Development Into Erythropoietic Fate by Recombinant Human VEGF-A_{165} and Erythropoietin

1. Recombinant human VEGF-A_{165} (1 µg lyophilized; R&D; cat. no. 293-VE). To prepare working solution (1 µg/mL): add 997 µL of D-PBS and 3 µL BSA (30% solution) to the vial. Aliquot 100 µL into Eppendorf tubes and store at –30°C up to 3 mo. Avoid repeated freeze-thaw cycles. Thawed aliquots are stable up to 1 mo at 4°C.

2. EPO (10,000 IU/mL; Amgen Inc.; donated).
3. Anti-human VEGF monoclonal antibody (MAb) (500-µg lyophilized; R&D, cat. no. MAB293). Reconstitute with 1 mL of D-PBS to get a concentration of 500 µg/mL, aliquot 25 µL into Eppendorf tubes, and store at –30°C up to 6 mo. Avoid repeated freeze-thaw cycles. Thawed aliquots are stable up to 1 mo at 4°C.

2.4. Derivation of Hematopoietic Cells From CD45^{neg}PFV Precursors

1. Collagenase B (100 mg; Roche Diagnostics GmbH, Mannheim, Germany; cat. no. 1088-807). To prepare stock solution: calculate the total amount of IU in each individual bottle, dilute with ko-DMEM to make 4.0 IU/mL and filter through a 0.22-µm membrane. Aliquot 1 mL stock solution into 15-mL polypropylene tubes and store at –30°C. Avoid repeated freeze-thaw cycles. To prepare working solution (0.4 IU/mL): add 9 mL of ko-DMEM to 1 mL of stock solution. The working solution can be stored at 4°C up to 1 wk.
2. 9500 BIT media, 5X (100 mL; Stem Cell Technologies, Vancouver, Canada; cat. no. 09500). Aliquot 4 mL per tube and store at –30°C. Thawed BIT media can be stored at 4°C up to 3 mo.
3. 96-well plates, fibronectin-coated (pack of five; VWR; cat. no. CACB354409).
4. 24-well plates, fibronectin-coated (pack of five; VWR; cat. no. CACB354411).
5. Serum-free hematopoietic conducive medium (BIT medium): the serum-free hematopoietic conducive medium consists of 9500 BIT media, 2 m*M* L-glutamine, 0.1 m*M* β-mercaptoethanol, 300 ng/mL SCF, 50 ng/mL G-CSF, 300 ng/mL Flt-3L, 10 ng/mL IL-3, 10 ng/mL IL-6 (***10***). To prepare 10 mL of medium: combine 2 mL of 5X BIT medium, 7.86 mL IMDM, 2 µL SCF, 12 µL Flt-3L, 4 µL IL-3, 2 µL IL-6, 1.64 µL G-CSF, 20 µL of 55 m*M* β-mercaptoethanol, and 100 µL L-glutamine. The medium is prepared before use.

2.5. Characterization of hEB-Derived Hematopoietic Cells In Vitro

2.5.1. Phenotypic Analysis of hEB-Derived Hematopoietic Cells by Fluorescence-Activated Cell Sorting

All antibodies must be protected from light and stored at 4°C.

1. Fluorescence-activated cell sorting (FACS) buffer. To prepare 500 mL of FACS buffer: combine 470 mL of D-PBS and 30 mL heat-inactivated FBS either in presence or absence of 0.05% NaN_3 (add 2.5 mL of 5% NaN_3 solution to 500 mL FACS buffer).
2. Allophycocyanin (APC)-conjugated anti-human CD45 MAb (0.5 mL; BD; cat. no. 340943).
3. PE-conjugated mouse anti-human PECAM-1 MAb (2 mL; BD; cat. no. 555446).
4. Fluorescein isothiocyanate (FITC)-conjugated anti-human CD34 MAb (2 mL; BD; cat. no. 555821).
5. FITC-conjugated anti-human CD36 MAb (1 mL; Research Diagnostics Inc., Flanders, NJ; cat. no. RDI-M1613016).

6. Phycoerythrin (PE)-conjugated anti-human CD33 MAb (2 mL; BD; cat. no. 347787).
7. PE-conjugated anti-human CD19 MAb (2 mL; Immunotech Beckman Coulter, Marseille, France; cat. no. IM1285).
8. PE-conjugated mouse IgG isotype MAb (2 mL; BD; cat. no. 340043).
9. APC-conjugated mouse IgG isotype MAb (0.5 mL; BD; cat. no. 340442).
10. FITC-conjugated mouse IgG isotype MAb (2 mL; BD; cat. no. 349041).
11. Mouse anti-human Glycophorin A-FITC MAb (2 mL; cat. no. IM2212) or mouse anti-human Glycophorin A-PE MAb (2 mL; cat. no. IM2211), both from Immunotech Beckman Coulter.
12. KS1/4 MAb, mouse IgG2a (0.1 mg; BD; cat. no. 554276).
13. Single chain anti-human VEGFR2 (KDR/Flk-1) scFvA7 MAb (0.5 mL; Research Diagnostics Inc.; cat. no. RDI-VEGFR2scX).
14. HRP-labeled mouse anti-E tag MAb (0.5 mg; Amersham Pharmacia Biotech, Piscataway, NJ; cat. no. 27-9413-01).
15. PE-conjugated $F(ab')_2$ fragment donkey anti-mouse IgG (H+L) MAb (1 mL; Research Diagnostics Inc.; cat. no. 715-116-150).
16. FITC-conjugated $F(ab')_2$ goat anti-mouse IgG (H+L) MAb (1 mg; Immunotech Beckman Coulter; cat. no. IM0819).
17. 7-Amino Actinomycin D (7-AAD) viability dye (3 mL ready to use solution; Immunotech Beckman Coulter; cat. no. IM3422).
18. FACS tubes, 5-mL polystyrene round-bottom tubes (BD; cat. no. 352054).

2.5.2. Morphology Assays of hEB-Derived Hematopoietic Cells

1. SCF, IL-3, EPO (*see* **Subheadings 2.2.** and **2.3.**).
2. Granulocyte macrophage colony-stimulating factor (Amgen; donated).
3. Methocult SF^{BIT} H4230, methylcellulose medium (MC) (80 mL, Stem Cell Technologies; cat. no. HCC-4230). MC is supplemented with growth factors. Thaw one 80-mL bottle overnight at 4°C and add 20 mL of sterile IMDM, 50 ng/mL SCF (3.4 µL at 1.5×10^3 ng/µL), 10 ng/mL IL-3 (40 µL at 25 ng/µL), 3 U/mL EPO (30 µL at 10,000 U/mL), and 10 ng/mL granulocyte macrophage colony-stimulating factor (3.4 µL at 1:10 dilution of 3×10^3 ng/µL). Shake vigorously and leave overnight to settle. Make 5- or 10-mL aliquots and store at −30°C. After thawing thawed, keep at 4°C for no longer than 2 wk.
4. 12-well plates, non-TC treated, flat bottom (BD; cat. no. 351143).
5. 1-mL syringes, slip tip (BD; cat. no. 309602).
6. 16-gage needle, 1.5-in. bevel needles (BD; cat. no. 305198).
7. IMDM medium (500-mL; Gibco; cat. no. 12200-028).
8. Sterilized water.
9. Wright Giemsa stain solution (500 mL; Sigma; cat. no. WG-16).
10. Xylenes (4 L; Sigma; cat. no. 53405-6).
11. Methanol (1 L; ICN, Montreal, Canada; cat. no. ICN155386). Acetone (500 mL; Fisher; cat. no. A18-500). To prepare 100 mL of 80:20 solution: combine 80 mL methanol and 20 mL acetone and store at room temperature in a fume cabinet.

Refrigerate the bottle at 4°C for 30 min prior to use as a fixative for Giemsa staining.
12. Buffer solution of D-PBS, dilute D-PBS 10 times with distilled water. Adjust to pH 6.8 (important for staining).

2.5.3. Detection of Embryonic (Primitive Erythropoiesis) and Adult (Definitive Erythropoiesis) Globins

1. FITC-conjugated mouse anti-human HbA (adult hemoglobin) MAb (400 µL; 250 µg/mL; PerkinElmer Life Sciences, Norton, Canada; cat. no. MBA-F).
2. FITC-conjugated mouse anti-human HbF (fetal hemoglobin) MAb (0.5 mL, 200 µg/mL; Research Diagnostics Inc.; cat. no. RDI-FHBabmFT).
3. Triton X-100 (500 mL; Bio-Rad, Hercules, CA; cat. no. 161-0407). To prepare 0.1% working solution: dilute 1000 times with D-PBS.
4. Glutaraldehyde (100 mL, 25% aqueous solution; Sigma; cat. no. G5882). To prepare 0.05% working solution: dilute 500 times with D-PBS.
5. RNase away (250 mL; Life Technologies, Carlsbad, CA; cat. no. 10328-011).
6. RNeasy Mini Kit (50) (Qiagen Inc., Mississauga, Canada; cat. no. 74104) and QIAshredder (50) (Qiagen Inc.; cat. no. 79654).
7. First-strand cDNA Synthesis Kit (Amersham Biosciences, Piscataway, NJ; cat. no. 27-9261-01).
8. 5 *M* sodium acetate, NH_4OAC (100 mL; Ambion, Austin, TX; cat. no. 9070G).
9. Glycogen 20 mg/mL aqueous solution (1 mL; Fermentas, Burlington, Canada; cat. no. R0561).
10. Distilled water, DNAse, and RNAse-free (500 mL; Gibco; cat. no. 10977-015).
11. 100% ethanol.

2.6. Characterization of hEB-Derived Hematopoietic Cells In Vivo

1. 3-mL syringes (BD; cat. no. CABD309585).
2. 27-gage needles, 0.5-in. bevel (VWR; cat. no. CABD305109).
3. 28-gage (.05 in., 0.3-mL) insulin syringes (VWR; cat. no. CABD309300).
4. Sterile eye ointment (from any pharmacy).
5. Gauze, 3 × 3 in., sterile, pack of two (Johnson and Johnson, Titusville, NJ; cat. no. 344-2339).
6. Proviodine detergent, 0.75% iodine (500 mL; Rougier, Mirabel, Canada; cat. no. DIN00172936).
7. Proviodine solution, 1.0% free iodine (500 mL; Rougier; cat. no. DIN00172944).
8. Tert-amyl alcohol (Sigma; cat. no. 246-3).
9. Avertin (10 g; Sigma; cat. no. 840-2). To prepare stock solution (50%): add 20 mL of Tert-amyl alcohol to dissolve Avertin. Wrap in foil in a glass jar and store at room temperature up to 6 mo. To prepare working solution (2.5%): combine 1 mL of Avertin stock solution with 19 mL D-PBS.
10. Buprenorphine (Buprenex) (Pack of 10 at 1 mL each; Burn Veterinary Supply, Inc., Farmers, Branch, TX; cat. no. 254-0251).

11. FITC-conjugated MAbs against human CD45 and CD36, PE-conjugated MAbs against human CD19, CD33, FITC-conjugated MAb against human glycophorin-A. All from BD (*see* **Subheading 2.5.1.**).
12. DNAzol reagent (100 mL; Gibco; cat. no. 10503-027).
13. 0.8% ammonium chloride solution (500 mL; Stem Cell Technologies; cat. no. 07850).
14. Hybond-N+ nylon membrane, 20 cm × 3 m (one roll; Amersham Bioscience; cat. no. RPN203B).

2.7. General Equipment and Plastics

The following are common equipment and plastic ware required in the experiments:

1. Humidified incubator at 37°C and 5% CO_2.
2. Inverted microscope.
3. Laminar flow cabinet.
4. 37°C water bath.
5. Freezers (−80°C, −30°C) and refrigerators (4°C).
6. Tabletop centrifuge (Eppendorf centrifuge 5417C).
7. Polymerase chain reaction (PCR) thermal cycler (we use Perkin-Elmer GeneAmp PCR system 9700 cycler).
8. GeneQuant II for quantification of DNA, RNA, and oligonucleotides (Pharmacia Biotech).
9. Electrophoresis equipment.
10. Gel reader system (we use Gel Doc and Quantity One software, Bio-Rad).
11. FACSCalibur (BD).
12. FACS Vantage SE sorter (BD).
13. Hematocytometer (VWR; cat. no. 15170-208).
14. Trypan blue (100 mL; Gibco; cat. no. 1691049).
15. FACS tube, 5-mL polystyrene tubes (BD; cat. no. 352054).
16. 15-mL polypropylene conical tubes (VWR; cat. no. CA21008-918).
17. 50-mL polypropylene conical tubes (VWR; cat. no. CA21008-940).
18. 5-mL pipets, sterile (Fisher; cat. no. CS004487).
19. 10-mL pipets, sterile (Fisher; cat. no. CS004488).
20. 25-mL pipets, sterile (Fisher; cat. no. CS004489).
21. Pipetmen (P2, P10, P20, P100, P200, and P1000 µL) and appropriate tips.

3. Methods

3.1. Formation of hEB (see Notes 2 and 3)

1. Culture hESCs for 7 d in a 37°C and 5% CO_2 humidified incubator. Add 3 mL MEF-CM supplemented with fresh bFGF to each well of six-well plates (addition of 8 ng bFGF to 1 mL MEF-CM just before feed). Change MEF-CM every day until d 7.

2. At d 7, aspirate MEF-CM. Add 0.5 mL of prewarmed Collagenase IV (200 IU/mL, working solution) to each well. Incubate at 37°C for 3–10 min until colony edges slightly pull away from the plate.
3. Aspirate Collagenase IV. Wash the residual Collagenase IV with 2 mL/well of prewarmed EB medium or ko-DMEM medium. Aspirate the medium.
4. Add 2 mL/well of EB medium. Scrape off the hESC colonies in strips using a 10-mL pipet.
5. Transfer 1.5 well of hESC to one well of a low attachment plate. Add 1 mL EB media to each well (total 4 mL/well).
6. Incubate overnight to allow for hEB formation; change EB medium 18–24 h later.

3.2. Promote hEB Development Into Hematopoietic Fate by Hematopoietic Cytokines and BMP-4

1. Transfer each well of hEBs to an individual 15-mL polypropylene tube 18–24 h after hEB formation.
2. Centrifuge at 129*g* for 5 s to separate hEBs from debris and single cells.
3. Aspirate supernatant. Add 4 mL of EB medium to each tube. Before use, the EB medium has been supplemented with hematopoietic cytokines (*see* **Subheading 2.1.2.**).
4. Transfer each tube of hEBs to the previous well. Culture hEBs in a 37°C and 5% CO_2 humidified incubator.
5. Change EB medium, hematopoietic cytokines, and BMP-4 every 5 d.

3.3. Promote Primitive Erythropoiesis From hEB by Addition of VEGF-A_{165} and EPO

1. Form hEBs as described in **Subheading 3.1.** Add VEGF-A_{165} (final concentration: 5 ng/mL) or EPO (final concentration: 3 U/mL) together with the cocktail of hematopoietic cytokines and BMP-4 (described in **Subheading 3.2.**).
2. Change EB medium and all factors every 5 d.
3. Add VEGF-A_{165} during d 1–10 of hEB differentiation (5 µL of working solution per mL, final concentration: 5 ng/mL), because there is no significant effect on promotion of the erythroid lineage when VEGF-A_{165} is added from d 10–15 of hEB differentiation. Optimal dose of VEGF-A_{165} is found between 1 and 20 ng/mL. Add EPO (final concentration: 3 U/mL).
4. Neutralize VEGF-A_{165} effects by adding 5 µL per well of anti-human VEGF MAb (final concentration: 2.5 µg/mL) simultaneously during changes of EB medium and growth factors.

3.4. Derivation of Hematopoietic Cells From CD45-PFV Precursors

A subpopulation of primitive endothelial-like cells purified from d 10 hEB that express PECAM-1, Flk-1, VE-cadherin, but not CD45 (CD45^{neg}PFV cells) is uniquely responsible for hematopoietic development *(7)*. To generate hematopoietic cells from hEB-derived CD45^{neg}PFV precursors: dissociate d

10 hEBs to produce single cells, isolate CD45^{neg}PFV precursors from these single cells, and derive hematopoietic cells by culture of CD45^{neg}PFV precursors in a hematopoietic conducive medium (BIT medium).

3.4.1. Dissociation of Day 10 hEB

1. Transfer each well of hEBs to an individual 15-mL polypropylene tube.
2. Centrifuge at 129*g* for 5 s. Aspirate supernatant.
3. Add 4 mL of Collagenase B (working solution, 0.4 IU/mL). Transfer to the previous well. Dissociate hEBs in a 37°C incubator for 2 h.
4. Transfer the cells to a 15-mL polypropylene tube. Centrifuge for 3 min at 453*g*.
5. Aspirate the Collagenase B supernatant.
6. Add 2 mL of cell dissociation buffer and incubate for 10 min in a 37°C water bath.
7. Centrifuge at 453*g* for 3 min. Aspirate supernatant. Add 0.5 mL of IMDM.
8. Gently triturate 50 times with a P1000 pipetman to dissociate hEB cells. Add 0.5 mL of IMDM to each tube.
9. Filter through a 40-μm cell strainer.
10. Count viable cells by trypan blue exclusion.

3.4.2. Purification of Hematopoietic Precursor CD45-PFV Cells

Isolation of CD45^{neg}PECAM1+Flk1+, CD45^{neg}PECAM1+, or CD45^{neg}Flk1+ cells from d 10 hEB demonstrates that VE-cadherin segregates with either of these populations and produces identical results, indicating that any of these sorting strategies isolated a similar population in which the majority of cells is CD45^{neg}PFV *(7)*. Therefore, purification of CD45^{neg} PECAM1+ subpopulation could be used as a strategy for purification of CD45^{neg}PFV cells. To purify CD45^{neg}PECAM1+ cells:

1. Resuspend single cells dissociated from day 10 hEB in D-PBS containing 3% FBS (2×10^6 cells/mL).
2. Precoat a few tubes (5-mL FACS tubes) with 0.5 mL of FBS for 30 min for collection of sorted cells.
3. Add 20 μL of anti-human PECAM-1-PE MAb and 20 μL of anti-human CD45-APC MAb to 1 mL of cell suspension. Add 2 μL of mouse IgG-PE and 2 μL IgG-APC MAbs into isotype control tubes containing 1×10^5 cells/200 μL. Incubate for 30 min at 4°C.
4. Centrifuge the tubes at 453*g* for 3 min. Aspirate supernatant. Wash twice with 3 mL of D-PBS containing 3% FBS.
5. Resuspend cells in D-PBS containing 3% FBS at a concentration of 2×10^6/mL. Add 20 μL of 7-AAD to 1 mL of cell suspension and stain for 10 min at room temperature to exclude dead cells.
6. Filter through a 40-μm cell strainer just prior to sort to avoid clogging of the sorting nozzle.

7. Set sorting gates, including histogram markers and dot plot quadrants, by use of respective IgG isotype controls. Sort CD45^{neg}PECAM-1+ subpopulation on a FACS Vantage SE (BD).
8. Determine purity immediately after sorting using the same sorting gate settings.

3.4.3. Hematopoietic Culture of hEB-Derived CD45-PFV Cells

To derive hematopoietic cells from CD45^{neg}PFV precursors:

1. Centrifuge CD45^{neg}PECAM1+ (containing the majority of CD45^{neg}PFV cells) cells at 453*g* for 3 min. Aspirate supernatant and add 2 mL of 1X BIT medium. Centrifuge at 453*g* for 3 min. Repeat this wash step once to get rid of residual FBS.
2. Resuspend cells in BIT medium containing 2 m*M* L-glutamine, 0.1 m*M* β-mercaptoethanol, 300 ng/mL SCF, 50 ng/mL G-CSF, 300 ng/mL Flt-3L, 10 ng/mL IL-3, and 10 ng/mL IL-6 (*see* **Subheading 2.4.1.**). Make a final cell concentration of 2.5×10^5 cells/mL.
3. Seed CD45^{neg}PECAM1+ cells in fibronectin-coated plates. Add 200 µL/well for a 96-well plate, 1 mL/well for a 24-well plate. Make a final cell density of 5×10^4 cells/cm^2.
4. At d 3 and 5, gently replace half the volume of medium with freshly prepared 1X BIT medium containing the hematopoietic cytokines. Single, bright round hematopoietic cells emerge around d 3. Avoid discarding these cells during medium replacement.
5. At d 7, transfer medium from each well to a 15-mL polypropylene tube.
6. Add 0.25% Trypsin/EDTA to each well (50 µL for 96-well plate, 200 µL for 24-well plate) and incubate at room temperature to dissociate the adherent cells. Examine the cells under the microscope until they round up (requires 1–5 min). Neutralize trypsin immediately with fivefold volume of IMDM containing 10% FBS.
7. Gently pipet the dissociated cells. Transfer the cells to the 15-mL polypropylene tube. Centrifuge at 453*g* for 3 min.
8. Aspirate supernatant. Wash once with IMDM medium. Count viable cells.
9. Prepare 25,000 viable cells for colony-forming unit assay, 20,000–100,000 viable cells/tube for FACS analysis.

3.5. Characterization of hESC-Derived Hematopoietic Cells In Vitro

3.5.1. Flow Cytometric Analysis of Cell Surface Markers

3.5.1.1. One-Step Staining With Fluorochrome-Conjugated MAbs

This method is used for detection of CD45, CD34, CD36, CD33, CD19, PECAM-1, and Glycophorin A on hESC-derived hematopoietic cells.

1. Resuspend the cells in 200 µL of FACS buffer containing 50,000 cells.
2. Add 2 µL of MAbs conjugated with APC, FITC, or PE fluorochromes to 200 µL of cell suspension and mix briefly by pipetting or vortexing. Incubate for 30 min at 4°C in the dark.

3. In the same conditions, stain 50,000 cells with 2 µL of the appropriate fluorochrome-conjugated matched isotype control MAbs.
4. Wash the cells twice with 2 mL of FACS buffer (453*g*, 3 min).
5. Resuspend cells in 300 µL of FACS buffer and stain with 4 µL of 7-AAD at room temperature for 10 min in the dark to exclude dead cells (*see* **Note 4**).
6. Analyze cells on a FACSCalibur. The percentages of positive cells are determined as compared with isotype controls that set up the background level of nonspecific staining.

3.5.1.2. Two-Step Staining to Detect Ep-CAM Expression on Erythroid Progenitors With KS1/4 MAb

1. Preincubate 2 µL of FITC-conjugated goat $F(ab')_2$ anti-mouse IgG MAb with 2 µL of KS1/4 MAb or its isotype control MAb in 100 µL FACS buffer for 30 min at 4°C in the dark.
2. Add the above 100 µL of MAb mixture to 100 µL of cell suspension. Incubate for 30 min at 4°C in the dark.
3. Proceed from **step 4** of **Subheading 3.5.1.1.**

3.5.1.3. Three-Step Staining to Detect VEGFR2 (KDR/Flk1)

1. Add 2.5 µL of anti-VEFGR2 MAb to 200 µL of cell suspension. Incubate for 30 min at 4°C in the dark.
2. Wash the cells twice with FACS buffer (453*g*, 3 min). Prepare working solution of HRP-labeled mouse anti-E tag MAb by adding 1 µL of MAb to 19 µL of FACS buffer. Add 2 µL of this solution to 200 µL of cell suspension. Incubate for 30 min at 4°C in the dark.
3. Wash the cells twice. Add 1 µL of PE-conjugated $F(ab')_2$ fragment donkey anti-mouse IgG to 200 µL of cell suspension. Incubate for 30 min at 4°C in the dark.
4. Proceed from **step 4** of **Subheading 3.5.1.1.**

3.5.2. Morphological Characterization of hEB-Derived Hematopoietic Colonies

3.5.2.1. Colony-Forming Unit Assay in MC

1. Collect and count cells from dissociated hEBs (*see* **Subheading 3.4.1.** for hEB dissociation), or hematopoietic cells derived from $CD45^{neg}$PFV. Plate 10,000–50,000 cells per well (12-well non-TC-treated plates).
2. Thaw MC supplemented with the hematopoietic cytokines at room temperature.
3. Resuspend the total number of cells to be plated in a final volume of 100 µL IMDM in a 1.5-mL Eppendorf tube.
4. Mix cell suspension.
5. Add 0.6 mL of MC supplemented with the cytokines using a 1-mL syringe coupled to a 16-gage needle.
6. Vortex the tube for a few seconds to completely mix the cells and MC.
7. Let the tube stand for 5 min to allow bubbles to move up.

8. Using a 1-mL syringe coupled to a 16-gage needle, transfer 0.5 mL of the preparation to 1 well of 12-well non-TC-treated plates. Move the plate slowly in order to distribute MC evenly in the well and to avoid bubbles.
9. Add sterilized water to the surrounding empty wells to maintain moisture.
10. Incubate the plate in a 37°C and 5%CO_2 humidified incubator. Examine the colonies (colony-forming units) up to 40 d under an inverted microscope.

3.5.2.2. Cytospin Preparation

1. Under a microscope, pluck individual colonies between 14 and 21 d using a P20 pipetman. Either use large single colonies or pool several smaller colonies with similar morphology.
2. Deposit cells in 1.5-mL Eppendorf tubes containing 1 mL of D-PBS. Gently pipet to release the cells from MC.
3. Wash the cells twice with 1 mL of D-PBS.
4. Resuspend the cells in 100 µL of D-PBS.
5. Position a slide, a filter card, and a sample chamber to the slide holder. Carefully align the hole on the filter card with the hole on the bottom of the chamber. Load it to a cytocentrifuge rotor.
6. Add 100 µL of cell suspension to the bottom of chamber.
7. Centrifuge at 129*g* for 5 min (*see* **Note 5**).
8. Gently separate the slide with the filter card and chamber. Leave slide at room temperature for 10 min.
9. Fix slide in cold methanol:acetone (80:20 v/v) for 5–10 min.

3.5.2.3. Giemsa Staining

1. Place slide on a horizontal staining rack over a sink. Flood the slide with Wright Giemsa solution and incubate for 1 min.
2. Add buffer solution (D-PBS diluted 10 times with distilled water, pH 6.8) in sufficient quantity to dilute staining two or three times and incubate for 3 min.
3. Wash off carefully with tap water, stand the slide on end to drain, and air-dry it at room temperature.
4. Clear in xylenes for 2 min. Cover with a cover slide and examine under a microscope.

3.5.3. Detection of Embryonic (Primitive Erythropoiesis), Fetal, and Adult (Definitive Erythropoiesis) Globins

3.5.3.1. Intracellular Detection of Fetal (HbF) and Adult (HbA) Globins

1. Dissociate hEBs or erythroid colonies (*see* **Subheadings 3.4.1.** and **3.5.2.2.; steps 1 and 2**, respectively).
2. Wash cells with 2 mL of D-PBS containing 0.1% BSA, centrifuge at 453*g* for 5 min.
3. By gentle pipetting, resuspend cells in 1 mL of cold 0.05% glutaraldehyde for 10 min at room temperature.

4. Wash cells with 2 mL of D-PBS containing 0.1% BSA, centrifuge at 453*g* for 5 min.
5. By gentle pipetting, resuspend cells in 0.5 mL 0.1% Triton X-100 and incubate for 5 min at room temperature.
6. Wash cells with 2 mL of D-PBS containing 0.1% BSA, centrifuge at 453*g* for 5 min.
7. By gentle pipetting, resuspend cells in 80 µL of D-PBS containing 0.1% BSA.
8. To detect HbA, combine 10 µL of 250 µg/mL FITC-conjugated anti-HbA MAb with 10 µL of D-PBS containing 0.1% BSA and add to 80 µL of cell suspension. To detect HbF, combine 10 µL of 200 µg/mL FITC-conjugated anti-HbF MAb with 10 µL of D-PBS containing 0.1% BSA and add to 80 µL of cell suspension.
9. Stain for 15 min at room temperature in the dark.
10. Wash the cells twice with 2 mL of FACS buffer (453*g*, 3 min).
11. Resuspend the cells in 200 µL of FACS buffer for immediate analysis.

3.5.3.2. Detection of Globin Expression by Endpoint Reverse Transcription PCR

3.5.3.2.1. Total RNA Extraction and Reverse Transcription Reaction (First cDNA Synthesis)

1. Extract total RNA from frozen cell pellets of hEBs or erythroid colonies (stored in liquid nitrogen) using the "mini protocol" for isolation of total RNA from animal tissues described in the RNeasy kit (**Subheading 2.5.3.**), according to the manufacturer's instructions.
2. Measure the total amount of RNA extracted (*see* **Note 6**).
3. Precipitate RNA by adding 0.5 vol of 5 *M* NH_4AC, 0.04 vol of glycogen, and 2.5 vol of 100% ethanol and store overnight at –80°C.
4. Centrifuge RNA for 1 h at 4°C and maximum speed in a tabletop centrifuge.
5. Gently aspirate the supernatant using P1000 then P200 pipetmen and add 400 µL of 70% ethanol (diluted in DEPC-treated water).
6. Centrifuge 15 min at 4°C and maximum speed.
7. Gently aspirate the supernatant as in **step 5**, and let the RNA air-dry (*see* **Note 7**).
8. Resuspend RNA by adding the desired volume of RNAse-free water as suggested in the kit.
9. Perform full-length synthesis of cDNA first strand from the desired amount of RNA using the appropriate kit (**Subheading 2.5.3.**), according to the manufacturer's instructions. Each reaction must stand at 4°C for at least 5 min before proceeding to PCR reaction.

3.5.3.2.2. PCR Reaction

1. All genes are amplified for 40 cycles on a Perkin-Elmer GeneAmp PCR system 9700 cycler.
2. The primer sets and cycle conditions used for the detection of embryonic (ε and ζ) and adult (β) globins as well as the internal invariant control (β-actin) are shown in **Table 1**.

Table 1
Primer Sets and Cycle Conditions

Gene	Forward primer	Reverse primer	Cycle conditions
ζ-globin (400 bp)	5′-CCAAGACTGAG AGGACCATCATTG	5′-AGGACAGAGGAT ACGACCGATAGG	2 min 94°C, 30 s 94°C, 30 s 64°C, 1.5 min 72°C, and 10 min 72°C
ε globin (212 bp)	5′-AAGATGAATGT GGAAGAGGCTGG	5′-TTAGCAAAGG CGGGCTTGAG	2 min 94°C, 45 s 94°C, 45 s 63°C, 1.5 min 72°C, and 10 min 72°C
β globin (394 bp)	5′-CACCAGCCA CCACTTTCTGA	5′-GTCTGCCGTT ACTGCCCTGT	2 min 94°C, 45 s 94°C, 45 s 62°C, 1.5 min 72°C, and 10 min 72°C
β-actin (222 bp)	5′-GATCCACATCT GCTGGAAGG	5′-AAGTGTGACG TTGACATCCG	2 min 96°C, 45 s 94°C, 45 s 60°C, 2 min 72°C, 10 min 72°C

The size of each PCR product is given between brackets. Detection of the amplified products is done by separation on 2% agarose gels with ethidium bromide (*see* **Note 8**).

3.6. Characterization of hESC-Derived Hematopoietic Cells In Vivo

Despite in vitro analysis, bona fide hematopoietic stem cells can only be functionally defined by sustained multi-lineage in vivo reconstitution upon transplantation. Experimentally, the NOD/SCID xenotransplant assay has provided a powerful tool to functionally define candidate human hematopoietic stem cells, defined as SCID-repopulating cells *(8)*. However, conventional intravenous transplantation of hESC-derived hematopoietic cells causes mouse mortality owing to emboli formed from rapid cellular aggregation in response to mouse serum *(9)*. IBMT is required for functional in vivo assessment of hESC-derived hematopoietic cells *(9)*.

3.6.1. IBMT of NOD/SCID-β2m-/- Mice

1. Collect $CD45^{neg}$PFV-derived hematopoietic cells as described previously. Resuspend the cells in IMDM medium.
2. Transfer 5×10^5–1×10^6 cells to 1.5-mL Eppendorf tubes. Prepare one tube for each mouse IBMT. Centrifuge the tubes for 2 min at 453*g*. Carefully discard supernatant without disturbing the cell pellet.
3. Resuspend the cells in 25 μL of IMDM medium, avoiding bubbles.
4. Sublethally irradiate (3.25 Gy) 8- to 12-wk old NOD/SCID-β_2m-/- mice.
5. Weigh the animal (*see* **Note 9**). Inject Avertin intraperitoneally at a dose of 0.014–0.018 mL/g of body weight. Use lower dosage for small females and higher dosage for large males. Leave the animal undisturbed until anaesthetic plane has been attained.
6. Fill a 3-mL syringe coupled to a 27-gage needle with 2 mL D-PBS. Rinse a 28-gage insulin syringe (0.5 in., 0.3 mL) with IMDM medium to fill the dead space, then carefully fill the barrel with the cell suspension.
7. Put the mouse on sterilized gauze. Gently place a small amount of sterile eye ointment on the mouse's eyes using sterile gauze.
8. Sterilize the knee with Proviodine detergent, alcohol wipe, and Proviodine disinfectant. Start sterilization at the incision site and move toward the periphery. Allow final solution to dry in order to create a bacteriostatic barrier.
9. Flex the mouse's leg to move the femur and tibia to a vertical position. Brace the animal's femur with your index finger, the tibia with your thumb, and use your middle finger for additional stabilization where needed. Make sure that your index finger can feel the femur and guide the direction.
10. "Drill" (do not push) a 27-gage needle vertically into the femoral cavity through the patellar tendon to form a channel.
11. Gently withdraw the 27-gage needle and keep the leg position unmoved. Insert a 28-gage insulin syringe into the channel made by the 27-gage needle. Inject 25 μL of cell suspension.
12. Gently remove the needle while straightening the leg and holding it in that position for several seconds to stop leakage of the injected cells.
13. Subcutaneously inject 0.3 mL buprenorphine (0.05–0.1 mg/kg of body weight) and 1 mL 0.9% D-PBS into the scruff.
14. Wrap the mouse in a sterile gauze. Return the mouse to the cage and keep it in a warm (under a heating lamp) and quiet environment until full recovery.
15. Administer the second dose of buprenorphine (0.3 mL) 24 h after IBMT. Monitor the mice daily for the first 3 wk (*see* **Note 10**).

3.6.2. Collect Bone Marrow of NOD/SCID-β2m-/- Mice Post-IBMT for Analysis of SCID-Repopulating Cells

1. Euthanize the mouse 8 wk after IBMT. Dip the mouse in 70% ethanol and dissect the mouse bones using sterile scissors and forceps.

2. Place injected femur, contralateral femur, or two tibias and two iliac crests separately into 35-mm dishes containing 2 mL of DMEM.
3. Flush bone marrow into a FACS tube with an insulin syringe containing 1 mL of DMEM.
4. For analysis of human white cells, lyse red cells with cold 0.8% ammonium chloride solution for 5 min at 4°C. Wash the remaining cells twice in D-PBS containing 3% FBS.

3.6.3. FACS Analysis of Engrafted Human Hematopoietic Cells in the Bone Marrow of NOD/SCID-β2m-/- Mice

1. Resuspend the cells in FACS buffer (D-PBS containing 3% FBS) at a concentration of 2.5×10^6 cells/mL. Distribute 200 µL of cell suspension (5×10^5 cells) to each FACS tube. To analyze human myeloid and lymphoid lineages, add 5 µL of FITC-conjugated MAb against human CD45 and 5 µL of PE-conjugated MAbs against either human CD19 (lymphoid) or CD33 (myeloid) to each tube. To analyze human erythroid lineage, add 5 µL of PE-conjugated MAb against human glycophorin-A and 5 µL of FITC-conjugated MAb against human CD36. Simultaneously set up corresponding IgG isotype MAb controls.
2. Incubate the tube for 30 min at 4°C.
3. Add 3 mL of FACS buffer to each tube. Centrifuge at 453*g* for 3 min. Aspirate the supernatant.
4. Repeat the wash step once. Leave 0.2 mL of supernatant after aspiration. Add 4 µL of 7-AAD. Incubate at room temperature for 10 min in the dark.
5. Analyze cell surface markers with a FACSCalibur and Cell Quest software (B&D).

3.6.4. Southern Blot and PCR Analysis to Detect Human DNA in Mouse Bone Marrow

1. In parallel, perform Southern blot and PCR analyses to detect human DNA in mouse bone marrow.
2. Isolate high-molecular-weight DNA using phenol/chloroform extraction or DNAzol reagent, according to the manufacturer's instructions.
3. Digest 1 µg of DNA with *Eco*RI restriction enzyme at 37°C overnight and separate on a 1.0% agarose gel.
4. Transfer the DNA to Hybond-N+ nylon membrane and hybridize with a ^{32}P-labeled human chromosome 17-specific α-satellite probe.
5. The level of human cell engraftment is quantified using a phosphorimager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA) by comparing the characteristic 2.7-kb band to human:mouse DNA mixture controls (limit of detection, approx 0.1% of human DNA).
6. If the level of human DNA is less than 0.1%, perform PCR to detect the human-specific chromosome 17-specific α-satellite. Forward primer 5′-ACACTCTTTTTGCAGGATCTA-3′ and reverse primer 5′-AGCAATGTGAAACTCTGGGA-3′ are used to amplify an 1171 bp sequence (40 cycles, 94°C 30 s, 60°C 30 s, 72°C 15 s + 72°C 10 min). The PCR products are separated on 1.0% agarose gels (*see* **Note 11**).

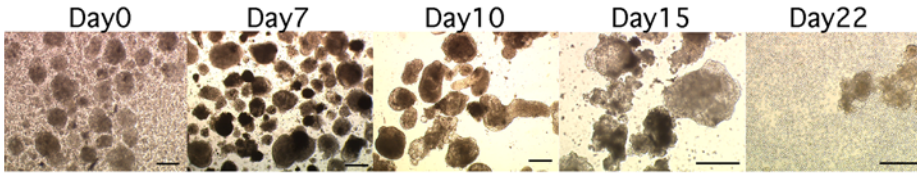

Fig. 1. Development of human embryoid bodies (hEBs) in the presence of hematopoietic cytokines and bone morphogenetic protein-4. Debris and single cells can be seen after hEB formation (d 0). Cystic hEBs can be observed after d 7 hEB development. Numerous bright single cells found in 22 hEBs are $CD45^+$ cells that have been confirmed by FACS analysis. Bar = 50 μm. (Please *see* the companion CD for the color version of this figure.)

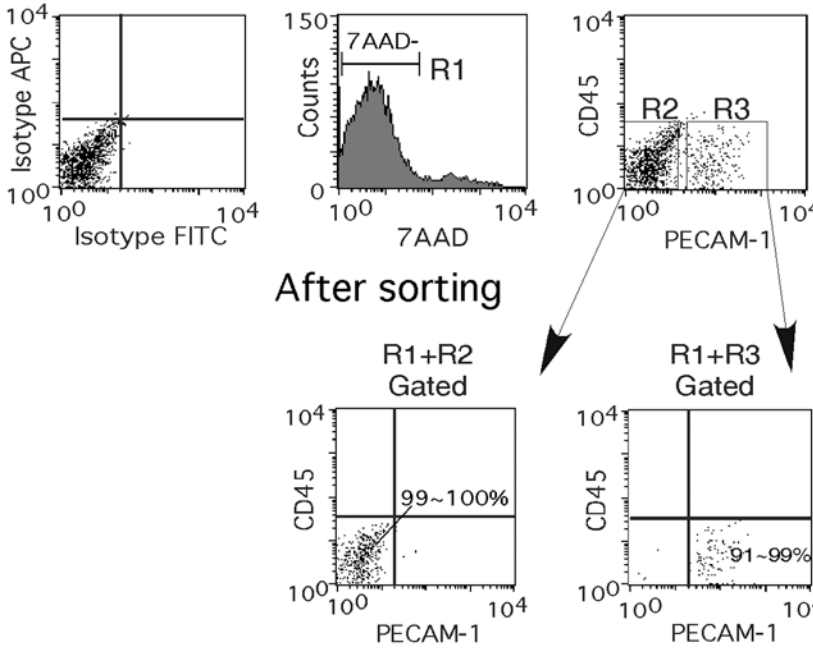

Fig. 2. Isolation of $CD45^-$PECAM1+ precursors. Hematopoietic precursor $CD45^-$ PECAM+ cells are isolated from d 10 human embryoid bodies by a fluorescence activated cell sorting Vantage SE. Dead cells are excluded by positive staining for 7AAD. The sorting purity are 99–100% for $CD45^-$PECAM1– (R1+R2), and 92–99% for $CD45^-$PFV+ (R1+R3) subpopulations determined using the same sorting gate settings.

4. Notes

1. MEF-CF quality is crucial for maintenance of undifferentiated hESC and subsequent formation and development of hEB. Because MEF-CM quality varies among different batches of MEF cells and MEF-CM preparations, assessing each batch of MEF-CM before use is required (*see* **Subheading 2.1.1.**).

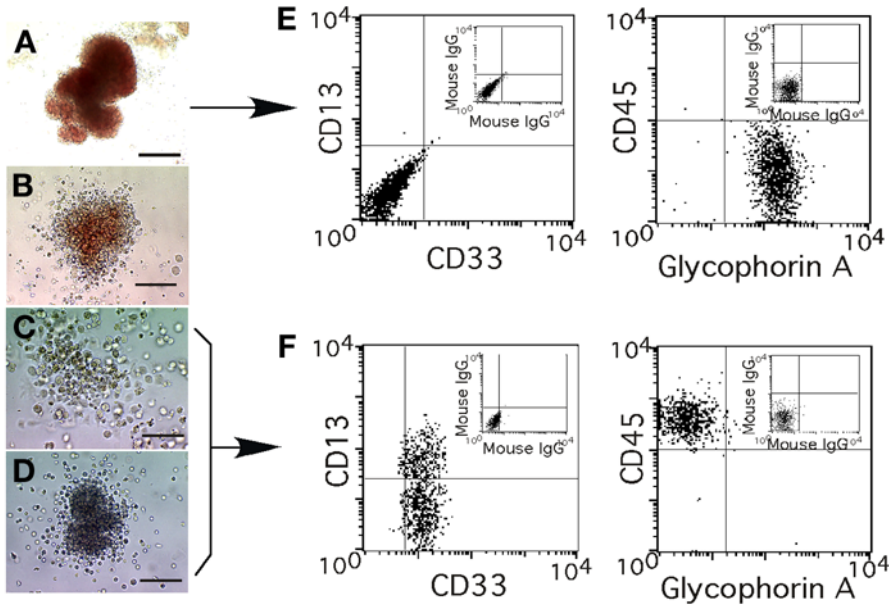

Fig. 3. Hematopoietic colonies from human embryoid bodies (hEB)-derived CD45$^+$ cells. Representative colonies from hEB-derived CD45$^+$ cells are depicted, including erythroid colony **(A)**, multipotent colony **(B)**, macrophage colony **(C)**, and granulocyte colony **(D)**. Cells comprising the erythroid colonies express erythroid marker Glycophorin A, but lack pan-leukocyte marker CD45 and myelomonocytic markers CD33 and CD13 **(E)**. Cells from the macrophage colonies or granulocyte colonies express CD45, CD33, and CD13, but lack glycophorin A **(F)**. (Please *see* the companion CD for the color version of this figure.)

2. All tissue culture protocols must be performed under sterile conditions. Prewarm all media required at 37°C before use. *Do not* warm unnecessary media, because certain components in the media might be sensitive to temperature. Keep all fluorochrome-conjugated MAbs in the dark. All staining steps using fluorochromes should be performed under protection from light. For all PCR experiments, wear gloves, clean bench and all equipment with RNase away solution before use, and employ new stocks of disposable RNase-free materials (tips, tubes) and reagents (*see* **Subheading 2.7.**).
3. Compact hESC colonies are crucial for hEB formation. When dissociation of hESC to form hEBs, determine the appropriate incubation time by examining the colonies under microscope, as incubation time will vary between different batches of collagenase IV and hESC lines. In addition, avoid vigorous pipetting (*see* **Subheading 3.1.**).
4. Do not fix the cells. Fixation step will increase background and cause artefacts (*see* **Subheading 3.5.1.1.**).

5. To avoid extrusion of the nucleus and distortion of cells' morphology, *do not* use higher speed or longer centrifugation time (*see* **Subheading 3.5.2.2.**).
6. We use GeneQuant II to measure total amount of RNA (*see* **Subheading 3.5.3.2.1.**).
7. Careful removal of supernatants and air-dry steps are critical to eliminate previously used reagents that could inhibit the reverse transcription reaction (*see* **Subheading 3.5.3.2.1.**).
8. For each PCR reaction, β-actin PCR product is amplified in parallel (*see* **Subheading 3.5.3.2.2.**).
9. Before injection, shake Avertin (2.5% working solution) vigorously because the solution is water insoluble (*see* **Subheading 3.6.1.**).
10. The IBMT technique requires practice. Most failures come from: inserting the needle when your index finger fails to identify the femur orientation; moving the 27-gage needle under the skin before "drilling" the femur (poor alignment of the entrance of skin and bone) causing the 28-gage needle to miss the bone entrance of the femur channel; or changing the femur position during the procedure (*see* **Subheading 3.6.1.**).
11. The criteria for mouse engraftment are the presence of human DNA in both the transplanted femur and nontransplanted bone marrows (*see* **Subheading 3.6.4.**).

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15

Retroviral Transduction of Hematopoietic Progenitors Derived From Human Embryonic Stem Cells

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Summary

It has been recently identified that cytokines and BMP-4 promote hematopoiesis from human embryonic stem cells (hESC) and that, before hematopoietic commitment, a rare subpopulation of cells lacking CD45, but expressing PECAM-1, Flk-1, and VE-cadherin (hereinafter termed $CD45^{neg}$PFV precursors), are exclusively responsible for hematopoietic cell fate on cytokine stimulation. Efficient strategies to stably transduce these hematopoietic precursors specifically generated from hESCs would provide a novel and desirable tool to study hematopoietic development through the introduction and characterization of candidate genes suspected to regulate self-renewal processes of hESC-derived hematopoietic cells or dynamically track hESC-derived hematopoietic stem cells in vivo. To date, only transient transfection and stable transduction using lentiviral vectors have been reported in undifferentiated hESC followed by random and spontaneous differentiation into different cell types. However, protocols for stable transduction of hematopoietic progenitors prospectively derived from hESC need to be developed yet. In the present chapter, we described detailed methods on the recently characterized and optimized GALV-pseudotyped retroviral gene transfer strategy to stably transduce the hematopoietic progenitor cells prospectively derived from $CD45^{neg}$PFV hemogenic precursors as a vital tool to study hematopoietic development and to characterize candidate genes suspected to eventually confer robust and sustained repopulating ability to hESC-derived hematopoietic cells.

Key Words: hESC; hematopoiesis; transduction; retrovirus; gene therapy.

1. Introduction

The ability to generate cells of the hematopoietic system has immense utility in several areas of clinical and experimental medicine and is now restricted to sources derived from human umbilical cord blood, bone marrow, and mobilized peripheral blood *(1)*. Therapeutic approaches involving the transplantation of

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hematopoietic stem cells (HSC) apply to a wide range of patients, prompting the search of an alternative source of hematopoietic cells *(2,3)*. Human embryonic stem cells (hESC) are considered totipotent cells derived from the inner cell mass of the developing blastocyst *(4,5)* capable of differentiation into embryoid bodies (EB) containing cell types representing endoderm, mesoderm, and ectoderm germ layers *(6–9)*. In this context, the establishment of hESC lines in 1998 *(4)* has given rise to a new and exciting research field in regenerative medicine that is expected to provide potential applications to cell replacement therapies and an immediate contribution to understanding the fundamental processes of human primitive hematopoietic developmental biology *(8,10–12)*.

Our group has recently identified that cytokines and BMP-4 promote hematopoiesis from hESC *(2,13)* and that, before the hematopoietic commitment, a rare subpopulation of cells lacking CD45, but expressing PECAM-1, Flk-1, and VE-cadherin (termed $CD45^{neg}PFV$ precursors) are exclusively responsible for hematopoietic cell fate potential on cytokine stimulation *(14)*. However, although the expansion potential of hESC is virtually unlimited, the ability to efficiently differentiate hESC into severe-combined immunodeficient (SCID) repopulating cells able to home and repopulate the bone marrow of NOD/SCID mice still remains to be overcome *(15,16)*. In this context, the genetic mechanisms dictating hematopoietic-lineage specification during human embryonic development are largely unknown *(17,18)*. In general, there is a strong evidence suggesting that the transition from primitive to definitive hematopoiesis seems to be tightly regulated by temporal and spatial transcription factors that act as molecular switches to activate or repress specific gene expression programs *(19–22)*. Before any clinical utility of hESC-derived hematopoietic cells can be applied for cell replacement strategies, reproducible methods to unravel these fundamental genetic mechanisms need to be developed. In this setting, efficient strategies to stably transduce these hematopoietic cells specifically generated from hESC become a novel highly desirable tool to study hematopoietic development, to characterize candidate genes suspected to regulate self-renewal processes of hESC-derived hematopoietic cells, and to in vivo dynamically track hESC-derived hematopoietic stem cells *(23,24)*.

To date, only transient transfection and stable transduction using lentiviral vectors have been reported in undifferentiated hESC followed by random and spontaneous differentiation into cell types comprised of the three germ layers *(10,25–28)*. However, protocols for stable transduction of hematopoietic progenitors prospectively derived from hESC will undoubtedly provide further insights into our understanding of hematopoietic development. In addition, despite to the higher efficiency and levels of transgene expression provided by lentiviral vector systems, to date, the retroviral long terminal repeat-driven vector system is the only one used in clinical trials for stable gene transfer into hematopoietic stem and progenitor cells *(29–31)*. In this context, our laboratory *(32)* has recently

characterized and optimized a GALV-pseudotyped retroviral gene transfer strategy to stably transduce the hematopoietic progenitor cells prospectively derived from CD45^{neg}PFV hemogenic precursors as a vital tool to study hematopoietic development and to characterize candidate genes suspected to eventually confer robust and sustained repopulating ability to hESC-derived hematopoietic cells *(23,33)*. In this chapter, we describe up-to-date protocols used to isolate CD45^{neg}PFV hemogenic precursors from EB as well as the retroviral gene transfer strategy developed to transduce hematopoietic progenitors emerging from these hESC-derived CD45^{neg}PFV hemogenic precursors.

2. Materials

2.1. Culture of Undifferentiated hESC and EB Formation

For the maintenance of hESC in undifferentiated state, human (hEB) formation and cytokine treatment of EB cultures refer to Chapter 15.

2.2. Preparation of Single Cells From hEB

1. Collagenase B (100 mg; Boehringer Mannheim, Burlington, Canada; cat. no. 1088-807). To prepare a stock solution: calculate the total amount of International Units (IU) in each individual bottle, dilute with knockout-Dulbecco's modified Eagle's medium (DMEM) to make 4.0 IU/mL and filter it through a 0.22-µm filter. Aliquot 1 mL of stock solution into 15-mL polypropylene conical tubes and store at –30°C. Avoid repeated freeze and thaw. To prepare working solution (0.4 IU/mL): thaw 1 mL aliquot of the stock solution and dilute it in 9 mL knockout-DMEM. The working solution can be stored at 4°C up to 1 wk.
2. Knockout-DMEM media (500 mL; Gibco, Burlington, Canada; cat. no. 10829-018).
3. 37°C water bath.
4. Cell dissociation buffer enzyme-free phosphate-buffered saline (PBS)-based (100 mL; Gibco; cat. no. 13151-014).
5. P-1000 pipetman and tips.
6. 40-µm nylon cell strainer (Becton Dickinson, Mississauga, Canada; cat. no. 352340).
7. Iscove's modified Dulbecco's medium (IMDM) (Gibco; cat. no. 12200-028).
8. 15-mL polypropylene conical tubes (Becton Dickinson; cat. no. 352096).

2.3. Isolation of CD45^{neg}PFV Precursors Cells

1. 1X PBS Ca^{2+} and Mg^{2+} free (PBS) (500-mL; Gibco; cat. no. 11965-092).
2. Nonheat-inactivated fetal bovine serum (FBS) characterized and screened for hESC growth (500 mL; Hyclone, Logan, UT; cat. no. 30071-03).
3. Allophycocyanin (APC)-conjugated anti-human CD45 monoclonal antibody (clone HI30; Becton Dickinson; cat. no. 555485). It should be used at 5 µg/mL. Protect from light.
4. PE-conjugated mouse anti-human PECAM-1-PE monoclonal antibody (clone WM59; Becton Dickinson; cat. no. 555446). It should be used at 5 µg/mL. Protect from light.

5. 7-aminoactinomycin D (7-AAD) (Immunotech, Marseille, France; cat. no. PN IM3422). Protect from light.
6. PE-conjugated mouse immunoglobulin (Ig)G isotype (Becton Dickinson; cat. no. 340043). Protect from light.
7. APC-conjugated mouse IgG isotype (Becton Dickinson; cat. no. 340442). Protect from light.
8. Trypan blue viability reagent (100 mL; Gibco; cat. no. 1691049).
9. FACS Vantage SE sorter (Becton Dickinson).
10. 5-mL polystyrene tubes (Becton Dickinson; cat. no. 352054).
11. Hematocytometer (Hausser Scientific, Horsman, CA; cat. no. 177569).

2.4. Retroviral Exposure of CD45^{neg}PFV Precursors Cells

1. Retroviral vector containing the gene of interest (GFP reporter) under the control of viral long terminal repeat promoter.
2. PG13 retroviral packaging cell line (ATCC, Manassas, VA; cat. no. CRL-10686) (*see* **Note 1**) stably expressing the provirus containing the gene of interest with an appropriate functional titer (*see* **Note 2**) ***(34–36)*** (*see* **Note 3**).
3. T-25 tissue culture flasks (Becton Dickinson; cat. no. 353109).
4. 96-well fibronectin-coated plates (Becton Dickinson; cat. no. 354409).
5. 96-well nontreated plates (Becton Dickinson; cat. no. 15705-066).
6. Fibronectin solution 1 µg/µL (5 mL; Sigma, Oakville, Canada; cat. no. F0895).
7. 5X serum-free BIT (bovine serum albumin-insulin-transferrin) media (Stem Cell Technologies, Vancouver, Canada; cat. no. 09500).
8. IMDM (Gibco; cat. no. 12200-028).
9. L-Glutamine 200 m*M* (Gibco; cat. no. 15039-027).
10. 14.3 *M* 2-betamercaptoethanol (100 mL; Sigma; cat. no. M7522).
11. 55 m*M* 2-betamercaptoethanol: prepare 1.43 *M* solution by diluting 1:10 in PBS the 14.3 *M* solution and then dilute 10 µL of 1.43 *M* solution in 250 µL of IMDM. 1.43 *M* and 55 m*M* solutions of 2-betamercaptoethanol can be stored at 4°C for 1 mo and 2 wk, respectively.
12. rhuSCF 2500 µg powder (Amgen Inc., Thousands Oaks, CA; donated). Prepare a working concentration of 1.5×10^3 ng/µL: dissolve the powder in 1.67 mL PBS + 2% FBS. Make 50-µL aliquots and store at –30°C. Once thawed, keep the vials at 4°C for no longer than 1 mo.
13. rhuFlt-3 Ligand 250µg powder (R&D Systems Inc, Minneapolis, MN; cat. no. 308-FK/CF). Prepare a working concentration of 250 ng/µL: dissolve the powder in 1 mL of PBS + 2%FBS. Make 100-µL aliquots and store at –30°C. Once thawed, keep the vials at 4°C for no longer than 1 mo.
14. rhuIL-3 50 µg powder (R&D Systems Inc; cat. no. 203-IL-050). Prepare a working concentration of 25 ng/µL: dissolve the powder in 2 mL of PBS + 2% FBS. Make 100-µL aliquots and store at –30°C. Once thawed, keep the vials at 4°C for no longer than 1 mo.
15. rhuIL-6 50 µg powder (R&D Systems Inc; cat. no. 206-IL-050). Prepare a working concentration of 50 ng/µL: dissolve the powder in 1 mL of PBS + 2% FBS. Make

50-µL aliquots and store at –30°C. Once thawed, keep the vials at 4°C for no longer than 1 mo.

16. rhuG-CSF 300 µg/mL (Amgen Inc; cat. no. 3105100). Make 100 µL aliquots and use as it is at 300 ng/µL. Aliquots must be stored at –30°C. Once thawed, keep them at 4°C for up to 1 mo.
17. 1X complete serum-free media for liquid culture of $CD45^{neg}$PFV precursors cells. The 1X complete serum-free media is composed of 1X BIT media made up in IMDM supplemented with 2 m*M* L-Glu, 0.1 m*M* 2-betamercaptoethanol, 300 ng/mL SCF, 300 ng/mL Flt-3L, 10 ng/mL interleukin (IL)-3, 10 ng/mL IL-6, and 50 ng/mL G-CSF. To prepare 10 mL of 1X complete serum-free media: combine 2 mL 5X BIT media, 7.86 mL IMDM, 2 µL rhuSCF at 1.5×10^3 ng/µL, 12 µL rhuFlt-3L at 250 ng/µL, 4 µL rhuIL-3 at 25 ng/µL, 2 µL rhuIL-6 at 50 ng/µL, 1.64 µL rhuG-CSF at 300 ng/µL, 20 µL 2-betamercaptoethanol at 55 m*M* and 100 µL L-Glu at 200 m*M*. The media must be prepared fresh.
18. Millex-GP 0.22-µm low binding protein filter (Millipore, Nepean, Canada; cat. no. SLGP033RS).
19. Millex-GP 0.45-µm low binding protein filter (Millipore; cat. no. SLHVR25LS).
20. 0.25% Trypsin-1 m*M* EDTA (100-mL; Gibco; cat. no. 25300-054).
21. 1-mL syringes (Becton Dickinson; cat. no. 309602).
22. 5-mL syringes (Becton Dickinson; cat. no. 309585).
23. 10-mL syringes (Becton Dickinson; cat. no. 309604).
24. Protamine sulfate (1 g; Sigma, cat. no. P40201). For a 1 µg/µL working solution: reconstitute 1 g in 1 L distilled water. The working solution is stored at 4°C for up to 1 yr.
25. Glucose 450 g/L solution (100 mL; Sigma; cat. no. G8769).
26. FBS (500 mL; Hyclone; cat. no. 30071-03).
27. α-minimum essential medium (α-MEM; Gibco; cat. no. 12200-028). α-MEM needs to be supplemented with 7% of FBS and 4.5 g/L glucose. To prepare 500 mL: mix 460 mL of α-MEM with 35 mL of FBS and 5 mL of 450 g/L glucose. α-MEM supplemented with FBS and glucose can be store at 4°C for up to 3 mo.
28. IMDM supplemented with 2.5% FBS. To prepare 500 mL: mix 487.5 mL of α-MEM with 12.5 mL of FBS.
29. IMDM supplemented with 10% FBS. To prepare 500 mL: mix 450 mL of α-MEM with 50 mL of FBS.
30. Flow buffer: PBS + 3% FBS. For 100 mL buffer preparation: mix 97 mL of 1XPBS and 3 mL of FBS.

2.5. Assessment of Transduction Efficiency Into hESC-Derived Hematopoietic Progenitor Cells

2.5.1. Gene Transfer Efficiency by Flow Cytometry

1. FACSCalibur (Becton Dickinson).
2. 5-mL polystyrene tubes (Becton Dickinson; cat. no. 352054).

3. PE-conjugated mouse IgG isotype (Becton Dickinson; cat. no. 340043). Protect from light.
4. APC-conjugated mouse IgG isotype (Becton Dickinson; cat. no. 340442). Protect from light.
5. 7-AAD (Immunotech; cat. no. PN IM3422). Protect from light.
6. PE-conjugated anti-human CD34 monoclonal antibody (clone HPCA-2; Becton Dickinson; cat. no. 555822). Use at 5 µg/mL and protect from light.
7. APC-conjugated anti-human CD45 monoclonal antibody (clone HI30; Becton Dickinson, cat. no. 340943). It should be used at 5 µg/mL. Protect it from light.
8. Flow buffer: PBS + 3% FBS (*see* **Subheading 2.4., item 30**).

2.5.2. Detection of Transduced Hematopoietic Progenitors by Colony-Forming Unit Assay

1. Hematocytometer (Hausser Scientific; cat. no. 177569).
2. 12-well nontreated plates (Becton Dickinson; cat. no. 351143).
3. 1-mL syringes (Becton Dickinson; cat. no. 309602).
4. 16-gage, 1.5-in. bevel needles (Becton Dickinson; cat. no. 305198).
5. Sterile water.
6. Trypan blue viability reagent (100 mL; Gibco; cat. no. 1691049).
7. IMDM (Gibco; cat. no. 12200-028).
8. Inverted microscope.
9. Fluorescence microscope.
10. SCF (*see* S**ubheading 2.4., item 12**).
11. EPO (Amgen Inc; donated).
12. GM-CSF (Amgen Inc; donated).
13. IL-3 (*see* **Subheading 2.4., item 14**).
14. H4230 methylcellulose culture (80 mL; Stem Cell Technologies; cat. no. 04230). H4230 has to be supplemented with growth factors. Thaw H4230 overnight at 4°C and add directly into the H4230 bottle: 20 mL of sterile IMDM, 50 ng/mL SCF (3.7 µL at 1.5×10^3 ng/µL), 10 ng/mL IL-3 (44 µL at 25 ng/µL), 3 U/mL EPO (33 µL at 10,000 U/mL) and 10 ng/mL GM-CSF (3.7 µL at 1:10 dilution of 3×10^3 ng/µL). Shake vigorously and leave overnight to settle. Make 5- or 10-mL aliquots and store at –30°C. Once thawed, keep at 4°C for no longer than 1 wk.
15. 1.5-mL microtubes (Sarstedt, Montreal, Canada; cat. no. 72690).

2.5.3. Polymerase Chain Reaction Detection of Proviral Integration

1. P-20 pipetman and tips.
2. 1.5-mL microtubes (Sarstedt; cat. no. 72690).
3. 1X PBS.
4. 1 *M* Tris-HCl, pH 7.6.
5. 0.5 *M* EDTA.
6. 5 *M* NaCl.

7. 20% SDS (1 L; Bio-Rad, Hercules, CA; cat. no. 161-0418).
8. DNA-A solution (10 m*M* Tris-HCl, 10 m*M* EDTA, 10 m*M* NaCl). To prepare: mix 1 mL 1 *M* Tris-HCl pH 7.6, 2 mL 0.5 *M* EDTA, 0.2 mL 5 *M* NaCl, and 96.8 mL H_2O.
9. Proteinase K 10 mg/mL (Sigma; cat. no. P2308). Make 100 µL aliquots and store at –30°C.
10. DNA-B solution: mix 90 mL DNA-A solution and 10 mL 20% SDS. Store at room temperature.
11. 37°C water bath.
12. Fume hood.
13. Tris-buffered phenol (100 mL; Invitrogen, Mississauga, Canada; cat. no. 15513-039). Store at 4°C.
14. Benchtop microcentrifuge.
15. Shaker for 1.5-mL microtubes.
16. Chloroform isoamyl alcohol 24:1 (1 L; Merck, Montreal, Canada; cat. no. CX1054-6). Store at room temperature.
17. 1:1 (v:v) phenol:chloroform isoamyl alcohol solution.
18. 100% ethanol.
19. 5 *M* sodium acetate.
20. Glycogen 20 mg/mL aqueous solution (1 mL; Fermentas, Burlington, Canada; cat. no. R0561).
21. TE buffer pH 8.0. To prepare: mix 100 m*M* Tris-HCl and 10 m*M* EDTA.
22. GeneAmp polymerase chain reaction (PCR) system 9000 (Applied Biosystems; Foster City, CA).
23. 0.2-mL PCR microtubes (Applied Biosystems; cat. no. N801-0840).
24. Distilled water DNAse, RNAse-free (500 mL; Gibco; cat. no. 10977-015).
25. 10X PCR buffer (Invitrogen; cat. no. Y02028).
26. 50 m*M* $MgCl_2$ (Invitrogen; cat. no. Y02016).
27. Taq DNA polymerase (Invitrogen; cat. no. 18038-042).
28. 100 m*M* dNTP set (Invitrogen; cat. no. 55082-85).
29. 20 m*M* dNTPs mixture (5 m*M* each): combine 20 µL of each dNTP at 100 m*M* with 320 µL of distilled water DNAse, RNAse-free water. Store at –30°C.
30. PCR primers diluted to 50 µ*M* with sterile distilled water DNAse, RNAse-free water. Primers used: eGFP (forward: 5′-GAC TTC AAG GAG GAC GGC AAC-3′; reverse: 5′-TAG AAA CTG CTG AGG GCG GG-3′); human CART-1 (forward: 5′-AGC TGC TGT GTG TGG AAT TG-3′; reverse: 5′-ACA GGG TTT GTG GAG ACT GG-3′).
31. Spectrophotometer.

3. Methods

3.1. Culture of Undifferentiated hESC and EB Formation

For the maintenance of hESC in undifferentiated state, hEB formation and cytokine-treatment of EB cultures refer to Chapter 15.

3.2. Preparation of Single Cells From hEB

1. Transfer each well of hEBs to an individual 15-mL polypropylene conical tube.
2. Centrifuge at 129*g* for 5 s. Aspirate off supernatant.
3. Add 4 mL Collagenase B (work solution, 0.4 IU/mL). Transfer hEBs back to the original low attachment plate and incubate them for 2 h at 37°C.
4. Transfer the cells to a 15-mL polypropylene tube. Centrifuge for 3 min at 453*g*.
5. Aspirate the Collagenase B and add 2 mL cell dissociation buffer.
6. Incubate for 10 min in a 37°C water bath.
7. Centrifuge at 453*g* for 3 min. Aspirate cell dissociation buffer and add 0.5 mL FBS-free IMDM.
8. Dissociate hEBs by gently pipetting up and down (approx 40 times) using a P1000 pipetman.
9. Add an extra 0.5 mL of FBS-free IMDM.
10. Pass the dissociated cells through a 40-μm cell strainer to get rid of the aggregates.
11. Count viable cells by trypan blue inclusion.

3.3. Isolation of CD45^{neg}PFV Precursor Cells

Isolation of CD45^{neg}PECAM1+Flk1+, CD45^{neg}PECAM1+, or CD45^{neg}Flk1+ cells from d 10 hEBs demonstrated that VE-cadherin segregated with either of these populations and produced identical results (*14*), indicating that any of these sorting strategies isolate a functionally identical subpopulation in which close to 90% of the cells are CD45^{neg}PFV^{pos}. Therefore, isolation of CD45^{neg}PECAM1+ cells represents a simpler and accurate strategy for purification of CD45^{neg}PFV precursors.

1. Resuspend single cells dissociated from d 10 hEBs in 1X PBS + 3% FBS in a concentration of 2×10^6/mL in a 5-mL polystyrene tube.
2. Add 20 μL of mouse anti-human PECAM-1-PE and 10 μL mouse anti-human CD45-APC into 1 mL cell suspension. In parallel and as a negative control add 2 μL of mouse IgG-PE and 2 μL of mouse IgG-APC into a separate 5-mL polystyrene tube containing 1×10^5 cells in 200 μL of 1X PBS + 3% FBS. Incubate for 30 min at 4°C.
3. Wash the cells with 3 mL 1X PBS + 3% FBS. Centrifuge for 5 min at 453*g*. Aspirate off the supernatant.
4. Repeat **step 3**.
5. Resuspend the cells in 1X PBS + 3% FBS at a concentration of 2×10^6/mL. Add 10 μL of 7-AAD to 1 mL cell suspension and stain for 10 min at room temperature to exclude dead cells.
6. Filter cells through a 40-μm cell strainer just before sorting to avoid clogging.
7. Isolate CD45^{neg}PECAM-1+ cells in a FACSVantage SE sorter. Set sorting gates based on respective IgG isotype controls.
8. Determine purity of the sorted population immediately after sorting using the same sorting settings (*see* **Note 4**).

3.4. Retroviral Exposure of CD45^{neg}PFV Precursors Cells (see *Notes 5 and 6*)

3.4.1. Day 0

1. As soon as you get the CD45^{neg}PECAM1+ cells back from the sorter, centrifuge them for 5 min at 250*g*.
2. Aspirate off supernatant and count them using a hematocytometer.
3. Seed 2 wells of a 96-well fibronectin-coated plate with 20,000 CD45^{neg}PFV cells in 200 µL of 1X complete serum-free media (*see* **Subheading 2.4., item 17**). Keep the cells growing and differentiating into hematopoietic cells for 10 d (*see* **Note 7**) with media changes every 3 d.
4. Thaw, using conventional techniques, two different aliquots of PG13 packaging cell line: one expressing the GOI (PG13-GOI) and a second one empty (not expressing any retroviral vector) (PG13-MOCK). Retroviral producers are grown in T-25 tissue culture-treated flasks to near confluence, by seeding 3×10^6 producers per flask with 5 mL α-MEM + 7% FBS + 4.5 g/L glucose (*see* **Subheading 2.4., item 27**).

3.4.2. Day 1

1. Aspirate off the media from producers (*see* **Note 8**) and replace with 5 mL of fresh α-MEM + 7% FBS + 4.5 g/L glucose media.
2. Keep growing the producers by passing them when they reach 90% confluence (*see* **Note 9**). To pass them, remove supernatant and add 0.75 mL of 0.25% Trysin-1 m*M* EDTA. After 2–4 min, all the cells should lift up. Then, add 9.25 mL of α-MEM + 7% FBS + 4.5 g/L glucose and pipet the cells several times to get a single cell suspension. Pass them 1:10 (1 mL of the single cell suspension plus 4 mL of α-MEM + 7% FBS + 4.5 g/L glucose into a new T-25 flask) approximately every 3 d.

3.4.3. Day 3

1. Gently replace half the volume of 1X serum-free complete media in each well with freshly prepared media. CD45^{neg}PFV cells grow loosely attached to the fibronectin-coated plate so the media can be easily removed from the top of the well without disturbing the cells using a P-100 pipetman.

3.4.4. Day 6

1. Replace the media as indicated above. From d 6 on, single, round, bright and nonadherent cells resembling hematopoietic morphology start to appear in the wells.

3.4.5. Day 9

1. Replace the media as indicated above. By d 9, most of the cultured cells should display hematopoietic-like morphology (*see* **Subheading 3.4.4.**).

2. Seed producers (PG13-GOI and PG13-MOCK) on d 9 at 3×10^6 cells in T-25 flask to have them near to confluence by d 10. Seed each producer in two different media:
   a. IMDM + 10% FBS: provides rich viral supernatant.
   b. IMDM + 2.5% FBS: provides partly depleted viral supernatant.

3.4.6. Day 10: First Retroviral Exposure

1. Precoat 2 wells of a 96-well nontreated plate with 5 μg/cm^2 fibronectin (*see* **Note 10**). Mix 6 μL of fibronectin at 1 μg/μL with 124 μL of 1X PBS and add 65 μL in each well.
2. Incubate at room temperature for 4 h to evenly cover the surface with the fibronectin.
3. Collect 10% FBS-containing viral supernatant from confluent producers (PG13-GOI and PG13-MOCK) and filter it through 0.45-μm filter.
4. Remove the fibronectin and preload one well with 100 μL (*see* **Note 11**) of 10% FBS-containing viral supernatant collected from PG13-GOI and the other well with 10% FBS-containing viral supernatant collected from PG13-MOCK. Incubate 30 min at 37°C.
5. Remove the supernatant and preload again as indicated in **step 4** (*see* **Note 11**).
6. During this incubation time, harvest the cells by gently pipetting up and down several times and transfer them into a 1.5-mL microtube (*see* **Note 12**).
7. Collect 2.5% FBS-containing viral supernatant from confluent producers (PG13-GOI and PG13-MOCK) and filter it through 0.45-μm.
8. Supplement this supernatant with 300 ng/mL SCF, 300 ng/mL Flt-3L, 10 ng/mL IL-3, 10 ng/mL IL-6, 50 ng/mL G-CSF, and 5 μg/mL protamine sulfate. To supplement 1 mL of viral supernatant, add 2 μL of a 1:10 dilution of rhuSCF at 1.5×10^3 ng/μL, 1.2 μL rhuFlt-3L at 250 ng/μL, 4 μL of a 1:10 dilution of rhuIL-3 at 25 ng/μL, 2 μL of a 1:10 dilution of rhuIL-6 at 50 ng/μL, 1.64 μL of a 1:10 dilution of rhuG-CSF at 300 ng/μL and 5 μL of protamine sulfate at 1 μg/μL.
9. Centrifuge the cells for 5 min at 201*g*, aspirate the supernatant and resuspend them in 200 μL (*see* **Note 11**) of supplemented 2.5% FBS containing viral supernatant.
10. Remove 10% FBS-containing viral supernatant from the wells and reseed the cells in 200 μL of supplemented 2.5% FBS-containing viral supernatant. Incubate 24 h at 37°C and 5% CO_2.
11. Seed producers (3×10^6 cells in T-25) on d 10 in 2.5% FBS-containing IMDM to collect viral supernatant on d 11.

3.4.7. Day 11: Second Retroviral Exposure (see ***Note 13***)

1. Collect 2.5% FBS-containing viral supernatant from confluent producers (PG13-GOI and PG13-MOCK) and filter it through 0.45-μm filter.
2. Supplement this supernatant with 300 ng/mL SCF, 300 ng/mL Flt-3L, 10 ng/mL IL-3, 10 ng/mL IL-6, 50 ng/mL G-CSF, and 5 μg/mL protamine sulfate (*see* **Subheading 3.4.6., step 8**).

3. Remove 150 µL of the old media using a P-100 pipetman. Special care should be taken to avoid disturbing the cells that are growing loosely attached to the fibronectin.
4. Add 150 µL of fresh 2.5% FBS-containing viral supernatant supplemented with cytokines and protamine sulfate. Incubate 24 h at 37°C and 5% CO_2.
5. Seed producers (3×10^6 cells in T-25) on d 11 in 2.5% FBS-containing IMDM to collect viral supernatant on d 12.

3.4.8. Day 12: Third Retroviral Exposure (see ***Note 13***)

1. Repeat **steps 1–4** of **Subheading 3.4.7.**
2. After the third retroviral exposure, discard the producers (*see* **Note 14**).

3.4.9. Day 13

1. Remove, without disturbing the cells, as much as you can of the old media using a P-100 pipetman.
2. Add 200 µL of 2.5% FBS-containing IMDM (*see* **Note 15**) and allow transgene expression for further 48 h.

3.4.10. Day 15

1. Harvest the cells and proceed to assess gene transfer transduction into $CD45^{neg}$PFV-derived hematopoietic progenitors.

3.5. Assessment of Transduction Efficiency Into hESC-Derived Hematopoietic Progenitor Cells

3.5.1. Gene Transfer Efficiency by Flow Cytometry

1. Collect cells 48 h after retroviral exposure, count them and determine how many cells you have available for flow cytometry (*see* **Note 16**). Stain 10,000–20,000 cells.
2. Resuspend 10,000–20,000 cells in 200 µL of PBS + 3% FBS in a 5-mL polystyrene tube.
3. Add 5 µL of mouse anti-human CD34-PE and 2 µL mouse anti-human CD45-APC. As a negative control, add 2 µL of mouse IgG-PE and 2 µL of mouse IgG-APC into a separate 5-mL polystyrene tube containing 5000 cells in 200 µL of 1X PBS + 3% FBS. Incubate for 30 min at 4°C.
4. Wash the cells with 3 mL 1X PBS + 3% FBS. Centrifuge the tubes for 5 min at 453*g*. Aspirate off the supernatant.
5. Resuspend the cells in 300 µL 1X PBS + 3% FBS. Add 2 µL of 7-AAD and stain for 10 min at room temperature to exclude dead cells.
6. Run the cells in a FACSCalibur flow cytometer. Set up quadrants based on mock retroviral exposure for GFP expression and based on respective IgG isotype controls for CD34 and CD45. The percent of GFP+ cells as compared to mock represents the overall transduction efficiency. The percent of GFP+CD45+ cells represents the transduction efficiency into $CD45^{neg}$PFV-derived hematopoietic cells and the

percent of GFP + CD45 + CD34+ indicates the transduction efficiency into $CD45^{neg}$PFV-derived cells with phenotype of hematopoietic progenitor (*see* **Fig. 1**).

3.5.2. Detection of Transduced Hematopoietic Progenitors by CFU Assay

1. Collect cells 48 h after retroviral exposure, count them, and determine the number of cells available for CFU assay (*see* **Note 16**). Plate 10,000 cells.
2. Using a 1-mL syringe coupled to a 16-gage needle, add 0.6 mL of H4230 methylcellulose media supplemented with growth factors (*see* **Subheading 2.5.2., item 13**) to 1.5-mL microtube.
3. Take 14,000 cells and bring them to a final volume of 100 µL in FBS-free IMDM. Mix cell suspension and 0.6 mL of H4230 methylcellulose media.
4. Vortex the tube vigorously and let it stand for 5 min to get rid of the bubbles.
5. Using a 1-mL syringe coupled to a 16-gage needle, plate 0.5 mL of the plating mixture in a nontreated 12-well plate.
6. Distribute methylcellulose over entire well surface avoiding bubbles.
7. Incubate cells at 37°C and 5% CO_2 in a humidified atmosphere.
8. Score the colonies after 12–14 d using standard morphological criteria in an inverted microscope. Then, use a fluorescence microscope to assess transduction into hematopoietic progenitors by calculating the frequency of GFP+ CFU (*see* **Fig. 2**).

3.5.3. Confirmation of Proviral Integration by PCR

3.5.3.1. Isolation of Individual Colonies From Methylcellulose

1. Pick a well-isolated GFP+ CFU under the microscope with a P-20 pipetman, and place into 300 µL PBS in 1.5-mL microtube, incubate for 20 min. Special care must be taken to ensure that cells from adjacent colonies do not contaminate the sample.
2. Spin at 453*g* for 5 min.
3. Aspirate off the PBS.
4. Snap freeze and store at –80°C until ready to extract DNA.

3.5.3.2. Genomic DNA Extraction From Individual Isolated Colonies

1. Thaw frozen colonies on ice.
2. Resuspend very well in 100 µL DNA A + 50 µg Proteinase K (5 µL of stock 10 mg/mL).
3. Add 100 µL DNA B and place in 56°C water bath for 1 h (*see* **Note 17**).
4. Add 200 µL Tris-buffered phenol and rotate the tube for 15 min (*see* **Note 18**). Do not vortex or pipet the DNA.
5. Spin tubes at maximum speed for 5 min.
6. Transfer the upper aqueous phase (containing genomic DNA) into clean 1.5-mL microtube. Ensure you do not transfer the white pellet containing the proteins.
7. Add 200 µL of 1:1 phenol:chloroform isoamyl alcohol solution and rotate the tubes for 15 min.

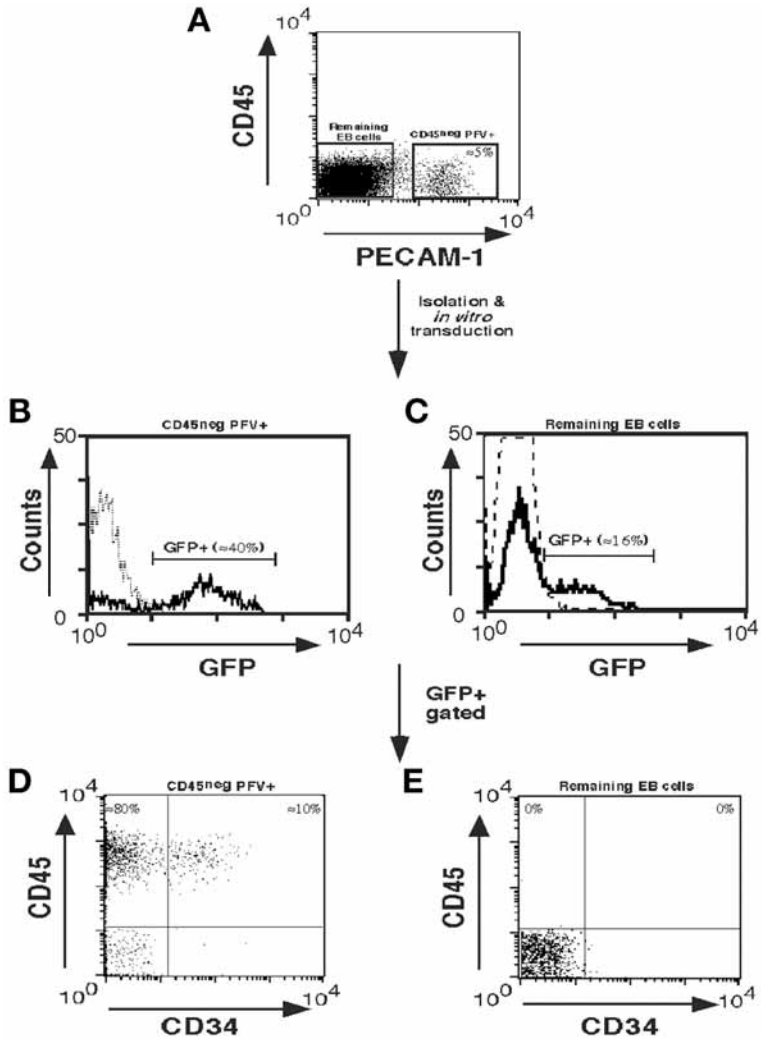

Fig. 1. Retroviral transduction of human embryonic stem cells (hESC)-derived hematopoietic cells. **(A)** Isolation of CD45^{neg}PECAM1+Flk1+, CD45^{neg}PECAM1+ or CD45^{neg}Flk1+ cells from hEBs demonstrated that VE-cadherin segregated with either of these populations and produced identical results *(14)*, indicating that any of these sorting strategies isolate a functionally identical subpopulation in which 90% of the cells are CD45^{neg}PFV^{pos}. Therefore, flow cytometric isolation of CD45^{neg}PECAM1+ cells represents a simpler and accurate strategy for purification of CD45^{neg}PFV precursors. **(B**,**C)** Flow cytometric analyses of transduction efficiency into CD45^{neg}PFV **(B)** and remaining EB cells **(C)**. Phenotype of the transduced (GFP+) progeny arising from CD45^{neg}PFV precursors **(D)** and remaining EB cells **(E)** showing that transduced hematopoietic cells exclusively arise from hESC-derived CD45^{neg}PFV precursors.

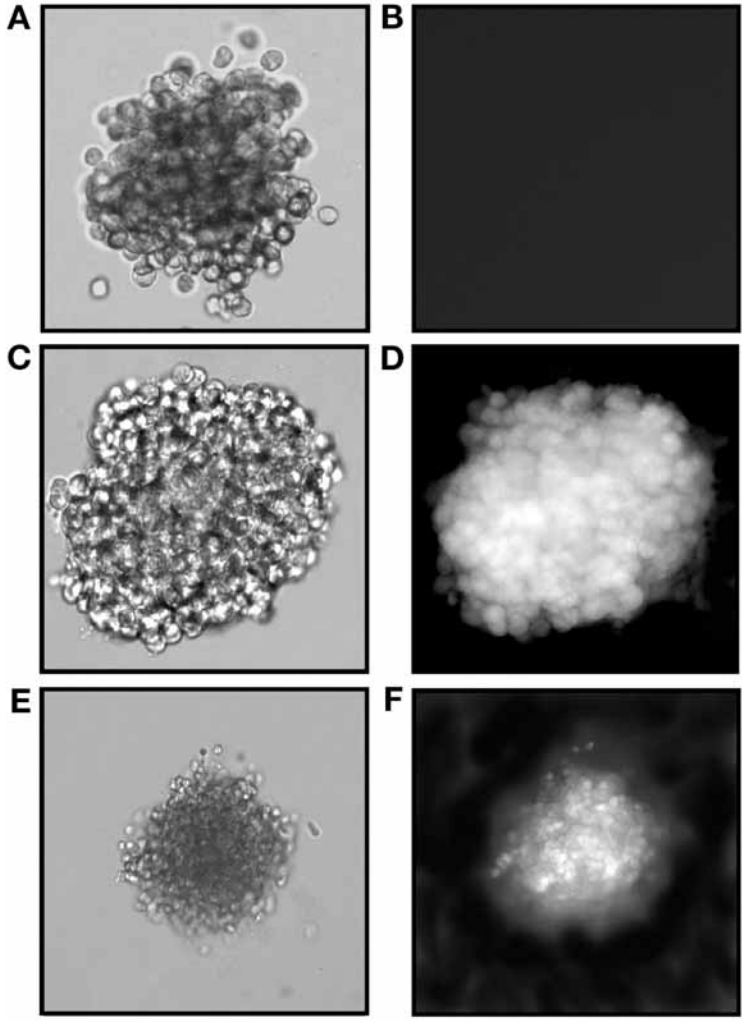

Fig. 2. Assessment of transduction efficiency into hematopoietic progenitors by using fluorescent microscopy. Bright-field (left panels) and fluorescence (right panels) microscopy pictures of representative colonies formed from CD45^{neg}PFV precursors following mock transduction **(A,B)** or GALV-pseudotyped retroviral transduction **(C–F)**.

8. Spin tubes at maximum speed for 5 min.
9. Transfer the upper aqueous phase (containing genomic DNA) into a clean 1.5-mL microtube.
10. Add 200 µL of chloroform isoamyl alcohol 24:1.
11. Spin tubes at maximum speed for 10 min.

12. Transfer the upper aqueous phase into a 1.5-mL microtube containing 30 µL 5 *M* NaCl and 20 µg glycogen at 20 mg/mL. Then add 500 µL 100% ethanol (two volumes).
13. Incubate overnight at –30°C.
14. Spin tubes at maximum speed for 30 min at 4°C.
15. Remove supernatant completely and dry the pellet in the fume hood.
16. Resuspend the pellet in 30 µL TE.
17. Measure DNA concentration using a spectrophotometer.
18. Store the DNA at 4°C until ready to use for PCR.

3.5.3.3. PCR

1. Prepare the PCR reaction mix (50 µL final volume): for GFP gene: 38.5 µL PCR dH_2O, 5 µL 10X PCR buffer, 1 µL 50 m*M* $MgCl_2$, 2 µL 20 m*M* dNTPs, 0.5 µL 50 µ*M* forward primer, 0.5 µL 50 µ*M* reverse primer, 0.5 µL Taq DNA polymerase, and 2 µL template DNA (approx 10 ng).
   For hCART-1 gene: 37.5 µL PCR dH_2O, 5 µL 10X PCR buffer, 2 µL 50 m*M* $MgCl_2$, 2 µL 20 m*M* dNTPs, 0.5 µL 50 µ*M* forward primer, 0.5 µL 50 µ*M* reverse primer, 0.5 µL Taq DNA polymerase, and 2 µL template DNA (approx 10 ng).
2. Run samples as per the following conditions: for GFP amplification: 1 cycle 96°C for 5 min, 35 cycles 96°C for 40 s, 65°C for 40 s, 72°C for 1 min, 1 cycle 72°C for 10 min, then hold at 4°C.
   For hCART-1 amplification: 1 cycle 96°C for 2 min, 35 cycles 94°C for 30 s, 60°C for 30 s, 72°C for 15 s, 1 cycle 72°C for 5 min, then hold at 4°C.
3. Resolve the PCR products on a 1% agarose gel. Only those colonies containing amplified hCART-1 template are used for determination of gene transfer efficiency into $CD45^{neg}$PFV-derived hematopoietic progenitors (*see* **Fig. 3**).

3.6. Future Directions

Being able to genetically manipulate hESCs and their derivatives opens new avenues in the study of human embryogenesis and the development of future cell therapies. Human ESCs provide a unique opportunity to study molecular mechanisms that regulate specification of the hematopoietic lineage in the human. Exploitation of this model using transgenic strategies depends on the ability to effectively target cells of the hematopoietic lineage and establish stable transgene expression. Mouse and hES derivatives are, so far, ineffective at repopulating hematopoiesis in lethally irradiated adults *(15,16)*. Previous data suggest that mouse primitive embryonic HSC can be induced to become definitive lymphoid-myeloid HSC if exposed to the proper microenvironment *(24)*. Recently, it has been shown that mouse primitive HSC can become definitive HSC by retroviral overexpression of either HoxB4 or Stat5 *(23,33)*. The generation of NOD/SCID repopulating cells (SRC) from hESC might be promoted by some of these genes as well as other potential factors capable of regulating primitive blood cells and inducing human stem cell expansion such as HoxB4

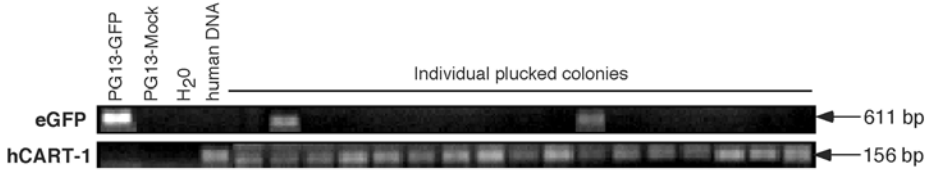

Fig. 3. Assessment of transduction efficiency into hematopoietic progenitors by using polymerase chain reaction (PCR) for proviral integration. Representative agarose gel stained with ethidium bromide containing the amplified PCR products from a GFP PCR done with individually plucked colonies. Positive DNA control can be extracted from PG13-GFP and an appropriate negative control is DNA extracted from PG13-Mock and mock-infected colonies. To ensure the availability of amplifiable template, DNA extracted from colonies should be subjected to a human-specific gene such as CART-1.

(23), Bmi-1 *(37,38)* and different members of Notch *(39,40)*, Wnt *(41)*, or Hedgehog pathways *(42)*. The successful delivery of a reporter gene (GFP) into $CD45^{neg}$PFV hemogenic precursors by means of a Gibbon ape leukemia virus (GALV)-pseudotyped retroviral gene transfer strategy paves the way for further gain-of-function studies conducted to address the potential role of different candidates genes in the generation of SRC from hESC and will provide a better understanding of the molecular mechanisms underlying cell fate.

4. Notes

1. PG13 is a retrovirus packaging cell line derived from TK- NIH/3T3 cells based on the GALV. Introduction of retroviral vectors by infection or transfection results in the production of retrovirus virions capable of infecting cells expressing GALV receptor 1 (also known Pit-1). $CD45^{neg}$PFV precursors express high levels of Pit-1 by RT-PCR *(32)*.
2. The functional titer of our GFP-expressing PG13 packaging cell line is 5×10^5 infectious virus particles per milliliter. Although many parameters may influence the titer, between 2×10^5 and 1×10^6 infectious virus particles per milliliter is usually considered a suitable titer when working with PG13 cell line.
3. Nontransduced PG13 retroviral packaging cell line should be used as mock in each experiment.
4. Purity average should be between 90 and 100%.
5. Although viruses produced by GALV-pseudotyped PG13 cell lines will no longer be able to replicate after they infect any target cell, they still are capable of infecting human cells. Caution should always be exercised in the production and handling of any recombinant retrovirus. For this reason, we highly recommend that you treat retroviral stocks generated by following Biosafety Level 2 guidelines. We also recommend consulting the health and safety guidelines and officers at your institution before starting your retroviral experiments.

6. The protocol detailed is optimized for 96-well plates. However, if you have enough cells you may scale up the procedure by using 48- to 6-well plates as long as you proportionally increase all the volumes (e.g., 1X serum-free complete media, virus-containing supernatants) as needed and maintain the concentration of all different cytokines and reagents.
7. 10-d prestimulation liquid culture is optimal to get the highest transduction efficiency into $CD45^{neg}$PFV-derived hematopoietic progenitors with CFU potential. Shorter prestimulation periods impair the transduction of hematopoietic progenitors with CFU capabilities although it slightly enhances the transduction efficiency of overall hematopoietic cells (CD45+).
8. It contains DMSO from the freezing procedure, which can negatively affect cell growth.
9. Virus production from freshly thawed PG13 cells is poor, so it is strongly recommended to have cells growing well approx 1 wk before collection of virus-containing supernatant.
10. Fibronectin facilitates colocalization of virus particles and target cells increasing the likelihood of infection of target cells.
11. The volume of viral supernatant used to preload the plate (2×100 µL) and to resuspend the cells (200 µL) is adjusted in order to have a multiplicity of infection (MOI) between 3 and 5: if virus titer equals 5×10^5/mL and each well contains 200 µL of viral supernatant it means that each well would have five times the input number of cells originally seeded (2×10^4). Although we have not tried to further increase the MOI, it is likely that higher MOI provides higher transduction levels.
12. It should be very easy to harvest the cells with no need of enzymatic digestion because they grow loosely attached to the fibronectin. If by any chance they do not lift up easily, just add 25 µL of 0.25% Trysin-1 m*M* EDTA and incubate for 1 min before blocking trypsin activity with 100 µL of IMDM + 10% FBS and harvest them.
13. We have experienced that isolated $CD45^{neg}$PFV precursors do not like to be repeatedly collected and transferred to a new fibronectin-coated, viral-preloaded well; resulting in a notable cell death. However, using the present retroviral gene transfer protocol we were able to get acceptable levels of transduction by precoating with fibronectin and preloading with viral supernatant just on the first day of retroviral exposure.
14. If no more experiments are scheduled for the next 7 d, it is strongly advised you discard the packaging cell line. Each laboratory should have enough frozen stocks of the packaging cell line for future retroviral gene transfer experiments. Prolonged culture of the PG13 cell line results in a drop of the titer and increases the likelihood for undesirable recombination events leading to the generation of helper-free infective virus *(34–36)*.
15. Data from our laboratory *(32)* indicate that the addition of FBS to cultures after retroviral exposure supported transgene expression resulting in hematopoietic progenitors derived from $CD45^{neg}$PFV hemogenic precursors.

16. At the end of the in vitro culture of CD45^{neg}PFV hemogenic precursors, we generally observe a twofold cell expansion as compared with the number of cells originally seeded *(32)*.
17. After this incubation you should not see any kind of cell pellet, debris, or white material floating in the tube.
18. All steps involving phenol or chloroform must be performed in a fume hood; and a lab coat, gloves, and safety glasses must be worn at all the times. Besides, an appropriate waste container for phenol and chloroform disposal is required.

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16

Genetic Manipulation of Human Embryonic Stem Cells by Transfection

Rachel Eiges

Summary

One of the great advantages of embryonic stem (ES) cells over other cell types is their accessibility to genetic manipulation. They can easily undergo genetic modifications while remaining pluripotent, and can be selectively propagated, allowing the clonal expansion of genetically altered cells in culture. Since the first isolation of ES cells in mice, many effective techniques have been developed for gene delivery and manipulation of ES cells. These include transfection, electroporation, and infection protocols, as well as different approaches for inserting, deleting, or changing the expression of genes. These methods proved to be extremely useful in mouse ES cells, for monitoring and directing differentiation, discovering unknown genes and studying their function, and are now being initiated in human ES (hES) cells. This chapter describes the different approaches and methodologies that have been applied for the genetic manipulation of hES cells and their applications. Specifically, two detailed protocols that can be used to generate clones of genetically modified hES cells by transfection will be described, with special emphasis on the important technical details that are required for this purpose.

Key Words: Human ES cells; genetic manipulation; transfection; overexpression; targeted mutagenesis; homologous recombination; knock-down by RNAi.

1. Introduction

1.1. Genetic Modification Approaches and Their Potential Applications

There are basically four types of strategies that can be applied for genetic engineering of hES cells: overexpression, knockout, knock-in, and knock-down experiments.

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1.1.1. Overexpression

Overexpression of genes is usually based on random integration of an exogenous DNA sequence into the genome. It can be applied for constitutive or facultative expression of either cellular or foreign genes. It may also be used for the introduction of reporter or selection genes, under the regulation of tissue specific promoters. These procedures allow us to label and track specific cell lineages following induced differentiation of human embryonic stem (hES) cells in culture. Moreover, it can be employed for the isolation of pure populations of specific cell types, by the use of selectable markers. The marker gene may either be a selectable reporter, such as green fluorescent protein (GFP), which can be selected for by fluorescent activated cell sorter (FACS), or a drug resistance gene *(1, 2)*. Likewise, the introduction of selectable reporters under the regulation of an inner cell mass-specific promoter, may allow the selection for or against undifferentiated cells in culture. This has been previously demonstrated by introduction of the *Rex1*-EGFP expression construct into hES cells, which is expressed in undifferentiated cells only *(3)*. The ability to isolate pure populations of specific cell types and eliminate undifferentiated cells prior to transplantation has great importance in cell-based therapy; this is because transplantation of undifferentiated cells may lead to teratoma formation.

Overexpression experiments may also be employed for directing the cell fate of differentiating ES cells in culture. This can be achieved by introducing master genes that play a dominant role in cell commitment, forcing the cells to differentiate into specific lineages that otherwise are rarely obtained among many other cell types in culture *(4– 6)*. In addition, overexpression may be employed for the generation of cell-based delivery systems by producing therapeutic agents at the site of damaged tissue. The use of ES-derived cells as therapeutic vectors has been previously shown to be possible in mice, where grafting of ES-derived insulin-secreting cells normalized glycemia in streptozotocin-induced diabetic mice *(7)*.

Apart from tagging, selecting, and directing the differentiation of specific cell types, it is possible to inactivate endogenous genes to study their function. This can be achieved either by disrupting both copies of the gene or by down-regulating its activity *in trans.*

1.1.2. Knockout

The most widely used technique for inactivating genes in ES cells is site-directed mutagenesis. This procedure involves the replacement of a specific sequence in the genome by a mutated copy through homologous recombination with a targeting vector. The targeting vector that contains the desired mutation and a selectable marker, flanked by sequences that are interchangeable with the genomic target, pairs with the wild-type chromosomal sequence and replaces it through homologous recombination. By targeting both alleles, using distinct selection markers, it is possible to create “loss-of-function” or so-called

knockout phenotypes in ES cells that can be used for functional studies of specific genes. This technology has been well practiced in mice for gene function studies, in which genetically altered cells are introduced into wild-type embryos, resulting in the creation of germ-line transmitting chimeras ***(8)***. The genetically manipulated animals can be further mutated to generate animals that are homozygous for the desired mutation. The creation of hES cells with a null genotype for specific genes may have great importance for modeling human diseases, and for the study of crucial developmental genes that in their absence are embryonic lethal. Recently, two independent reports demonstrated the successful targeting of the X-linked gene hypoxanthine phosphoribosyl transferase 1 (HPRT-1) in hES cells. Mutations in the HPRT-1 are the cause for Lesch-Nyhan syndrome ***(9, 10)***. In both cases, this was performed by introducing a large deletion at the HPRT-1 locus in hES cells of an XY karyotype. The resulting cell lines recapitulate the major biochemical defect that characterizes Lesch-Nyhan affected individuals, which involves the accumulation of uric acid ***(10)***. Thus, these cells should be valuable for basic research, but more importantly for exploration of new gene therapy-based treatments and drug discovery.

1.1.3. Knock-In

Similar to the knockout strategy, it is possible to generate clones of hES cells in which the gene of interest is deleted by inserting a promoterless reporter gene through homologous recombination. The method, termed knock-in, allows the positioning of a reporter gene under the regulation of a native gene. Therefore, it can be applied to monitor the expression of a target gene *in situ* during ES cell differentiation. Accordingly, Zwaka and Thomson have created human knock-in ES cell lines that express either GFP or a neomycin resistance gene under the regulation of the endogenous OCT4 promoter ***(9)***. The OCT4 gene encodes for a transcription factor that is specifically expressed by pluripotent stem cells. Thus, by replacing OCT4 with such reporters, the authors were able to monitor and select for undifferentiated hES cells in culture.

The relative ease by which ES cells can be genetically manipulated has made them particularly useful for the search of unknown genes whose pattern of expression suggests that they might have developmental importance. The identification of such genes is performed by the gene trap method, which is based on the random disruption of endogenous genes (reviewed by Stanford et al., 2001) ***(11)***. As opposed to targeted mutagenesis, it involves the random insertion of a reporter gene that lacks essential regulatory elements into the genome. Because the expression of the reporter gene is conditioned by the presence of an active endogenous regulatory element, it may serve to identify only transcribed sequences. Using this method, a large-scale gene disruption assay is possible, allowing the discovery of new genes and the creation of wide variety of mutations.

1.1.4. Knock-Down

Downregulation of particular genes can also be achieved by overexpressing specific RNA molecules that inhibit the activity of a given gene through the generation of small interfering RNA molecules (siRNAs). Because siRNAs operate *in trans* and are not involved in the modification of the targeted gene, it is relatively simple to achieve transient or conditional gene silencing using this method. The use of RNA interference (RNAi) was demonstrated to be feasible in mouse ES cells to inactivate genes and was shown to be equally effective as the knockout models in the generation of null mutant embryos *(12)*. Downregulation by RNAi in hES cells was recently demonstrated for the HPRT and OCT4 genes *(13, 14)*. Applications of this loss-of-function approach will have widespread use, not only to study developmental roles of specific genes in human, but also for their utility in modulating hES cell differentiation in vitro.

1.2. Methods for Genetic Manipulation

There are many factors that may influence transfection efficiency: phase of cell growth, number of passages, size and source of the transgene, vector type and size, and the selection system. However, the most important factor is the transfection method. Several gene transfer techniques are now available for manipulating gene expression in hES cells. The latter include chemical-based (transfection), physical (electroporation), and viral-mediated (infection) techniques.

1.2.1. Transfection

Transfection is probably the most commonly used method for introducing transgenes into hES cells. It is straightforward, relatively easy to calibrate, provides a sufficient number of cells for clonal expansion, can be performed on adherent cell cultures, and allows the insertions of constructs of virtually unlimited size. This system is based on the use of carrier molecules that bind to foreign nucleic acids and introduce them into the cells through the plasma membrane. In general, the uptake of exogenous nucleic acids by the cell is thought to occur through endocytosis, or in the case of lipid-based reagents, through fusion of lipid vesicles to the plasma membrane. The first study to describe stable transfection in hES cells *(3)* was based on the use of a commercially available reagent, ExGen 500, which is a linear polyethylenimine (PEI) molecule that has a high cationic charge density. The unique property of this molecule is due to its ability to act as a "proton sponge," which buffers the endosomal pH, leading to endosome rupture and DNA release. This method routinely produces transient transfection rates of approx 10–20% and stable transfection efficiencies of $1{:}10^{-5}$–10^{-6} *(3)*. Since then, other chemical-based transfection methods have been found to be equally effective. The calcium phosphate precipitation method

is a widely used method for transfecting many different cell types. It is also based on negatively charged molecules that interact with DNA to form precipitates that are incorporated by the cells. The calcium phosphate transfection system seems to be slightly more efficient in gene delivery in comparison to ExGen 500. Lipofectamine 2000 reagent is a positively charged cationic lipid compound that forms small unilamellar liposomes and was recently shown to be useful in obtaining transient and stable transfections in hES cells as well *(13, 14)*.

1.2.2. Electroporation

Electroporation is a method that employs the administration of short electrical impulses that create transient pores in the cell membrane, allowing foreign DNA to enter into the cells. Although efficient and most popular in mouse ES cells, this procedure gave poor results in hES cells, both in transient and stable transfection experiments. This was most probably because of the low survival rates of hES cells after the voltage shock. Recently, Zwaka and Thomson managed to increase the yield of electroporation 100-fold, thereby achieving an integration rate of approx $1:10^{-5}$ *(9)*. This was performed by carrying out the procedure on cell clumps rather than on single cell suspension, and altering the parameters of the protocol used in mouse ES cells. Using this method, 2–40% homologous recombination events were reported, subject to vector properties. A substantial number of hES clones obtained by homologous recombination has been created thus far using different constructs, demonstrating the feasibility of this technique for site directed mutagenesis in hES cells.

1.2.3. Infection

Unlike in all nonviral-mediated methods (transfection and electroporation), gene manipulation by viral infection can produce a very high percentage of modified cells. To date, genetic manipulation of hES cells by viral infection has been reported by several groups using adeno- as well as lenti-viral vectors *(15– 18)*. Infection studies with RNA and DNA viruses have demonstrated that these viral vectors have two distinct advantages over other systems: high efficiency of DNA transfer (almost 100% efficiency) and single-copy integrations. However, integration occurs randomly and cannot be targeted to a specific site in the genome. In addition, the vector size is limited. Yet, because of its high efficiency, this method could prove useful for bypassing the need for selection and time consuming clonal expansion, as well as for experiments that aim for random insertion mutagenesis or gene trap.

1.2.4. Short- vs Long-Term Expression

Gene transfer experiments can be subdivided into short-term (transient) and long-term (stable) expression systems. In transient expression, the foreign

DNA is introduced into the cells and its expression is examined within 1–2 d. The advantage of this assay is its simplicity and rapidity. Furthermore, because the foreign DNA remains episomal, there are no problems associated with site of integration and the copy number of the transgene. Yet, it does not allow conducting experiments over long periods. Moreover, transfection efficiency usually does not exceed 20%. For short-term induction, efficient transient expression can be achieved through the insertion of supercoiled plasmid DNA rather than the linear form. Transient expression in hES cells usually peaks roughly 48 h after transfection, and frequently results in high expression levels attributed to the high copy number of plasmid DNA molecules that occupy the cell.

During long-term assays, one isolates a clone of hES cells that has stably integrated the foreign DNA into its chromosomal genome. The major advantage of this method is the ability to isolate stable ES cell lines that have been genetically modified and can be grown indefinitely in culture. In this type of experiment, it is important to linearize the vector, leading to greater integration and targeting efficiencies. When the target gene is nonselectable, one must introduce also a positive selection marker under the regulation of a strong constitutive promoter. This can be performed either by cotransfecting the selectable marker on a separate vector, or as is frequently done, by fusing the selectable marker to the targeting vector. Selection should not be carried out immediately after transfection but at least 24 h later, giving the cells time to recover, integrate the foreign DNA and express the resistance conferring gene.

2. Materials

2.1. Tissue Culture (see Notes 1 and 2)

1. Knockout DMEM-optimized Dulbecco's modified Eagle's medium for ES cells (Gibco BRL, Carlsbad, CA; cat. no. 10829-018).
2. DMEM 4.5 g/L glucose (Sigma, Dorset, UK; cat. no. D5796).
3. 1 *M* β-mercaptoethanol (Sigma; cat. no. M7522).
4. Nonessential amino acids 100X stock (Biological Industries, Kibutz Beit-Haemek, Israel; cat. no. 01-340-1B).
5. Insulin-transferrin-selenium 100X (Gibco BRL; cat. no. 41400-045).
6. Bovine serum albumin (Sigma; cat. no. A-4919).
7. Mitomycin C (Sigma; cat. no. M-0503).
8. 0.1% gelatin (Sigma; cat. no. G-1890).
9. Hygromycin B (Sigma; cat. no. H-3274).
10. 6-thioguanine (Sigma; cat. no. A-4660).
11. KnockOut SR—serum-free formulation (Gibco BRL; cat. no. 10828-028).
12. Fetal calf serum (Biological Industries).
13. L-glutamine 100X stock (200 m*M*/L, Biological Industries; cat. no. 03-020-1).
14. Penicillin (10,000 U/mL) and streptomycin (10 mg/mL) 100X stock (Biological Industries; cat. no. 03-031-1B).

15. Human basic fibroblast growth factor (bFGF) stock solution (2 ng/μL) (human recombinant; Gibco BRL; cat. no. 13256029).
16. Trypsin-EDTA: 0.25% trypsin and 0.05% EDTA (Biological Industries; cat. no. 03-052-1).
17. G418 (Geneticin; Sigma; cat. no. G-9516).
18. Puromycin (Sigma; cat. no. P8833).
19. Dimethylsulfoxide (DMSO; Sigma; cat. no. D-2650).
20. 1X phosphate-buffered saline (PBS) without Ca^{2+}/Mg^{2+}. For 1 L: mix 3.58 g sodium phosphate ($Na_2HPO_4 \cdot 12H_2O$), 0.24 g potassium phosphate monobasic (KH_2PO_4), 8 g sodium chloride (NaCl), 0.2 g potassium chloride (KCl), in a final volume of 1 L of double-distilled water (ddH_2O). Aliquot 200 mL solution per bottle and autoclave, store at room temperature.
21. 10 m*M* β-mercaptoethanol: dilute 1:100 in PBS, filter, sterilize, and store at 4°C.
22. 50X Mitomycin-C: dissolve 2 mg in 4 mL MEF medium, store in 4°C.
23. bFGF solution: add 10 μg of bFGF solution to 5 mL of filter-sterilized 0.1% bovine serum albumin dissolved in 1X PBS (with Ca^{2+}/Mg^{2+}), to give a final concentration of 2 μg/mL, store 1-mL aliquots in –20°C.
24. 0.1% gelatin solution: add 0.1 g of gelatin into a bottle containing 100-mL distilled water and autoclave immediately. The gelatin is dissolved while boiling in the autoclave, store at 4°C.
25. MEF media: add to a 500-mL bottle of DMEM (high glucose and L-glutamine) 50-mL fetal calf serum and 2.5 mL penicillin/streptomycin.
26. hES medium: add to a 500-mL bottle of Knockout DMEM: 75 mL KnockOut SR, 6 mL nonessential amino acids, 6 mL glutamine (2 m*M*), 3 mL insulin-transferrin-selenium, 60 μL β-mercaptoethanol (0.1 m*M*), 3 mL penicillin/streptomycin, and 1 mL bFGF. ES media should be protected from light (*see* **Note 3**), and stored in 4°C up to 1 mo.
27 Freezing medium: add 1 mL of DMSO to 9 mL of appropriate media (either hES or MEF media). Media should be prepared fresh.
28. Leishman's stain (BDH, Poole, England) in 100% methanol.

2.1.1. Equipment and Supplies for Tissue Culture

1. Laminar flow hood.
2. Humidified incubator set at 37°C and 5% CO_2.
3. Phase contrast microscope (objective range from ×10 to ×40).
4. Liquid nitrogen storage tank.
5. Refrigerator (4°C) and freezers (–20°C, –70°C).
6. 37°C water bath.
7. Swing-out centrifuge for conical tubes (15- and 50-mL).
8. Cell counter.
9. Pipetmen (2, 10, 20, 200, and 1000 μL) designated for tissue culture use only.
10. Sterile forceps and scissors for dissecting mouse embryos.
11. Falcon tissue culture plates (100 × 20 mm) and 6-, 12-, and 24-multiwell trays (Falcon, Bedford, MA; cat. no. 353047, 353047, 353043, 353046).

12. Falcon 15-mL and 50-mL (Falcon; cat. no. 352097, 352098) polypropylene conical tubes.
13. Cryo vials (1.8-mL CryTube; Nunc, Roskilde, Denmark; cat. no. 363401).
14. Plastic pipets (1-, 2-, 5-, and 10-mL).
15. Tips for 2-, 10-, 20-, 200- and 1000-µL pipetmen.
16. Eppendorf tubes (1.5-mL).

2.2. Transfection

1. 2X HBS: 50 m*M* HEPES and 280 m*M* NaCl; dissolve 1.57 g NaCl, and 1.19 g HEPES in approx 80 mL sterile ddH_2O. Adjust pH to 6.8 and bring to a final volume of 100 mL with ddH_2O. Filter-sterilize and store in 15-mL aliquots at –20°C.
2. 70 m*M* Na_2HPO_4: dissolve 2.5 g of $Na_2HPO_4 \cdot 12H_2O$ in 100 mL of ddH_2O. Filter-sterilize and store in 15-mL aliquots at –20°C.
3. Transfection buffer: mix 485 µL of 2X HBS with 15 µL of 70 m*M* Na_2HPO_4.
4. 2 *M* $CaCl_2$: dissolve 27.75 g $CaCl_2$ in ddH_2O to a final volume of 100 mL. Filter-sterilize and store 15-mL aliquots at –20°C.
5. Humidified incubator set at 34°C, 3% CO_2.
6. Tips for 2-, 10-, 20-, 200- and 1000-µL pipetmen.
7. 10-mL tubes.
8. Eppendorf tubes (1.5-mL).
9. ExGen 500 (Fermentas, Hanover, MD; cat. no. R0511).
10. Vortex.
11. Swing out centrifuge for microplates.

2.3. Colony Picking

1. hES medium (*see* **Subheading 2.1., item 26**).
2. G418 (200 µg/mL).
3. Puromycin (0.5–1 µg/mL).
4. Hygromycin (100 µg/mL).
5. 6-Thioguanine (1 µg/mL).
6. 6-, 12-, and 24-well Falcon tissue culture plates (*see* **Subheading 2.1.1., item 11**).
7. Mouth apparatus consisting of an aspirator mouthpiece, tubing and Pasteur pipet pulled on flame for collecting single colonies (*see* **Note 4**).

3. Methods

3.1. Tissue Culture (see Notes 5 and 6)

3.1.1. MEFs

The special growth conditions that are required for supporting undifferentiated growth of hES cells in culture rely mostly on the presence of inactivated fibroblasts, serving as a feeder layer. The feeder layer sustains undifferentiated growth by secreting unknown growth factors, and by serving as a growth matrix that allows the cells to adhere and grow as monolayer culture.

So far, primary mouse embryonic fibroblasts (MEFs) were the most commonly used in the propagation and derivation of hES cells. However, STO cells *(19)*, fetal muscle *(20)*, foreskin fibroblasts *(21, 22)*, and marrow cells *(23)* were also reported to be equally effective in supporting undifferentiated growth. The feeders are prepared only from early passage MEFs (up to passage 5). Their mitotic inactivation is carried out by the treatment with mitomycin-C *(24)*, but can also be achieved through irradiation *(25)*.

Normally we prepare MEFs from 13.5-d-old ion cyclotron resonance -derived embryos. However, inactivated primary fibroblasts are required not only for routine maintenance of ES cells in culture, but also for stable transfection experiments, where drug selection is applied. Therefore, it is a prerequisite that feeder cells be resistant to the drug employed. For this purpose, one must separately prepare MEFs from different strains of mice that bear resistance to the desired drug or alternatively, use feeders that carry multidrug-resistant genes by intercrossing between different strains. For instance, the transgenic strain of mice DR-4, expresses four different drug-selected genes and can be used for the production of MEFs, which confer resistance to G418, puromycin, Hygromycin, and 6-thioguanine drugs *(26)*. The DR-4 strain, therefore, represents a suitable and an economical donor for the production of drug-resistant MEFs, and is especially advantagous for gene targeting experiments, which normally involve sequential selection for multidrug-resistant markers.

There may be a significant variability between various batches of MEFs, with respect to their capacity for supporting undifferentiated proliferation of hES cells. To overcome this problem, the competence of different batches of MEFs to support undifferentiated growth can be assessed by testing their ability to maintain undifferentiated proliferation of mouse or primate ES cell lines before their use.

3.1.1.1. Isolation of MEFs

1. Coat plates with 0.1% gelatin by incubation for 1 h at room temperature.
2. Collect 13.5-d-old fetuses from pregnant mice using sterile equipment: sacrifice pregnant mice and dissect the embryos by removing the uterus and transferring it into a sterile PBS-containing Petri dish.
3. Rinse twice in PBS and relocate all work to laminar flow hood.
4. Using sterile tweezers and scissors, remove the fetuses from the uterus, separate them from extraembryonic tissues (amniotic and yolk sacs) and transfer them to a clean Petri dish with PBS.
5. Count the number of collected fetuses and prepare, for later use, 1X 10-cm gelatin-coated tissue culture dish for every three fetuses.
6. Remove head and internal parts (liver, heart, kidney, lung, and intestine) with sterile tweezers under a stereomicroscope.
7. Cut the remaining tissues into small pieces in a minimal volume of PBS (1–2 mL) and transfer into a sterile 50-mL Falcon tube.

8. Disaggregate the cell clumps obtained by passing them through a 5-mL syringe with an 18-gauge needle, no more than 10 times.
9. Add MEF media to reach 10 mL per three embryos, distribute cell suspension evenly into 10-cm tissue culture dishes and incubate.
10. Change media the following day. When plates are confluent (2–3 d after dissection) split 1:3 by trypsinization.
11. Change media (10 mL) every 2 d. When cell density reaches confluence, trypsinize the cells and freeze each 10-cm plate in one cryovial, store in liquid nitrogen.

3.1.1.2. Mitomycin-C Inactivation of MEFs

1. Thaw contents of one cryotube into 3X 10-cm culture dishes.
2. Grow the cells to confluence by changing the media every other day.
3. Further propagate the cells by splitting them twice at a 1:3 dilution (sums to 27 plates).
4. To inactivate the cells, add 40 µL of mitomycin-C stock solution (1 mg/mL) to 5 mL culture media (final concentration of 8 µg/mL) and incubate at 37°C, 5% CO_2, for 3 h.
5. Aspirate the mitomycin-containing medium and wash the plates twice with 6 mL PBS.
6. Tripsinize cells by adding 1 mL of trypsin-EDTA and incubate at 37°C, 5% CO_2, for 5 min.
7. Add 5 mL medium and suspend the cells by vigorous pipetting.
8. Collect cell suspension into a 50-mL Falcon tube.
9. Centrifuge mitomycin-treated cell pool at 1000*g* for 5 min.
10. Aspirate supernatant and add fresh medium to reach a final cell concentration of $4 \cdot 10^6$ cells/10-cm dish. Feeder plates can be stored in the incubator for 3–4 d, but should be examined under the microscope before use.
11. It is possible to freeze mitomycin-C treated MEFs and keep them for later use. For this purpose freeze $1.5–7 \times 10^6$ cells in each cryotube and later thaw and plate to give 1–5X 10-cm dishes, respectively.

3.1.2. Maintenance of hES Cells and Genetically Modified Clones

The maintenance of hES cells in culture relies on the continuous and selective propagation of undifferentiated cells. Controlling culture conditions and minimizing the effect of spontaneous differentiation, which constantly occurs, can achieve this. When passing the cells, care must be taken so that the cell number will not drop below a certain density, because this increases their tendency to differentiate, possibly from a lack of autocrine signaling. The differentiation status of the cultures should be followed daily by observation through a phase-contrast microscope. Undifferentiated colonies are easily recognized by their typical appearance, which includes small and equal-sized cells that are defined by a discrete border, pronounced nucleus and clear cellular boundaries. As differentiation begins, the cells at the

periphery of the colonies lose their typical morphology. At that stage, splitting must be performed (*see* **Note 7**).

3.1.2.1. Subculture of hES Cells

1. Remove culture media and rinse with 6 mL PBS.
2. Add 1 mL of trypsin-EDTA and incubate for 5 min.
3. Add 5 mL growth medium and suspend the cells by vigorous pipetting.
4. Collect suspension into a conical tube and pellet by centrifugation 1000*g* for 5 min.
5. Resuspend with fresh media and plate on mitotically inactivated feeders prepared the previous day.

3.1.2.2. Freezing hES Cells

1. Trypsinize hES cells and pellet them, as described in **Subheading 3.1.2.1., steps 1–4**.
2. Resuspend cells in an appropriate amount of growth media supplemented with 10% DMSO.
3. Mix the cells are gently by pipetting up and down and place in a properly marked cryotube.
4. Store at –70°C in a low temperature vial container filled with isopropanol for at least 1 d.
5. For long-term storage, vials must be kept in liquid nitrogen.

3.1.2.3. Thawing hES Cells (*see* **Note 8**)

1. Incubate the frozen cryovial in a 37°C water bath until it is completely thawed.
2. Transfer and resuspend the cells with 5 mL growth media in a conical tube.
3. Pellet the cells by centrifugation at 1000*g* for 5 min.
4. Resuspend again in an appropriate amount of fresh media.
5. Plate cells and incubate overnight.

3.1.2.4. Mouse ES Cells Clonal Assay to Test Competence and Quality of KO-Serum Batch

Batch-to-batch variability in the competence of the KO-serum replacer to support undifferentiated proliferation may be remarkable. Clonal assays with mouse ES cells may be used to test the quality of the serum substitute batch before its use. An established culture of mouse ES cells is used as previously described *(27)* and all medium components should be those that will be used to culture the hES cells (*see* **Note 9**).

1. Trypsinize mouse ES cells *(27)* and plate individual cells in pre-gelatinized 6-cm Petri culture dishes at a low density (1000 cells per plate).
2. Culture either with the medium that was in current use or the new tested medium at 37°C in a 5% CO_2 atmosphere.

3. Change medium once on the fifth day after plating.
4. On the seventh day, rinse the cultures with PBS and stain for 5 min with 0.15% Leishman's fix and stain.
5. Wash the stained cultures thoroughly with water and let them air-dry.
6. Compare the number of colonies per plate as well as the size and degree of differentiation and select the batch of serum with the best performance compared with the batch in use.

3.2 Transfection

3.2.1. DNA Preparation for Transfection

1. Prepare DNA vector by any commonly used technique to obtain OD_{280}/OD_{260} absorption ratio value of 1.8 or greater (*see* **Note 10**).
2. To linearize the vector by digesting it with the appropriate restriction enzyme.
3. Assess the completion of the restriction digest by electrophoresis of a small aliquot on a 1% gel agarose.
4. Ethanol precipitate the DNA and resuspend in a small volume (20–50 µL) of TE or sterile water. Adjust concentration to 1 µg/µL.

3.2.2. Growing hES Cells for Transfection

1. Split (1:2 or 1:3) a morphologically undifferentiated and confluent hES cell culture 2 d before transfection (*see* **Note 11**).

3.2.2.1. Transfection by Calcium Phosphate (*see* **Note 12**)

1. Harvest hES cells and split 1:4 into 10-cm culture dishes containing MEFs that were plated the previous day.
2. Prepare for each 10-cm plate transfection buffer and DNA in separate tubes. Dilute 10–20 µg of DNA in 240 m*M* $CaCl_2$ by bringing the DNA to a final volume of 0.5 mL with DDW and then slowly adding 60 µL of 2 *M* $CaCl_2$ (and not the reverse order).
3. Add very slowly the DNA solution (one to two drops/s) to the transfection buffer, while gently mixing by generating small air bubbles with a sterile disposable tip.
4. Incubate 10 min at room temperature (*see* **Note 13**).
5. Add the 1-mL solution dropwise on to the cells without swirling or rotating the dish.
6. Incubate at 34°C, 3% CO_2, for 4 h and then change the growth media by aspirating it and washing twice with PBS. Add fresh media and return to the incubator.
7. Apply selection the following day by adding the appropriate drug to the growth media.
8. Refeed the cells with selection media when the medium starts to turn yellow, usually every day during the first 5 d and then every other day. By d 10–12 of selection, colonies should visible and large enough to be picked for further expansion and analysis.

3.2.2.2. Transfection by Exgen 500 (*see* **Note 14**)

1. Two days before transfection by Exgene 500, harvest and split hES cells into six-well trays containing inactivated and drug resistant MEFs.
2. About 1 h before transfection, change the growth media by rinsing the cells with PBS and adding 1 mL of fresh media to each well.
3. For each well of a six-well tissue culture tray prepare a tube containing 2 µg of DNA to a final volume of 50 µL of 150 m*M* NaCl and vortex.
4. In a separate tube mix 10 µL ExGen 500 to 40 µL of 150 m*M* NaCl and vortex.
5. Mix DNA and transfecting agent by rapidly adding diluted ExGen 500 to DNA (not the reverse order). Vortex-mix the solution immediately for 10 s and then incubate for 10 min at room temperature.
6. Add 100 µL of ExGen/DNA mixture to each well.
7. Gently rock the plate back and forth to equally distribute the complexes on the cells.
8. Centrifuge culture trays immediately for 5 min at 280*g*.
9. Incubate at 37°C, 5% CO_2, for 30 min.
10. Wash twice with PBS and return to incubator (*see* **Note 15**).

3.3. Colony Picking and Expansion

After 10–12 d in selection media, individual hES cell-resistant clones become visible and are big enough to be isolated for expansion.

1. Screen transfected culture plates using an inverted microscope for the presence of resistant clones and mark their location at the bottom of the dish.
2. Manually pick selected hES cell colonies (*see* **Note 16**).
3. Disconnect the cell colony from the feeders by dissociating it into small cell pieces using the sharp edge of the glass micropipet while collecting them by aspiration into the tip of the pipet.
4. Plate the small cell clumps on fresh drug-resistant feeder layer, in a single well of a 24-well culture tray and return to incubator for further growth. The replated cell clumps, which have originated from a single cell clone, give rise to round flat colonies with well-defined borders in 3–5 d, while changing the selection media as necessary (*see* **Note 17**).
5. Scale up the clone population by splitting 1:2 with trypsin, twice.
6. When the wells (2 × 12-well) are approaching confluence, freeze each well in individual cryovial. The remaining cells can either be further expanded (**Fig. 1C**), by splitting 1:4, or directly used for DNA, RNA, or protein extraction (*see* **Note 18**) (**Table 1**).

4. Notes

1. **Subheading 2.1., items 1–10** are stored at 4°C, **items 11–18** at –20°C, and **item 19** at room temperature. As a rule, all tissue culture protocols must be performed under sterile conditions, in a laminar flow hood, using sterile disposable plastics and clean, detergent-free, glassware.

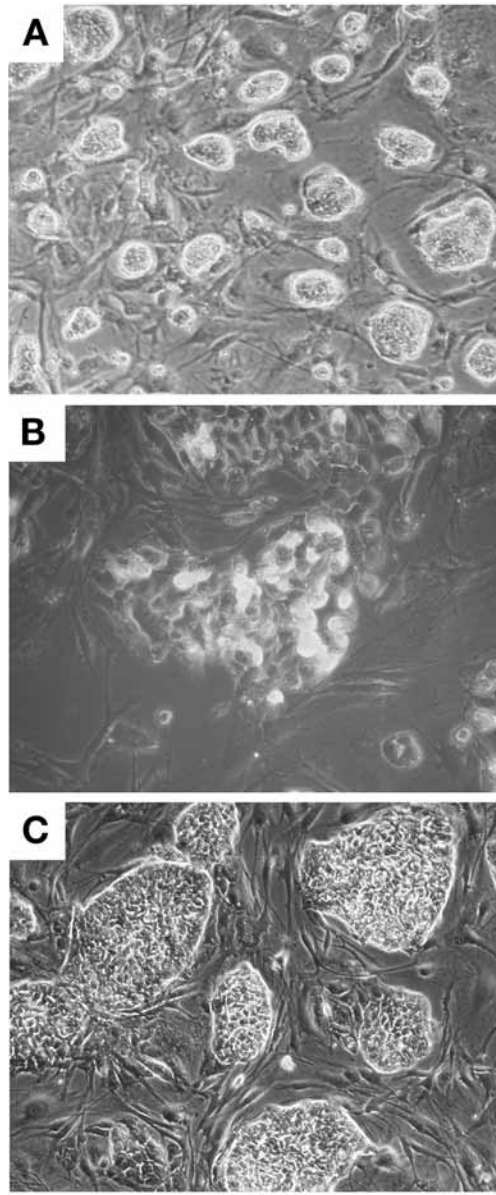

Fig. 1. **(A)** Human embryonic stem (hES) cell culture on day of transfection. The culture should be composed of many small (8–32 cells) colonies. **(B)** Transient expression of CMV-EGFP in hES cells after 48 h to transfection. **(C)** Established cell line of hES cells after transfection, selection, and clonal expansion of genetically modified cells.

Table 1
Transfection Protocol Timetable

d	
1	Plate MEF resistant cells
2	Split/thaw a vial of hES to high density
4	Transfect hES cells (high density cultures of 8–32 cells/colony)
5	Begin selection
6–10	Change selection media every day
11–15	Change selection media every other day
16–18	Screen for resistant colonies
	Pick up selected colonies and plate them on MEF resistant feeder in 1X 24-well tissue culture trays
20–30	Split 1:2 and plate on MEF resistant feeder in 1X 12-well twice
	Freeze and/or screen/further propagate in 1X six-well trays

MEF, mouse embryonic fibroblasts; hES, human embryonic stem cell.

2. Media should be stored in 4°C and can be used for up to 1 mo.
3. Serum replacement is sensitive to light. Protect supplemented hES media by covering it with aluminum foil.
4. The mouth-controlled device is the same as the one that is commonly used for handling oocytes and preimplantation embryos in mice. The mouthpiece is available as a part of an aspiration tube assembly from Drummond (model no. 2-000-0001). Sterile glass Pasteur pipets are pulled on a flame to create long tubing with a narrow opening. Soften the glass tubing by rotating it in a fine flame until the glass becomes soft. Then, withdraw the glass quickly from the heat and pull both ends smoothly to produce a tube with an internal diameter of about 200 μm. Neatly break the tube and fire polish its tip by quickly touching the flame.
5. All tissue culture procedures are performed under sterile conditions, using prewarmed media and gelatin-precoated plates.
6. Protocols for cell freezing, thawing and splitting are basically the same for all cell types (feeders and hES cells).
7. As in other cell lines growing in vitro, chromosomal aberrations may occur. Working with cells of low passage number can minimize this. Thus, it is advisable to monitor the karyotype of the cells following prolonged growth in culture and subsequent to stable transfection.
8. Cell thawing must be performed as quickly as possible.
9. The culture medium is supplemented with 10% of the tested batch of knockout-serum substitute (instead of 15%) and mouse recombinant LIF at 1000 U/mL.
10. The purity of the DNA is very critical for successful transfection.

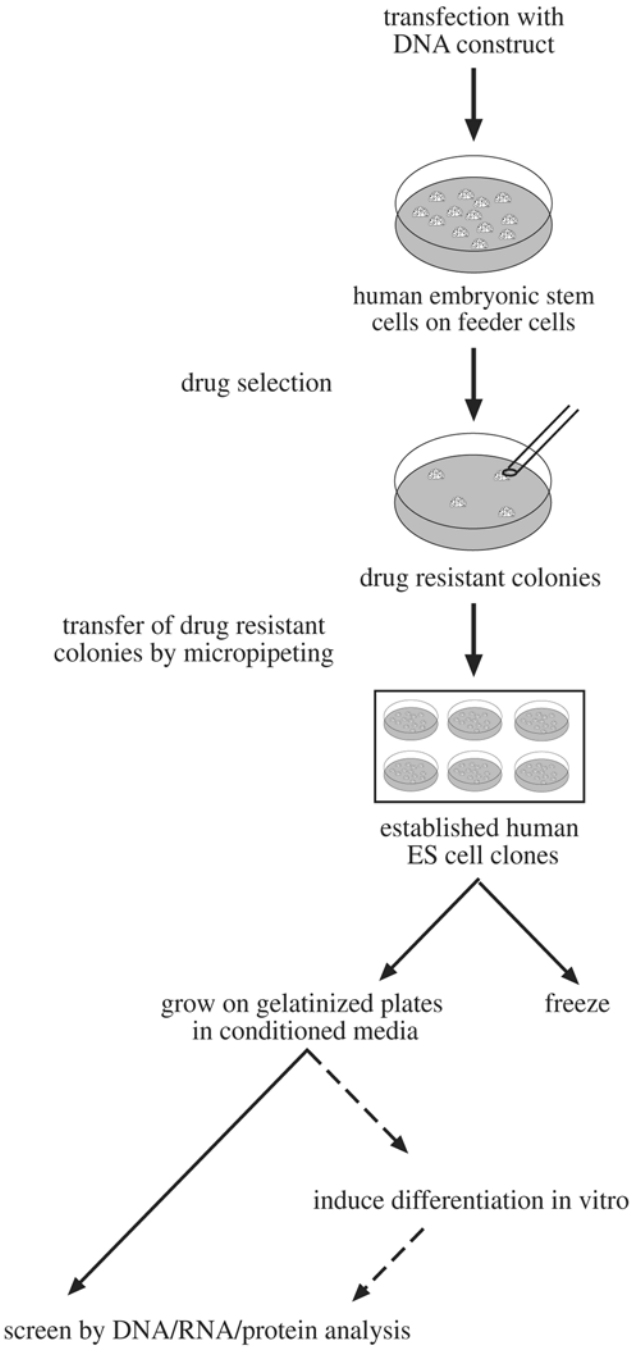

Fig. 2. Schematic illustration describing the methods for generating genetically modified hES cells by transfection.

11. The cells should be transfected during the lag phase of cell division. The transfection rate is most efficient when the cell density reaches 50–70% and the colonies are small (8–32 cells per colony) (**Fig. 1A**). The colonies should have discrete borders and be composed of similar sized cells, with a pronounced nucleus.
12. The calcium phosphate ($CaPO_4$) transfection protocol is basically similar to the protocols used for other cell types.
13. At this time, fine DNA-calcium phosphate precipitates should be formed without agitation.
14. ExGen 500 (polyethylenimine, PEI) is a cationic polymer, which is capable of transfecting a wide range of cell types at relatively high efficiency. It interacts with the negatively charged DNA molecules by forming small, stable, and highly diffusible particles, which settle on the cell surface by gravity and absorb into the cell by endocytosis.
15. In parallel to the experiment, one may consider to carrying out transient transfection on a small number of cells with a construct carrying a constitutive expressed reporter gene, such as CMV-EGFP, to asses transfection efficiency before applying selection (**Fig. 1B**).
16. The colonies are picked up by the aid of a mouth apparatus connected to a sterile pulled and fire polished paster pipet, as is commonly used for handling oocytes and preimplantation embryos (*see* **Note 4**).
17. We find this pickup method more suitable and efficient for isolating single hES colonies than the method applied in mouse, where individual ES colonies are collected with a disposable tip, trypsinzed, and then plated.
18. In some cases, it is crucial that no feeders will be present during the screen. For this purpose, cells must be propagated in feeder-free gelatinized plates, for at least one passage. Under such conditions the cells must be grown in conditioned media (CM, hES cell media conditioned by MEFs for 24 h), preventing from differentiation and consequently culture loss.

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17

Designing, Testing, and Validating a Microarray for Stem Cell Characterization

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Summary

Microarray technology is a powerful tool that allows for simultaneous assessment of the expression of thousands of genes and identification of gene expression patterns associated with specific cell types. Here we describe a protocol using this method to examine stem cells.

Key Words: Focused cDNA microarray; oligo-array; neural stem cells; human ES cells.

1. Introduction

Evaluating the purity and state of neural stem cell populations relies on using a battery of markers. Different laboratories use some subset, often nonoverlapping, of known markers. Given the amount of tissue or cells required for the variety of tests and the difficulty in obtaining, maintaining and validating immunostaining, Western blot, polymerase chain reaction (PCR), or Northern blot analysis, consistent evaluation of stem cell populations between different laboratories and even between different batches of cells has been difficult.

Traditional assay techniques such as Northern blot, ribonuclease protection assay or more recent techniques, such as quantitative real-time reverse transcriptase (RT)-PCR, are suitable for studying one or a few genes in a single assay. The ideal tool required would be one that is capable of evaluating gene expression of related gene sets simultaneously. If such a reliable tool existed then not only could one study cell type specific markers, but additional candidate genes known to be expressed at the appropriate stage of development could be rapidly evaluated as well. Such genes might be growth factors,

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extracellular matrix molecules, chemokines, or key regulators of cell proliferation or apoptosis.

Microarray technologies, in which some or a large number of genes, clones or oligonucleotides are spotted on membranes, glass slides, or chips, allows for simultaneous assessment of the expression of thousands of genes, and would be a candidate assay method. This approach has been used successfully to identify gene expression patterns associated with specific biological functions *(1,2)* and has been proven to be reliable and reproducible *(1,2)*. However, there are several technical limitations and problems that are associated with most of the commercially available microarrays that may preclude their use for assays such as the one we have proposed.

The first limitation is gene coverage. Although a high-density microarray contains thousands of genes on a single glass slide, they usually fail to provide full gene coverage for specific gene groups necessary for a particular application. This is especially true for stem cells that are rare, populations for which libraries of clones have not been prepared. For example, only 49 of the 70 known interleukin and receptor-related genes are present on one commercial array. Likewise few of the 23 known fibroblast growth factor (FGF) genes are present on any of the large microarrays that are available.

The second problem with large-scale arrays is cDNA fragment selection and quality. The cDNA fragments used in the microarrays are usually 3′ end-biased because they are generated from oligo-dT primed cDNA synthesis. The 3′ end region is not necessarily the most gene specific region for some genes and is often a poor choice for a gene specific probe. The third problem is the complexity of the data collection and analysis. In fact, the cost and the special equipment requirement and complex data analysis are barriers that prohibit most research laboratories from using microarray technology as a routine research tool. Thus, a general high-density cDNA array may not be the best tool for rapid gene expression profiling of stem cells.

A complementary approach to large-scale microarrays that can bypass some of these problems is to prepare cell type-specific focused arrays. These arrays can be rapidly generated, updated easily, and prepared in multiple formats. Careful selection of a limited number of genes can allow tighter and more uniform hybridization conditions and more stringent washes. Cross-reactivity can be minimized by careful selection and direct testing, control hybridization patterns can be generated and positive/negative controls can be prepared for the entire set if necessary.

In this chapter, we describe two approaches to access stem cells. We describe the preparation of a focused membrane based array that can be used to rapidly assess the overall state of a cell population, an approach that is ideal for routine use. In the second section, we describe the development of a large-scale,

oligonucleotide-based array set that spans approx 50% of the genome. The issues of quality control, verification, and data analysis are discussed.

2. Materials

2.1. Mouse and Human Stem Cell-Focused cDNA Array

2.1.1. Array Membrane Preparation

1. Cartesian SynQuad Prosys dispenser (Genomis Solution, Ann Arbor, MI).
2. UV crosslinker (e.g., commercially available Fisher, VWR, Stratagene).
3. Primers and cDNA clones (available from SuperArray Bioscience Corp., Frederick, MD).
4. RedTaq DNA polymerase (Sigma, St. Louis, MO; cat. no. D-5684).
5. 10 m*M* dNTP (Invitrogen, Gaithersburg, MD; cat. no. 1297018).
6. NaOH (Sigma; cat. no. S-8045).
7. Bromophenol blue (Sigma; cat. no. B-8026).
8. Nytran$^+$ membrane (Amersham Bioscience, Piscataway, NJ; cat. no. RPN137B).
9. Mouse neural stem cell-focused cDNA array (SuperArray Bioscience Corp.; cat. no. MM-601.2).
10. Human neural stem cell-focused cDNA array (SuperArray Bioscience Corp.; cat. no. HS-601.2).

2.1.2. Probe Preparation

1. ThermoCycler (e.g., commercially available from Applied Biosystems).
2. Moloney murine leukemia virus (MMLV) RT labeling kit (SuperArray Bioscience Corp.; cat. no. L-01N). This kit contains following items: (1) RNase-free H_2O; (2) 5X GEA labeling buffer (BN); (3) RNase inhibitor (RI); (4) RT; (5) 10X Stop solution (C); (6) 10X denaturing buffer (D); (7) 2X Neutralization buffer (E); and (8) Buffer A, containing gene-specific primers.
3. Ampolabeling kit (SuperArray Bioscience Corp.; cat. no. L-03N). This kit contains following items: (1) RT primer (P); (2) RNase-free H_2O; (3) 5X GEA labeling buffer (BN); (4) RNase inhibitor (RI); (5) RT; (6) LPR buffer (L); (7) Buffer AF (blue tube), containing gene specific primers; (8) DNA polymerase (LE); and (9) 10X Stop solution (C).
4. Biotin-16-dUTP (Roche, Basel, Switzerland; cat. no. 1-093-070).

2.1.3. Hybridization and Washing

1. Hybridization Oven (e.g., commercially available Fisher, VWR, Stratagene, Thomas Scientific).
2. GEAhyb hybridization solution (SuperArray Bioscience Corp.; provided free when arrays are ordered).
3. Salmon Sperm DNA (Ambion, Austin, TX; cat. no. 9680).
4. 20X SSC (Biosource International, Camarillo, CA; cat. no. 357-000).
5. 20% SDS (Quality Biological, Inc., Gaithersburg, MD; cat. no. 351-066-101).
6. Molecular Biology Grade Water (Biosource International; cat. no. 384-000).

2.1.4. Chemiluminescence Detection of Image

1. Chemiluminescence image capture equipment (e.g., commercially available FluorChem [Alpha Innotech Corp.]).
2. GEA chemiluminescence detection kit (SuperArray Bioscience Corp.; cat. no. D-01). The kit provides the following items: (1) GEA blocking solution Q; (2) 5X Washing buffer F; (3) alkaline phosphatase (AP)-conjugated streptavidin; (4) AP-assay buffer Q; and (5) CDP-Star chemiluminescent substrate.

2.2. Large-Scale Human Oligo-Microarray

2.2.1. Fabrication of Human 16K Microarray on Glass Slides

1. Human genome set version 1.1 (Qiagen/Operon, Valencia, CA).
2. Biomek FX liquid handling system (Beckman Coulter, Inc., Fullerton, CA).
3. 20X SSC (Biosource International; cat. no. 357-000).
4. Glass microscope slides (Sigma; cat. no. S8902).
5. Poly-L-lysine (Sigma; cat. no. P 8920). Poly-L-lysine solution: add a stir bar to 720 mL double-distilled water (ddH_2O) and begin stirring before adding first 90 mL Dulbecco's phosphate buffered saline, then 90 mL poly-L-lysine (90 mL); stir for 2–3 min.
6. Tissue culture phosphate buffered saline (Sigma; cat. no. D 8537).
7. Slide box (VWR, West Chester, PA; cat. no. 48443-806).
8. OminiGrid Arrayer (GeneMachine, San Carlos, CA).
9. Stealth micro spotting SMP3 pins (TeleChem International, Inc., Sunnyvale, CA).
10. Centrifugator, Sorvall Super T21 (e.g., commercially available from Kendro Laboratory Products, Asheville, NC).
11. Stratalinker (Stratagene, La Jolla, CA).
12. Diamond scriber (VWR; cat. no. 52865-005).
13. Slide rack and chamber (Shandon Lipshaw, Inc., Pittsburgh, PA; cat. no. 121).
14 Succinic anhydride (Sigma-Aldrich; cat. no. 23969-0).
15. 1-Methyl-2-pyrrolidinone (Sigma-Aldrich; cat. no. 32863-4).
16. Sodium borate (Fisher, Pittsburgh, PA; cat. no. A73-500).
17. Cleaning solution. For 895 mL: dissolve 80 g NaOH in 335 mL ddH_2O; add 560 mL 95% ice-cooled ethanol and stir until completely mixed. The solution should not turn cloudy if using ice-cold EtOH. If the solution becomes cloudy, ice solution to drop temperature and solution will clear up.
18. Succinic anhydride blocking solution: measure out a 8.95-mL aliquot of 1 *M* sodium borate pH 8.0, which was prepared from solid boric acid and pH adjusted to 8.0 with NaOH pellets or liquid; combine 200 mL 1-methyl-2-pyrrolidinone and 3.68 g succinic anhydride in a dry, clean Pyrex dish with a stir bar; mix in the sodium borate solution as soon as the last flake of succinic anhydride dissolves.

2.2.2. Probe Labeling

1. Thermo Savant DNA SpeedVac Systems (e.g., commercially available Thomas Scientific, Telechem International, Inc.).

2. FairPlay Microarray Labeling Kit (Stratagene; cat. no. 252002).
3. Cy3 dye (Amersham; cat. no. PA23001).
4. Cy5 dye (Amersham; cat. no. PA25001).
5. MinElute PCR purification kit (Qiagen; cat. no. 28004).
6. Universal Human Reference RNA (BD Biosciences, Clontech, Palo Alto, CA; cat. no. 64115-1).
7. Cy3-labeled 9mer (IDT, Coralville, IA).
8. Dimethylsulfoxide (Sigma; cat. no.D-8418).

2.2.3. Hybridization and Washing

1. Hyb chamber (Genomic Solutions; cat. no. JHYB20004).
2. COT-1 DNA (Invitrogen; cat. no. 15279-011).
3. Poly(dA) (Amersham; cat. no. 27-7988-01).
4. Yeast tRNA (Invitrogen; cat. no. 15401-029).
5. 20X SSC (Biosource; cat. no. 357-000).
6. 20% SDS (Quality Biological, Inc.; cat. no. 351-066-101).
7. BSA (Sigma; cat. no. 9048-46-8).
8. Isopropanol (J.T. Baker, Phillipsburg, NJ; cat. no. UN1219).

2.2.4. Scanning and Image Collection

1. Scanner (e.g., commercially available Axon Instruments Inc.; PerkinElmer Life and Analytical Sciences, Inc.; Agilent).

3. Methods

3.1. Preparation of Mouse and Human Neural Stem Cell-Focused Array Membranes

1. Select known genes that are expressed in neural stem and progenitor cells *(3–5)*. As shown in **Table 1**, **Figs. 1**, and **2**, around 260 known genes were chosen for array construction (*see* **Note 1**).
2. Prepare PCR in 96-well format. Each well has 20 µL reaction solution containing 2 µL 10X PCR buffer, 150 µmol $MgCl_2$, 10 nmol dNTP, 20 pmol primer, 1 µL 10X diluted plasmid, and 1 U RedTaq DNA polymerase.
3. Run PCR as follows: 2 min at 95°C for denaturation, 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, and a final extension for 10 min at 72°C.
4. Take 1 µL of PCR products each to run 1.2% agarose gel to check purity of the product (*see* **Note 2**).
5. Concentrate and adjust the remaining PCR products to 150 µg/mL in 0.08 *N* freshly prepared NaOH and bromophenol blue (0.001%).
6. Print the cDNA solution (10–15 nL per spot) onto Nytran$^+$ membrane using a Cartesian SynQuad Prosys dispenser in an 18 × 16 format as shown in **Figs. 1** and **2**. All array spots are arranged in a rectangular area (23 × 35 mm). Spot diameter is 0.7–0.9 mm. The spot to spot distance is 1.25 mm.

Table 1
Gene List for Mouse Stem Cell Chip

Groups	Subgroup	No. genes	Gene name
Markers	ES	13	*CER1; FGF4; Lif R; LIF; Pou5f1; Sox2; gp130; Utf1; Zfp42; Prdc; Gcm2; Tert; GATA4.*
	Ectoderm	5	*k14; k15; Krt1-17; MHR a-1; Krt2-8.*
	NEP	14	*Prom; Sox1; Glucose transporter 1; Numb; Abcg2; Fabp7; FGFR4; Fzd9; Sox10; Nes; Brn-1; Brn-2; Gjb5; Gja7.*
	Mesoderm	18	*MLC-1; a-MHC; SM-MHC; Fabp4; Cd34; Cd45; VEGFR2 (FLK 1); Thy1; Actc1; Csx; ANF; Gata2; Foxa1; Actin, alpha 2; Col6a2; Afp; M-cadherin.*
	Endoderm	5	*Pdx1; Actg2; Gcg; Insulin I ;Isl1*
	Neuronal	9	*Map5; Mtab2; Tubb3; Prox1; Brn5; Nfl; Ncam; Ncam2; Syn1.*
	Astro/Radial glia	8	*EGFR; Gjb4; Vim; GFAP; MGLT1; Glt4; S100B; CD44.*
	Oligo	9	*Nkx2-2; Olig1; Olig2; PDGFRa; Cnp1; Mbp; Plp; Pax6; Gjb1.*
	Crest	6	*DRG11; Krox-20; SCG1; Slug; Sna; Ngfr.*
Growth factors		96	*ALK-3, 6; BMP1, 2, 3, 4, 5, 6, 7, 8a; BMP8b/OP-3, 10, BMP15/GDF9B; BMPR2; aFGF; FGF5, 6, 8, 10, 11, 12A, 14, 15, 16, 17; 18; 20, 21, 22, 23, Int-2; KGF; FLG; FGF9; bFGF; KGFR; FGFR3; Fzd1, 3, 4, 7; IGF-1; IGF-II; Igf1r; Igf2r; Insrr; Igfbp3; Ntrk2, 3; CNTF; CNTFR; NGF b; Neurotrophin 3; TGFb1; TGF b2; TGF b3; ALK-5; TGFbR2; Betaglycan; Wnt2; Wnt3a; Wnt4; Wnt5b; Wnt6; Wnt7b; Wnt8a; Wnt11; GDF1; BMP9/GDF2; GDF3; GDF5; GDF6; GDF8; GDF9; BMP11/GDF11; Notch1, 2, 3, 4. 4; DLK; EGF; Shh; Erbb4; PDGF a, b; INHBA; PDGFRb; NODAL; Patched; TSK-7L; Patched 2; ACVR2; ALK1; Nog.*
Cytokines		4	*IL-6; IL-6R; BDNF; VEGF (VEGI).*
Cell cycles		6	*Cyclin G2; p27Kip1; p19; p21Waf1 (p21cip); PTEN.*
ECMs		37	*Cadherin 2, 3, 4, 5; E-cadherin; Ctnnd2; Catna1; Catna2; Catnal1; b catenin; ICAM-1, 5; Jam; VCAM-1; LFA1b; Integrin-α 2b, a3, a4 (VLA-4), a5, a6, a7, a8; Integrin b1, b2, b3(CD61), b4, b5, b6, b7; Itgae; LFA1a /(CD11A); Integrin aM, aV, aX;Cystatin C; PECAM1; Tenascin C.*
Telomerase		3	*Tebp; Tep1; Terf1.*
Transcription		14	*Hfhbf1; Fast2; MPP2; FKHR1; Sox3, 4, 5, 6, 13, 15, 17, 18; Nrg4; Neurod3.*
Others		10	*Gjb3;. Fabp4; Nrg1; Erbb2ip; Erbb3; DNMT1; Dnmt2; Dnmt3a; DNMT13b; Dnmt3l.*
Controls		4	*Ppia; Rpl13a; Actb; Gapdh.*

Cer1	**Fgf4**	Lifr	Pou5f1	Sox2	Utf1	Zfp42	Prdc-pending	**Gcm2**	Tert	Il6ra	Pecam	Actg2	Acta2	Gcg	Ins1
1	**2**	3	4	5	6	7	8	**9**	10	11	12	13	14	15	16
blank	Pdx1	Myh11	Cd34	Ptprc	Kdr	Thy1	Actc1	Nkx2-5	Pnd	Gata2	Gata4	Foxa1	Col6a2	Afp	Cdh15
17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
Myla	Myhca	Fabp4	Krt1-14	Krt1-15	Krt1-17	Krt1-5	Krt2-8	Ina	Abcg2	Fabp7	Fgfr4	Fzd9	Nes	Pou3f3	Pou3f2
33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
Prom	Sox1	S1c2a1	Numb	Il6st	Drg11	Egr2	Ngfr	Scgn10	Slug	Sna	Gjb4	Gja7	Gjb3	Gjb5	Gjb1
49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64
Isl1	**Mtap1b**	**Mtab2**	**Tubb3**	**Prox1**	Pou6f1	**Nfl**	Ncam1	**Ncam2**	Syn1	Pax6	Vim	Gfap	Slc1a2	Slc1a6	S100B
65	**66**	**67**	**68**	**69**	70	**71**	72	**73**	74	75	76	77	78	79	80
Egfr	Cd44	Nkx2-2	Neurod3	Olig1	Olig2	Pdgfra	Cnp1	Mbp	Plp	**Cst3**	Tnc	Sox10	Fzd9	blank	blank
81	82	83	84	85	86	87	88	89	90	**91**	92	93	94	95	96
Nrg1	blank	Nrg4	Egf	Egfr	Erbb2ip	Erbb3	Erbb4	Igf1	Igf2	Igf1r	Igf2r	Insrr	Igfbp3	Vegfa	blank
97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112
Fgf1	Fgf2	Fgf3	**Fgf4**	Fgf5	Fgf6	Fgf7	FGF8	Fgf9	Fgf10	Fgf11	Fgf12	Fgf13	Fgf14	Fgf15	Fgf16
113	114	115	**116**	117	118	119	120	121	122	123	124	125	126	127	128
Fgf17	Fgf18	blank	Fgf20	Fgf21	Fgf22	Fgf23	Fgfr1	Fgfr2	Fgfr3	Fgfr4	Ngfb	Bdnf	Ntf3	Ngfr	Ntrk2
129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144
Ntrk3	Il6	Lif	Cntf	Cntfr	Shh	Nog	Pdgfa	Pdgfb	Pdgfra	Pdgfrb	Ptch	Ptch2	Notch1	Notch2	Notch3
145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160
Notch4	Tgfb1	Tgfb2	Tgfb3	Bmp1	Bmp2	BMP3	Bmp4	Bmp5	Bmp6	Bmp7	Bmp8a	Bmp8b	Bmp10	Bmp15	Gdf1
161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176
Gdf2	Gdf3	Gdf5	Gdf6	Gdf8	Gdf9	Gdf11	blank	Inhba	Inhbb	Nodal	Acvr1	blank	Acvr2	blank	Acvrl1
177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192
Bmpr1a	Bmpr1b	Bmpr2	Tgfbr1	Tgfbr2	Tgfbr3	Fzd1	Fzd3	Fzd4	Fzd7	Fzd8	Wnt11	Wnt2	Wnt3a	Wnt4	blank
193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208
Wnt5b	Wnt6	Wnt7b	Wnt8a	Wnt11	Tebp-pending	Tep1	Terf1	Foxg1	Foxh1	Foxm1	Foxo1	Sox3	Sox4	Sox5	Sox6
209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224
Sox13	Sox15	Sox17	Sox18	**Ccng2**	**Cdkn1b**	Cdkn2d	Dlk1	Cdkn1a	Pten	**Dnmt1**	Dnmt2	Dnmt3a	Dnmt3b	Dnmt3l	Kdr
225	226	227	228	**229**	**230**	231	232	233	234	**235**	236	237	238	239	240
Cdh2	Cdh3	**Cdh4**	Cdh5	Cdh1	Ctnnd2	Catna1	Catna2	Catnal1	Catnb	Icam1	Icam5	Jcam1	Ncam1	**Ncam2**	Vcam1
241	242	**243**	244	245	246	247	248	249	250	251	252	253	254	**255**	256
Itga2	Itga3	Itga4	Itga5	**Itga6**	Itga7	Itga8	Itgal	Itga2b	Itgam	Itgav	Itgax	Itgb1	Itgb2	Itgb3	Itgb4
257	258	259	260	**261**	262	263	264	265	266	267	268	269	270	271	272
Itgb5	Itgb6	Itgb7	Itgae	Ppia	**Rpl13a**	**Actb**	**Gapd**	Ppia	**Rpl13a**	**Actb**	**Gapd**	Ppia	**Rpl13a**	**Actb**	**Gapd**
273	274	275	276	277	**278**	**279**	**280**	281	**282**	**283**	**284**	285	**286**	**287**	**288**

Fig. 1. Distribution of genes in the mouse stem cell chip. The chip consists of 260 genes useful for identification of developmental stages of stem cells. The polymerase chain reaction fragment (approx 500 bp) of each gene was printed on a Nytran^{+} membrane with a rectangular area (23×35 mm). There are 288 spots including 10 duplicated gene spots, 10 blank negative controls, and 4 positive controls in a triplicate repeat (last row). Many potential molecular markers for embryonic stem and neural progenitors are located on the first and fifth row, respectively.

ABCG2 1	ACTA2 2	ACTC 3	ACTG2 4	ACVR1 5	ACVR2 6	ACVRL1 7	AFP 8	BDNF 9	BMP1 10	BMP10 11	BMP15 12	BMP2 13	BMP3 14	BMP4 15	BMP5 16
BMP6 17	BMP7 18	BMP8 19	BMPR1A 20	BMPR1B 21	BMPR-II 22	C3orf4 23	CCNE1 24	CCNE2 25	CCNG2 26	CD24 27	CD34 28	CD44 29	CD9 30	CDH1 31	CDH15 32
CDH2 33	CDH3 34	CDH4 35	CDH5 36	CDK4 37	CDKN1A 38	CDKN1B 39	CDKN2A 40	CDKN2D 41	CER1 42	CNP 43	CNTF 44	CNTFR 45	COL6A2 46	CST3 47	CTNNA1 48
CTNNA2 49	CTNNAL1 50	CTNNB1 51	CTNND2 52	CXCL12 53	CXCR4 54	DLK1 55	DNMT1 56	DNMT2 57	DNMT3A 58	DNMT3B 59	DNMT3L 60	EGF 61	EGFR 62	EGR2 63	ESG1 64
FABP4 65	FABP6 66	FABP7 67	FGF1 68	FGF10 69	FGF11 70	FGF12 71	FGF14 72	FGF16 73	FGF17 74	FGF18 75	FGF19 76	FGF2 77	FGF20 78	FGF21 79	FGF23 80
FGF3 81	FGF4 82	FGF5 83	FGF6 84	FGF7 85	FGF8 86	FGF9 87	FGFR1 88	FGFR2 89	FGFR3 90	FGFR4 91	FLJ10314 92	FLJ21195 93	FOXG1A 94	FOXH1 95	FOXM1 96
FOXO1A 97	FZD1 98	FZD2 99	FZD3 100	FZD4 101	FZD7 102	FZD8 103	FZD9 104	GATA2 105	GATA4 106	GCG 107	GCM2 108	GDF1 109	GDF11 110	GDF2 111	GDF3 112
GDF5 113	GDF8 114	GDF9 115	GFAP 116	GJA7 117	GJB1 118	GJB3 119	GJB5 120	HSPA9B 121	ICAM1 122	ICAM5 123	IGF1 124	IGF1R 125	IGF2 126	IGF2R 127	IL6 128
IL6R 129	IL6ST 130	INA 131	INHBA 132	INHBB 133	INS 134	INSRR 135	ISL1 136	ITGA2 137	ITGA2B 138	ITGA3 139	ITGA4 140	ITGA5 141	ITGA6 142	ITGA7 143	ITGA8 144
ITGAE 145	ITGAL 146	ITGAM 147	ITGAV 148	ITGAX 149	ITGB1 150	ITGB2 151	ITGB3 152	ITGB4 153	ITGB5 154	ITGB6 155	ITGB7 156	KDR 157	KRT14 158	KRT15 159	KRT17 160
KRT8 161	LIF 162	LIFR 163	LOC145957 164	MAP1B 165	MAP2 166	MDM2 167	MGC21116 168	MYH11 169	MYH6 170	MYL4 171	NCAM1 172	NCAM2 173	NEFL 174	NES 175	NEUROG1 176
NGFB 177	NGFR 178	NKX2-5 179	NKX2B 180	NOG 181	NOTCH1 182	NPPA 183	NRG1 184	NRG2 185	NTF3 186	NTRK2 187	NTRK3 188	NUMB 189	OLIG1 190	OLIG2 191	PAX6 192
PDGFA 193	PDGFB 194	PDGFRA 195	PDGFRB 196	PDX1 197	PECAM1 198	PLP1 199	POU3F2 200	POU3F3 201	POU5F1 202	POU6F1 203	PROML1 204	PROX1 205	PTCH 206	PTCH2 207	PTEN 208
PTPRC 209	PUM1 210	PUM2 211	RB1 212	RBL1 213	RBL2 214	S100B 215	SHH 216	SIAT8A 217	SLC1A2 218	SLC1A6 219	SLC2A1 220	SNAI1 221	SNAI2 222	SOX1 223	SOX10 224
SOX13 225	SOX15 226	SOX17 227	SOX18 228	SOX2 229	SOX3 230	SOX4 231	SOX5 232	SOX6 233	SYT1 234	TEP1 235	TERF1 236	TERT 237	TGFB1 238	TGFB2 239	TGFB3 240
TGFBR1 241	TGFBR2 242	TGFBR3 243	TGFBR3 244	THY1 245	TINF2 246	TNC 247	TP53 248	UTF1 249	VCAM1 250	VEGF 251	VIM 252	WNT11 253	WNT2 254	WNT3 255	WNT4 256
WNT5A 257	WNT5B 258	WNT6 259	WNT7A 260	WNT7B 261	WNT8A 262	ZFP42 263	Blank 264	Blank 265	Blank 266	Blank 267	Blank 268	Blank 269	Blank 270	Blank 271	Blank 272
PUC18 273	PUC18 274	PUC18 275	PUC18 276	GAPD 277	GAPD 278	GAPD 279	PPIA 280	PPIA 281	PPIA 282	RPL13A 283	RPL13A 284	RPL13A 285	ACTB 286	ACTB 287	ACTB 288

Fig. 2. Gene list and distribution of the human stem cell chip. Similar to the mouse stem cell chip, the human stem cell chip consists of 263 known genes useful for the identification of developmental stages of stem cells. The polymerase chain reaction fragment (approx 500 bp) of each gene was printed on a Nytran^{+} membrane with a rectangular area (23 × 35 mm) with an 18 × 16 format (row × column).

7. Dry the printed membranes at room temperature overnight and then subject to 1200 J ultraviolet crosslinking. Perform quality control of the arrays (*see* **Subheadings 3.3.1.** and **3.3.2.**). After passing the quality control test, store the array at –20°C until use (*see* **Note 3**).

3.2. Neural Stem Cell-Focused Microarray and Data Analysis

A flow chart for microarray procedures is presented in **Fig. 3**. These steps include generation of gene-specific dUTP labeled cDNAs, hybridization, and chemiluminescence detection of the image.

3.2.1. Labeling Probes

The biotin dUTP-labeled cDNA probes were specifically generated by following protocols of either the AmpoLabeling (LPR) kit or conventional MMLV RT method (*see* **Note 4**).

3.2.1.1. LPR Amplification Protocol

1. Anneal the primers and RNAs: warm up the LPR kit on ice. Take a sterile 0.5-mL PCR tube, and mix the following for each sample: 0.5–5.0 μg total RNA (*see* **Note 5**); 1 μL buffer P; and RNase-free water to final volume 10 μL. Briefly centrifuge the tube and heat the mixture at 70°C for 3 min. Cool to 37°C and keep it at this temperature for 10 min.
2. Prepare the RT cocktail: for each array the following mixture is made while the annealing mixture is incubating at 37°C. Take a sterile 0.5-mL PCR tube, and mix the following (total volume 10 μL): 4 μL buffer BN; 4 μL RNase-free water; 1 μL RNase inhibitor; and 1 μL RT.
3. Run RT reaction: transfer the 10 μL of RT cocktail to the Annealing solution and mix well. The RT reaction is conducted at 37°C for 25 min and then stopped at 85°C for 5 min. The solution is then kept on ice for the following labeling step.
4. Prepare the LPR cocktail: for each array, the following cocktail is made in a sterile 0.5-mL PCR tube (total volume 30 μL): 18 μL buffer L; 9 μL buffer AF; 2 μL biotin-16-dUTP; and 1 μL DNA polymerase.
5. Perform LPR: add 30 μL of the LPR cocktail to each RT reaction tube, mix well, and conduct the reactions as follows in the thermal cycler: 85°C, 5 min; 30 cycles of (85°C, 1 min; 50°C, 1 min; 72°C, 1 min); then 72°C, 5 min.
6. Stop the reaction by adding 5 μL of buffer C.
7. Denature the probes at 94°C for 5 min and quickly chilling on ice. This biotin-labeled cDNA probe is ready for hybridization.

3.2.1.2. RT-Labeling Protocol

1. Anneal the primers and RNAs: warm up the MMLV RT labeling kit in ice. Take a sterile 0.5-mL PCR tube, and mix the following for each sample: 2.0–5.0 μg total RNA (*see* **Note 5**); 3 μL buffer A; and RNase-free water to final volume 10 μL. After mixing, briefly centrifuge the tube and heat the mixture at 70°C for 3 min. Cool to 42°C and keep it at this temperature for 2 min.

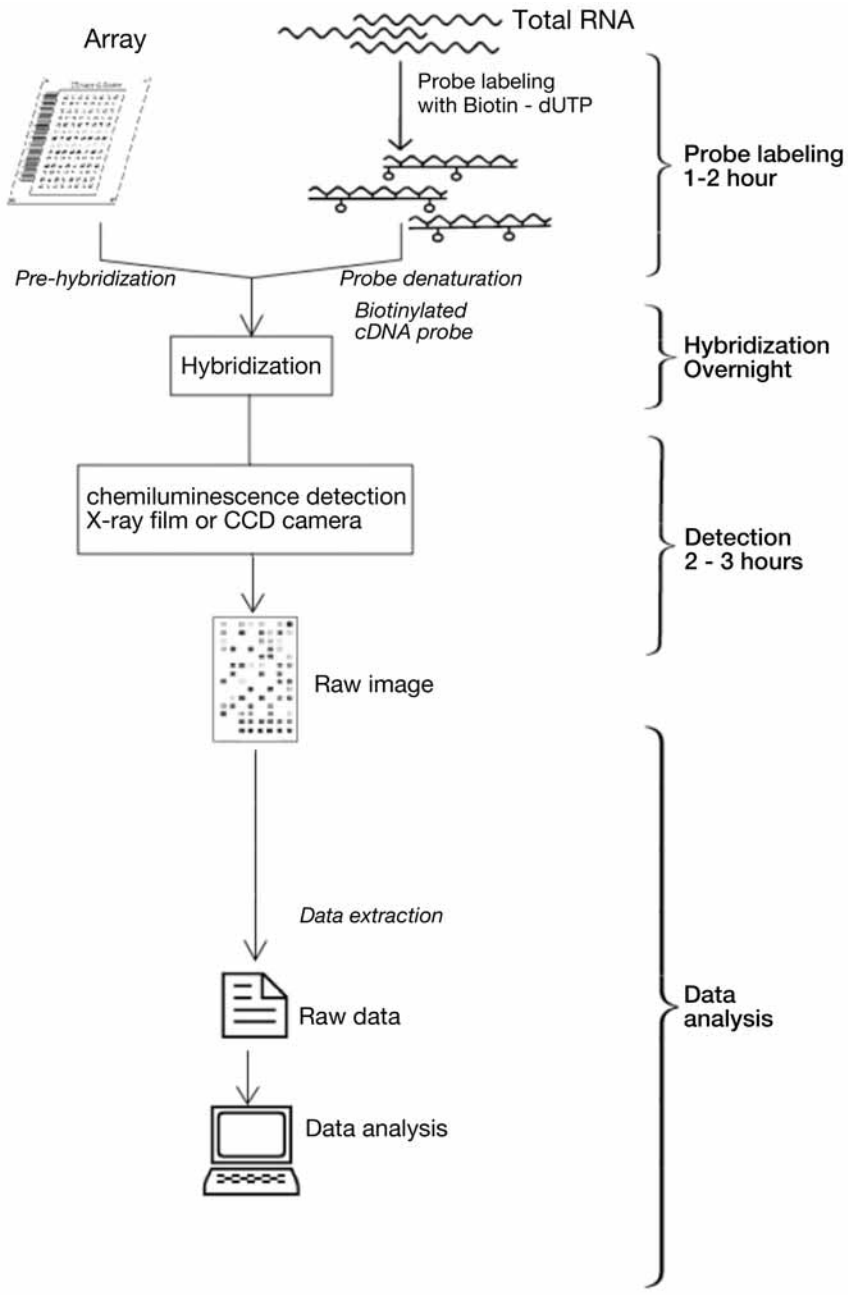


Fig. 3. Array procedures. Steps include preparation of gene-specific biotin dUTP labeled cDNA probes, hybridization, chemiluminescence detection, and data analysis.

2. Prepare the RT cocktail: while annealing mixture is incubating at 70°C, the following mixture is made in a sterile 0.5-mL PCR tube for each array (total volume 10 µL: 4 µL buffer BN; 2 µL Biotin-16-dUTP; 2 µL RNase-free water; 1 µL RNase Inhibitor (RI); and 1 µL RT.
3. Run RT reaction: prewarm the RT cocktail at 42°C, transfer 10 µL of prewarmed cocktail to the 10 µL of annealing mixture, mix it well, and incubate at 42°C for 90 min.
4. Stop the reaction by adding 2 µL of buffer C.
5. Denature the probes by adding 2 µL of buffer D and incubate at 68°C for 20 min.
6. Neutralize the solution by adding 25 µL of buffer E and incubate at 68°C for 10 min. Now the probes are ready for hybridization.

3.2.2. Prehybridization, Hybridization, and Washing

1. Set up temperature of hybridization oven at 60°C.
2. Prewarm GEAhyb hybridization solution in the 60°C oven and make sure that the solute is completely dissolved.
3. Denature the salmon sperm DNA at 100°C for 5 min and chill quickly on ice.
4. Make 100 µg salmon sperm DNA per mL with the prewarmed hybridization solution and keep it at 60°C. For each array, 2 mL of such solution is required.
5. Wet the array membranes by adding 5 mL of molecular biology grade water to the hybridization tube containing the membrane. After the membrane is completely wet, drain and discard the water.
6. Prehybridize the wet membranes with 2 mL of the hybridizing solution containing salmon sperm DNA at 60°C for 2 h in a hybridization oven with continuous agitation at 5–10 rpm.
7. Pour off the prehybridization solution into a sterile tube.
8. Take 0.75 mL of the prehybridization solution and mix with the denatured probes made in above probe labeling procedures.
9. Hybridize the membranes with the probes at 60°C overnight (*see* **Note 6**).
10. Prepare 1 L of 2X SSC containing 1% SDS and 0.1X SSC containing 0.5% SDS with molecular biology grade water and incubate at 60°C overnight.
11. On the second day, wash the membranes twice with 5 mL of prewarmed 2X SSC containing 1% SDS solution at 60°C (15 min per wash at 15 rpm).
12. Wash the membranes twice with 5 mL of prewarmed 0.1X SSC containing 0.5% SDS solution at 60°C (15 min per wash at 15 rpm).

3.2.3. Chemiluminescence Detection of Image

1. Prewarm the GEA blocking solution Q, 5X washing buffer F, and AP-assay buffer Q at 37°C; make sure that no precipitation occurs and keep them in room temperature.
2. Set up temperature of hybridization oven to 25°C.
3. Block the washed membranes with 2 mL of solution Q for 40 min at 5–10 rpm.
4. During blocking time, prepare 1 L of 1X buffer F with molecular biology grade water.

5. Prepare 1:10,000 dilution of AP-streptavidin with 1X washing buffer. Each array needs 2 mL of such solution.
6. Incubate each array with 2 mL of 1:10,000 AP-streptavidin for 10 min.
7. Wash the membranes with 5 mL of 1X washing buffer four times at 15 rpm. Vortex each time after the addition of the washing buffer.
8. Rinse the membranes with 2 mL of AP-assay rinsing buffer Q for 3 min.
9. Incubate membranes with 1 mL of CDP-Star Chemiluminescent substrate for 5 min at 5–10 rpm.
10. Place the membranes into a small zippered plastic bag or between plastic sheets, smooth out bubbles, remove excess CDP-Star solution with a filter paper, and acquire images using a digital imaging system (such as FluorChem). The image is stored in a TIF format file.

3.2.4. Data Analysis

1. Qualify the spots: open the image file with ImageQuant 5.2 software, identify the positive and negative spots by eye (verified by at least two people) and record the results in the corresponding gene list file that is usually stored as an Excel file.
2. Quantify the spot: measure intensity of spots by using ImageQuant 5.2 software.
3. Transfer the intensity of spot into the corresponding gene list file that contains qualified data.
4. Average intensities derived from blank spots.
5. Subtract the blank average intensities and then divide by the mean glyceraldehyde-3-phosphate dehydrogenase (GAPDH) intensity to obtain a relative intensity for each spot.
6. These relative intensities can be used to compare expression levels in different groups for further statistical analysis. The identification of spots by eye helps to verify results derived from comparison.

3.3. Evaluation of Mouse Neural Stem-Focused Array and Quality Control

To assess quality of the membranes one needs to evaluate the quality of the DNA, the accuracy of the sequence information, the quality of printing and the specificity of hybridization.

3.3.1. Quality Control of Printed Membranes

1. Observe the spots that have been stained by tracking day (0.001% bromophenol blue) by eye during printing (*see* **Note 7**).
2. Perform array hybridization and analysis by using a mixture of total RNA from mouse D3 ES cells and adult rat hippocampus that covers at least 90% of the genes in the chip (*see* **Subheading 3.3.3.**) and following procedures described under **Subheading 3.2.**
3. Perform data analysis (*see* **Subheading 3.2.4.**). Results are shown in **Fig. 4.** Gene expression scored as existing (positive) or absent (negative) shows a high reproducibility irrespective of the probe preparation technique used (**Fig. 4C**) even

when different batches of RNA were used. All 10 blank spots were consistently negative, duplicates showed consistent levels of expression and virtually all positives remained positive.

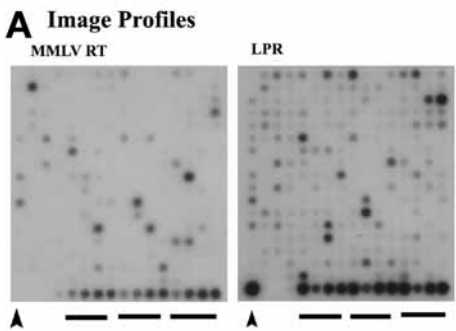
3.3.2. Cross Hybridization Assessment

It is important to determine that arrayed genes can be detected in a specific fashion without cross hybridization.

1. Randomly select 17 genes to test the specificity of hybridization. For example, we used *fgf4*, *Gcm2*, *Mtap1b*, *Mtab2*, *Tubb3*, *Prox1*, *NFL*, *NCAM2*, *Cst3*, *Ccng2*, and *CDKn1b*, *DNMT1*, *Cdh4*, *Itga6*, *Rpl13a*, *Actb*, and *GAPDH*. In addition, spot some genes in duplicate or triplicate. The location of each gene in the array is bolded in **Fig. 1**.
2. Label probes by PCR in the presence of biotin-dUTP. Each reaction (10 μL) contains 1 μL 10X PCR buffer, 150 μmol $MgCl_2$, 0.8 μL buffer BN, 20 pmol primer, 1 μL 100X diluted specific gene inserted plasmid, 1 U RedTaq DNA polymerase, and 0.2 pmol biotin-dUTP.
3. Run PCR in 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, and a final extension for 10 min at 72°C.
4. Take 3 μL of each labeled cDNA fragment to check probe quality by running it in 1.2% agarose gel (**Fig. 5A**).
5. Pool the labeled probe for each gene and perform an array (*see* **Subheading 3.2.**).
6. Perform data analysis (*see* **Subheading 3.2.4.**). As shown in **Fig. 5B**, the biotin-labeled probe of each gene identified only its corresponding arrayed gene fragment, suggesting limited or no cross-hybridization. This is clearly illustrated with *FGF4*, a member of the *FGF* family. A total of 22 members were spotted on the chip that share varying degrees of homology. The *FGF4* probe gave a strong signal on the *FGF4* spot and showed no cross-hybridization with any of other *FGFs*, suggesting a high specificity for this gene hybridization under our conditions. Moreover, the chip has a high quality in printing. All duplicates (*FGF4* and *NCAM2*) and triplicates (*Rpl13a*, *Actb*, and *GAPDH*, underline in **Fig. 5B**) were evenly detected.

3.3.3. Assessments of Detection Sensitivity by Comparison of Labeling Protocols Between AmpoLabeling (LPR) Kit and Conventional MMLV RT Method

1. Choose a RNA source that expresses the majority of genes printed on the focused membranes. We used a mixture of RNA from mouse D3 ES cells and (0.6 μg) adult rat hippocampus (0.2 μg)/μL because PCR amplification could readily detect 234/260 genes in this pooled RNA.
2. Perform array analysis by using this pooled RNA to determine how many of the 260 arrayed genes are detectable using either standard MMLV RT or LPR protocol (*see* **Subheading 3.2.**).
3. Perform data analysis (*see* **Subheading 3.2.4.**). A representative imaging profile and results are shown in **Fig. 4** (*see* **Note 8**).



B Confirmation by PCR

Groups	Array+ PCR+	Array- PCR+	Array- PCR-	Array+ PCR-
MMLV RT	78	156	24	2
LPR	176	58	18	8

C Reproducibility in Quantity

MMLV RT Method

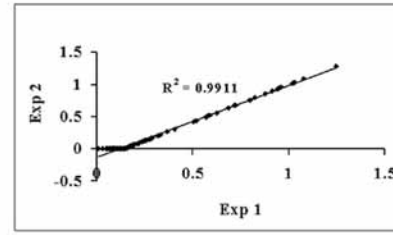
Groups	Exp.1 Numbers of Positives	Exp.1 Numbers of Negatives	Exp. 2 Numbers of Positives	Exp. 2 Numbers of Negatives
Blanks		10		10
Positive & House Keeping Controls	12		12	
Samples	184	72	180	76
Duplicated genes	6	4	6	4
Total	202	86	198	90

LPR Method

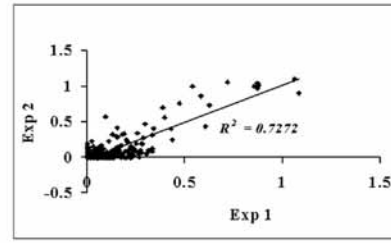
Groups	Exp.1 Numbers of Positives	Exp.1 Numbers of Negatives	Exp. 2 Numbers of Positives	Exp. 2 Numbers of Negatives
Blanks		10		10
Positive & House Keeping Controls	12		12	
Samples	184	72	180	76
Duplicated genes	6	4	6	4
Total	202	86	198	90

D Reproducibility in Quantification

MMLV RT Method



LPR Method



E Semi-quantitative RT-PCR

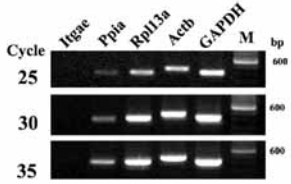


Fig. 4. Quality control of the chips and comparison of results derived from moloney murine leukemia virus reverse transcriptase (MMLV RT) and linear polymerase reaction (LPR) or Ampolabeling methods. Total RNAs from mouse D3 ES cells and adult rat hippocampus were first isolated and then mixed together with a ratio of 3:1. This mixed RNA (0.8 μg/membrane) was used to prepare probes using MMLV RT or LPR methods. Labeled probes were used to perform array hybridization. **(A)** Shows distinct

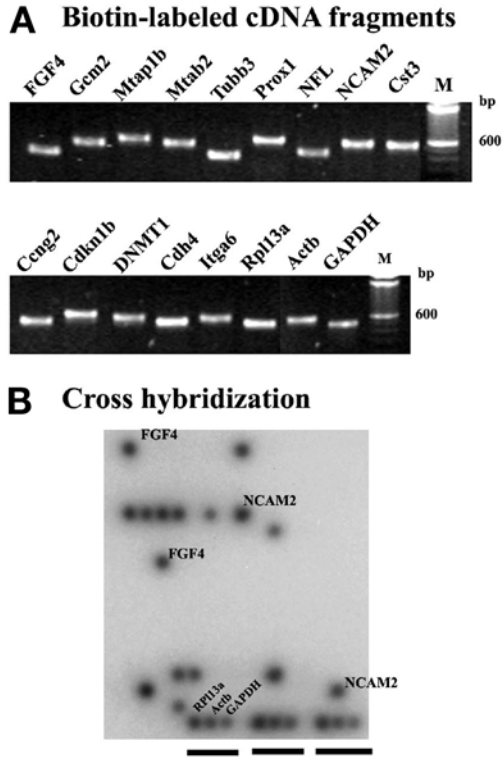
3.3.4. Array Test for Specific Tissues

A real evaluation of quality of the array is to determine if the arrayed genes were selective and sufficient in number to differentiate between different cell populations.

1. Choose cell types. We examined embryonic stem cells (undifferentiated mouse D3 ES cells) that are developmentally closely related to neural cells and share many antigens ***(6,7)***, and fetal neural cells (rat E14.5 neural tubes) that consist of predominantly neural stem cells and neural progenitors ***(8–10)***. We included liver cells (derived from 6-mo-old male Sprague-Dawley rat) as an endodermal derivative that is distinct from the other two populations.
2. Isolate total RNA by using TRIzol.
3. Perform array analyses by a standard MMLV-RT labeling protocol (*see* **Subheading 3.2.**).
4. Perform data analysis (*see* **Subheading 3.2.4.**). As shown in **Fig. 6A**, the gene expression profile could be easily distinguished between these cell types. There were nine ES markers detected in mouse D3 ES cells that were absent or expressed at low/undetectable levels in neural cells or in liver (a list in **Fig. 6C**). Six of these ES markers were easily identified in the first row. *Dnmt1* and *Itga6* were located in rows 15 and 17, respectively, and appeared to be specific to ES cells though they were not initially included on the array as cell type specific markers. The relatively specific expression in D3 cells was confirmed by RT-PCR. As shown in **Fig. 6B**,**C**, *Sox2* is present in D3 (ES cells) as well as E14.5 neural tubes (neural stem and progenitor cells) consistent with published data of *Sox-2* expression in ES and neural stem cell populations ***(4,11)***. In addition to positive expression of ES cell markers, we also found 17 neural markers that were expressed by neural cells with little or no expression in ES cells and the liver (*see* list in **Fig. 6C**). Some of them were easily identified in row 5 (as indicated by the long arrow in **Fig. 6A**). RT-PCR analysis for expression of neural markers was in agreement with the array results (**Fig. 6B**). Interestingly glial markers present on

Fig. 4. (Continued)

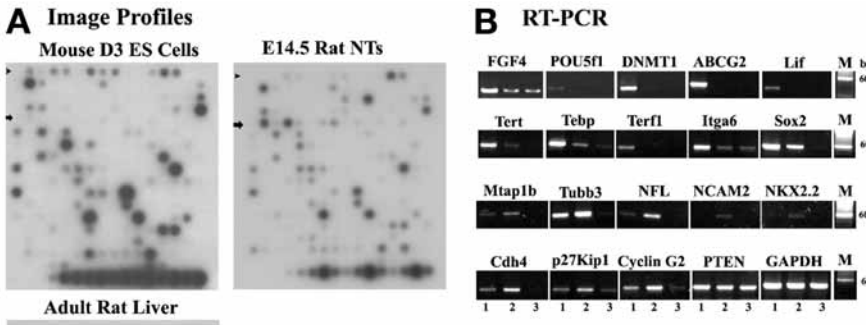
image profiles obtained by these two methods. The LPR method (71%, 184/260) also reveals higher detection rate than MMLV RT method (31%, 80/260). Short arrow: spot for integrin b5. Underline: four positive controls in triplicate. **(B)** Summarizes results comparing array data and RT-polymerase chain reaction (PCR) amplification. Both methods show high agreement (96–98%) between positive results in array and RT-PCR. The false-positive rate is 2.5–4.3%. Tables in **(C)** summarize the results of positive and negative hybridization from two experiments. A relative intensity of each spot was calculated and plotted for these repeated experiments. These scatter plots are shown in **(D)**. The data suggest a larger variability for probe prepared by the LPR method compared with MMLV RT labeled probe. Further analysis of expression of five genes by semi-quantitative RT-PCR is shown in **(E)**. Results from this RT-PCR is in agreement with those obtained by MMLV RT method (*see* underline in **A**).



C Intensities of duplicate and triplicate spots

Gene Name	Spot 1	Spot 2	Spot 3	Average	SD	CV (%)
RPL13A	24134078	26932504	22908457	24658346	2062615	8.4
GAPDH	19380352	22473332	17791351	19881678	2380910	11.9
Beta-Actin	22843892	26130430	22678396	23884239	1947017	8.2
NCAM2	22599535	19475958				
FGF4	20888423	20629357				

Fig. 5. Cross-hybridization test. cDNA fragments of 17 genes, as indicated in **(A)**, were biotin-labeled, pooled, and used to probe the array. **(A)** Amplification quality of the PCR amplified and labeled probes. **(B)** An array image profile obtained by hybridization of the array with the mixture of 17 labeled probes. Location of each gene in array is indicated in bold letter in **Fig. 1**. Spots for *FGF4* and *NCAM2* were duplicated, and genes for *Rpl13a*, *Actb*, and *GAPDH* were spotted in triplicates. The results clearly indicate no cross hybridization using these 17 randomly genes. Spot intensities for genes present as duplicates and triplicates were also measured by using ImageQuan 5.2 and are presented in panel C. The data suggest a high reproducibility for duplicates and triplicates. CV: coefficient variance; SD/mean.



C Comparison between Array and RT-PCR

Markers	Gene Names	Array			PCR		
		D3 ES	E14.5 NT	Liver	D3 ES	E14.5 NT	Liver
ES	Dnmt1	0.40	0.05	0.00	0.62	0.20	0.13
	Fgf4	0.37	0.11	0.19	0.80	0.37	0.43
	Itga6	0.39	0.01	0.10	0.67	0.21	0.19
	Pou5f1	0.29	0.21	0.14	0.23	0.15	0.21
	Sox2	0.15	0.13	0.07	0.98	0.71	0.13
	Lifr	0.08	0.07	0.29			
	Zfp42	0.40	0.06	0.03			
	Gcm2	0.22	0.15	0.25			
	Prdc	0.16	0.23	0.09			
Neural Progenitors	Nkx2-2	0.38	0.83	0.64	0.10	0.14	0.07
	Mtap1b	0.10	0.77	0.30	0.09	0.19	0.04
	Tubb3	.07	0.73	0.17	0.53	0.85	0.07
	Cdkn1b	0.07	0.24	0.06	0.22	0.40	0.14
	Nfl	0.04	0.20	0.15	0.12	0.48	0.09
	Ccng2	0.00	0.13	0.03	0.34	0.63	0.13
	Cdh4	0.01	0.01	0.08	0.23	0.49	0.08
	Ncam2	0.00	0.01	0.00	0.04	0.12	0.07
	Ncam1	0.00	0.42	0.13			
	Mtab2	0.04	0.34	0.28			
	Cdh2	0.00	0.24	0.24			
	Fabp7	0.00	0.68	0.00			
	Vim	0.04	0.05	0.11			
	Syn1	0.04	0.10	0.14			
	Cst3	0.01	0.05	0.07			
	Cdh5	0.09	0.02	0.04			
	Prox1	0.00	0.20	0.38			

Fig. 6. Evaluation of the array by using different types of tissues. Total RNAs (3 μg/membrane) derived from mouse D3 ES cells, rat E14.5 neural tubes and adult rat liver were used to make biotin dUTP-labeled cDNA probes with the moloney murine leukemia virus reverse transcriptase (RT) method. The array filters were hybridized with these probes and their images were recorded. **(A)** A representative image profile of three experiments. A short arrow indicates ES markers in the first row, whereas a longer arrow points to neural markers in the fifth row. **(B)** An image of RT- polymerase chain reaction (PCR) confirmation. Amounts of cDNAs from different tissues were adjusted to be nearly equal by using GAPDH as a control. **(C)** A summarized result derived from the array analysis and RT-PCR. Relative intensities of each gene expression relative to that of GAPDH were determined and are presented. 1, mouse D3 cells; 2, E14.5 rat NTs; 3, adult rat liver.

Table 2
A Partial List of Sources for Large Scale Genome Microarrays

Affymetrix; New York, NY	http://www.affymetrix.com/products/arrays/index.affx
Yale University; New Haven, CT	http://keck.med.yale.edu/dna_arrays.htm
Agilent; Foster City, CA	www.chem.agilent.com/Scripts/pds.asp?lPage=2433
MWG Biotech; High Point, NC	http://www.mwg-biotech.com/html/d_arrays/d_catalog_arrays.shtml
Amersham; Piscataway, NJ	http://www5.amershambiosciences.com/APTRIX/upp01077.nsf/Content /codelink_human_bioarrays?OpenDocument&hometitle=codelink
NIAID Microarray Research Facility, NIH; Rockville, MD	http://www.niaid.nih.gov/dir/services/rtb/newmicro/overview.asp
NCI LMT microarray laboratory, NIH; Rockville, MD	http://web.ncifcrf.gov/rtp/LMT/Microarray/default.asp
Qiagen; Valencia, CA	http://omad.qiagen.com/download/index.php
Compugen; Jamesburg, NJ	http://www.labonweb.com/chips/libraries.html

the array did not hybridize to probe prepared from neural cells at E14.5. It has been shown that glial progenitor cells appear late compared to neuronal progenitors in neural tubes (*12*). The stem array analysis also failed to detect glial markers, such as S100β, and GFAP, at this stage. Thus, this stem cell array can distinguish stem cells and progenitor cells at different development stages.

3.4. Large-Scale Human Oligo-Microarrays

Although small focused arrays are useful for routine use, in other circumstances, large-scale genomic coverage is required. We have evaluated several array formats and have elected to use an oligonucleotide-based array using long oligonucleotides to enhance binding specificity and to reduce cross hybridization issues. The design and validation and use of such an array is described in the following section (*see* **Note 9**). We have included a partial list for sources of large-scale genome microarrays (**Table 2**).

Table 3
Process of Production of Human Oligonucleotide Arrays

Step	Process
Preparing 16K oligonucleotides for array fabrication	Suspension of lyophilized oligos in deionized dH_2O, transfer of oligonucleotides from 96-well plates to 384-well plates, and addition of 20X SSC to get printing concentration of 25 μ*M* in 4XSSC.
Coating slides with poly-L-lysine	Cleaning and dipping glass slides in poly-L-lysine solution for 1 h followed by drying at room temperature.
Designing, testing, and printing program	Calibration of sample plate, slide offsets, sonicator, rinse station, and wash station.
Deposition of oligonucleotides on slides	Cleaning pins and pinhead, and printing oligonucleotides on slides.
Generation gene array list (gal file)	Adjustment of plate sample list in consoli dated tab-delimited format and completing the gal file deconvolution.
Post-printing processing	Cross-linking using UV cross-linker followed by chemical blocking of arrays.
Quality control	Scanning and hybridization of arrays with Cy3-labeled nonamer oligonucleotide and Cy3- and Cy5-labeled total RNA from two glioblastoma cell lines.

3.4.1. Preparing 16K Human Oligo-Microarray

Human genome set (version 1.1) containing 16,659 of 70mer oligonucleotides was purchased from Qiagen/Operon. All 260 genes on the focused stem cell array were present on the large-scale array providing an independent measure of control. The process of production of human oligo-arrays is illustrated in **Table 3**.

3.4.1.1. Coating Poly-L-Lysine Substrate Slides (*see* **Note 10**)

1. Place slides in four slide racks.
2. Place two slide racks in each of the two chambers.
3. Pour the cleaning solution (*see* **Subheading 2.2.1., item 17**) into the two chambers with slides; cover the chambers; mix on orbital shaker for 2 h.
4. Quickly transfer racks to fresh chambers filled with ddH_2O; rinse vigorously by plunging racks up and down 36 times; repeat rinses six times with fresh ddH_2O; shake on orbital shaker for 5 min on the second and fourth rinse (*see* **Note 11**).
5. Pour poly-L-lysine solution (*see* **Subheading 2.2.1., item 5**) onto slide chamber. Put slides in and shake for 1 h.

6. Transfer rack to fresh chambers filled with ddH_2O (*see* **Note 12**). Leave them on the orbital shaker until the rinse water is ready; plunge up and down 10–20 times to rinse.
7. Centrifuge slides on microtiter plate carriers (*see* **Note 13**) for 5 min at 1000*g*.
8. Dry slide racks in a 45°C vacuum oven for 10 min (this step is optional).
9. Store slides in closed slide box for at least 3 wk before printing (*see* **Note 14**).

3.4.1.2. Fabrication of 16K Human Oligo-Microarray

1. Check poly-L-lysine-coated slides: the poly-L-lysine coating should not be opaque and there is no visible sign of the poly-L-lysine coating cracking.
2. Prepare 70mer 16,659 oligonucleotides solutions with 4X SSC to the concentration of 25 μ*M* using Biomek FX liquid handling system.
3. Use an OminiGrid Arrayer equipped with 32 stealth microspotting SMP3 pins to spot oligos. Program the array format as 4×8 blocks with 23×23 spots in each block.
4. Adjust printing conditions as 40–45% humidity and a room temperature of less than 22.2°C.
5. After spotting, leave the printed slides on the arrayer platform at 60% humidity for 5 min to be rehydrated and at 30–40% humidity (room humidity) overnight.
6. Crosslink the chips with a Stratalinker at 65 mJ to allow electrostatic attachment of DNA to the substrates.
7. Label slides and mark array area using diamond pen (*see* **Note 15**).
8. Place the slides back in the rack (usually 20–30 slides).
9. Block the chips with succinic anhydrite solution (*see* **Subheading 2.2.1., item 18**): quickly and evenly plunge the slide rack up and down in the solution for 1 min (*see* **Note 16**); let the slides sit in the solution with gentle agitation on an orbital shaker for 15–20 min.
10. Remove the slide rack and plunge the slides up and down in a slide dish with room temperature distilled water to rinse off the blocking solution and incubate for 1 min.
11. Transfer the slide rack to a dish containing 95% ethanol and plunge up and down several times.
12. Dry the slides by spinning in a tabletop centrifuge at 1000*g* for 5 min.
13. Store the slides at room temperature in a dust-free box until use.

3.4.1.3. Quality Control by Cy3-Labeled 9mer

1. Prepare 20 μL Cy3-labeled 9mer probe (*see* **Note 17**) mix as following: 4 μL of 20X SSC, 2 μL of 10 mg/mL poly-dA, 1 μL of 1 *M* Tris-HCl pH 7.5, 0.4 μL of 10% SDS, 150 pmol Cy3 random 9mer, and DH_2O to final 20 μL volume.
2. Briefly heat probe to 90°C.
3. Cool by spinning in microcentrifuge in room temperature (*see* **Note 18**).
4. Pipet probe onto slide and use a cover slip as for a normal hybridization.
5. Allow incubation at room temperature for 3 to 5 min.
6. Wash slide in 2X SSC 0.2% SDS for 2 min at room temperature.
7. Wash slide in 0.05X SSC for 2 min at room temperature.
8. Dry slide by spinning slide rack or by placing slide in a 50-mL Falcon tube, and spinning at 800–1000*g* for 5 min.

9. Scan the slide with Axon GenePix scanner. A representative image of Cy3-labeled 9mer oligonucleotide is shown in **Fig. 7A** (*see* **Note 19**).

3.4.2. Labeling Probe

An indirect labeling method that adapts the procedures of FairPlay Microarray Labeling Kit is used. Protocols using both 20 and 5 µg of total RNAs are described.

3.4.2.1. Probe Preparation

1. Add 1 µL oligo-dT primer (1 µg/µL) into 20 µg total RNA (*see* **Note 5**) in 19 µL DEPC H_2O (total 20 µL volume), incubate at 70°C for 5 min, and then quickly chill in ice for 3 min (in case of 5 µg total RNA: add 1 µL of amino allyl dT primer [1 µg/µL] into 5 µg total RNA in 12 µL DEPC water (total 13 µL volume]).
2. Add the following RT labeling reaction mix to each tube. For 20 µg total RNA: 3 µL of 10X first strand buffer, 2 µL of 20X aa-dT/(NTP) mix, 3 µL of 0.1 *M* DTT, and 2 µL of Stratascript RT. For 5 µg total RNA: 2 µL of 10X first strand buffer, 1.5 µL of 20X aa-dT/(NTP) mix, 2 µL of 0.1 *M* DTT, and 1.5 µL of Stratascript RT.
3. Vortex reaction mix and do a quick spin.
4. Incubate at 42°C for 90 min.
5. After incubation, add 30 µL DEPC water (add 40 µL DEPC water in case of 5 µg total RNA) to make up total 60 µL volume.

3.4.2.2. cDNA Purification (Qiagen MinElute PCR Purification Kit)

1. Add 300 µL of binding buffer PB to reaction mixture and mix well.
2. Apply the mixture to the MinElute column, and spin for 1 min at maximum speed.
3. Discharge flow-through, repeat **steps 1** and **2**.
4. Add 600 µL of washing buffer PE to the column, and spin for 1 min at maximum speed, discharge flow-through.
5. Repeat **step 4**.
6. After discharging flow-through, spin 1 min at maximum speed to avoid carrying over PE buffer.
7. Place the column into a fresh 1.5-mL tube.
8. Add 15 µL elution buffer to the center of the membrane, incubate for 1 min.
9. Spin for 1 min at maximum speed, put the flow through back into column, wait for 1 min and spin for 1 min at maximum speed (*see* **Note 20**).
10. Discard column, dry flow-through in SpeedVac for 19 min (*see* **Note 21**).

3.4.2.3. Dye Coupling

1. Resuspend cDNA pellet in 5 µL 2X couple buffer.
2. Add 6 µL aliquot Cy3 and 6 µL of Cy5 dye (*see* **Note 22**) into the tube, and mix well by pipetting up and down (final concentration of coupling reaction: 0.1 *M* $NaHCO_3$, pH 9.0; total reaction 11 µL). Place tube in a dark box at room temperature for 90 min (*see* **Note 23**).

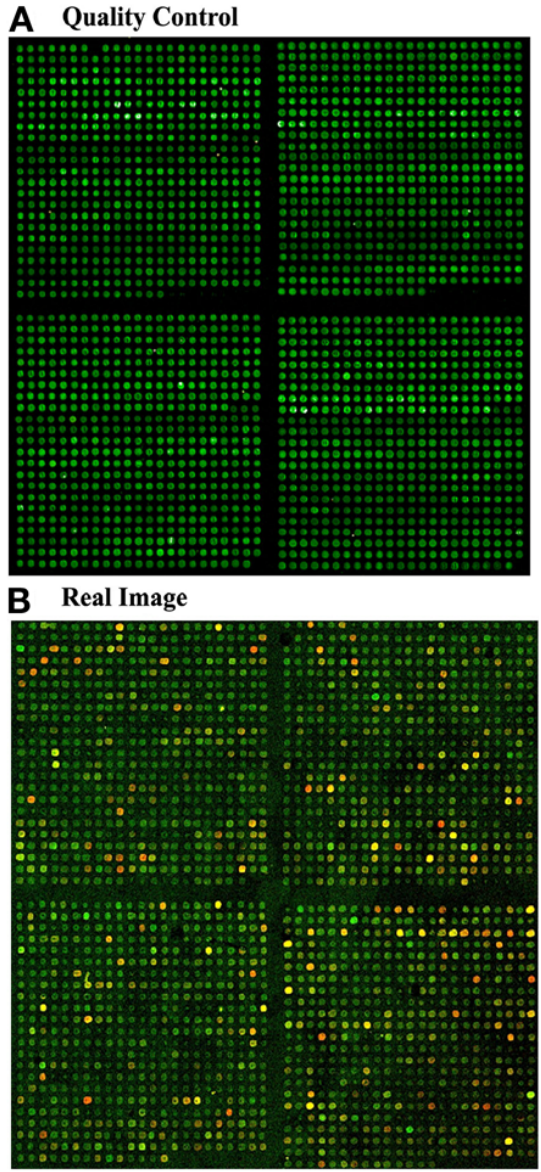


Fig. 7. Quality control and real images of large-scale microarray. **(A)** A representative image obtained by hybridization of the printed array with Cy3-labeled-9mer oligonucleotides. This image shows four blocks with 23 spots in each row and column, which can be termed as good spots (*see* **Note 19**). **(B)** Four blocks as a representation of a real hybridization image using Cy5 probe prepared with RNA from human BG02 ES cell line and Cy3 probe prepared with Human Universal Reference RNA (*see* **Note 28**). Five micrograms of total RNA are used in both samples. (Please *see* the companion CD for the color version of this figure.)

3.4.2.4. Coupled Probe Cleanup (Qiagen MinElute Kit)

1. Purify the labeled Cy3 or Cy5 probes separately. Add molecular grade dH_2O to each tube to make 60 µL volume.
2. Add 300 µL of binding buffer PB to the coupled cDNA, apply the mixture to the MinElute column, and spin for 1 min at maximum speed.
3. Discharge flow-through, repeat **step 2**.
4. Add 600 µL of washing buffer PE to the column, and spin for 1 min at maximum speed, discharge flow-through.
5. Repeat **step 4**.
6. Spin 1 min at maximum speed, eliminate carrying over PE buffer.
7. Place the column into a fresh 1.5-mL tube.
8. Add 13 µL elution buffer to the center of the membrane, incubate for 1 min.
9. Spin for 1 min at maximum speed, put the flow-through back into column, wait for 1 min and spin for 1 min at maximum speed (*see* **Note 20**).
10. Combine the separately purified probes together; increase total volume up to 26.75 µL with dH_2O.

3.4.3. Prehybridization and Hybridization

3.4.3.1. Prehybridization

Set up prehybridization reaction.

1. Make 100 µL of prehybridization buffer: 25 µL 20X SSC; 20 µL 5% BSA (*see* **Note 24**); 1 µL 10% SDS; and 54 µL dH_2O.
2. Apply 50 µL of prehybridization buffer to array, add a cover slip (*see* **Note 25**).
3. Add 20 µL water to the wells at the ends of the hybridization chamber.
4. Place array in hybridization chamber and incubate at 42°C for 60 min.
5. Remove the cover slip by swishing slide in dH_2O.
6. Wash the array in dH_2O for 2 min with shaking.
7. Wash the array in isopropanol for 2 min with shaking.
8. Spin to dry the array slide and store in a clean box.
9. Use the prehybridized array within 1 h.

3.4.3.2. Hybridization

1. Make a final volume of 36 µL for hybridization, add 1 µL (10 µg) human COT-1 DNA, 1 µL (8–10 µg) poly(dA), 1 µL yeast tRNA (4 µg), 5.25 µL 20X SSC (for 3.5X final), and 1 µL 10% SDS (for 0.3% final).
2. Heat for 2 min at 100°C, spin 1 min at maximum speed.
3. Add total volume of probe (36 µL) to the center of a processed array and quickly add a cover slip (22 × 40 mm) (*see* **Note 25**).
4. Place slide in a hybridization chamber, add 20 µL water at both ends of the slide (to maintain humidity), and incubate overnight (10–16 h) at 65°C (*see* **Note 26**).

3.4.3.3. Hybridization Washes

1. Carefully remove hybridization chamber from incubator. Remove slide from chamber, and submerge in staining dish filled with 2X SSC and 0.1% SDS. Plunge gently until the cover slip falls away from the slide. Place slide into the rack, and wash for 2 min.
2. Wash for 2 min in 1X SSC, plunging gently.
3. Wash for 2 min in 0.2X SSC, plunging gently.
4. Quickly spin dry in a centrifuge set at 50–100*g* for 5–10 min.
5. Scan as soon as possible and save the image.

3.4.4. Data Filtration, Normalization, and Analysis

1. Scan microarray slides in both Cy3 (532 nm) and Cy5 (635 nm) channels using Axon GenePix 4000B scanner with a 10-micron resolution.
2. Export scanned microarray images as TIFF files to GenePix Pro 3.0 software for image analysis. Collect the raw images at 16-bit/pixel resolutions that displayed all pixels in a 0 to 65,535-count dynamic range. A representative view of image is shown in **Fig. 7B**.
3. Use the area surrounding each spot image to calculate a local background and subtract from each spot before Cy5:Cy3 ratio calculation. The average of the resulting total Cy3 and Cy5 signal gave a ratio that was used to normalize the signals.
4. Globally normalize each microarray experiment to make the median value of the log2-ratio equal to zero. The normalization process corrects for dye bias, PMT (photo multiplier tube) voltage imbalance, and variations between channels in the amounts of the labeled cDNA probes hybridized. The data files representing the differentially expressed genes were then created.
5. For advanced data analysis, import data files (in gpr format) and image (in jpeg format) files into mAdb (microarray database), and analyze by software tools provided by National Institutes Health Center for Information Technology (http://madb.nci.nih.gov, *see* **Note 27**).

4. Notes

1. A detailed gene list including Unigene, AA no., and description can be accessed on the web (http://www.superarray.com). Purchase all designed plasmids and primers from SuperArray Bioscience Corp.
2. The PCR product should appear as a single band of expected size if stained with ethidium bromide.
3. These arrays are now commercially available from SuperArray Bioscience Corp with cat. no. MM-601.2 and HS-601.2 for mouse and human neural stem cell, respectively.
4. *See* **Subheading 3.3.3.** to choose the method designed for your experiments.
5. A poor-quality RNA will not generate a good quality image and the data obtained from such hybridizations should not be considered reliable. Therefore, the quality of RNA should be assured before any hybridization reaction is performed.

6. Make sure the hybridization tube is sealed with Parafilm and does not leak.
7. The spot sizes and stained intensities should be uniform and consistent if the amounts of cDNA printed were similar.
8. It is noted that, although LPR increased sensitivity and was quite reproducible from experiment to experiment irrespective of the probe concentration used, not all messages were amplified equally. We suggest using MMLV RT method to compare gene expression between two types of tissues.
9. Arrays of this kind are available from Agilent (Palo Alto, CA). Operon (Valencia, CA) will also provide oligos for the entire set of gene list.
10. This protocol is adapted from Qiagen and worked for pretreatment of 100 slides. Scale-up is necessary if more slides are needed.
11. After slides are clean, they should be exposed to air as little as possible because dust particles will interfere with coating and printing.
12. Avoid allowing the slides to sit idle.
13. Place paper towels below rack to absorb liquid.
14. This will assure that the coating is optimal and will produce small tight spots and low background. A mild desiccation environment may help. Slides are usually good for up to 6 mo. Slides may be good for longer but we recommend checking visually and doing a test print.
15. Because the arrayed spots will disappear upon processing, a couple of etch marks on the back of the slide can guide cover slip placement. This is easily accomplished by placing two plastic pipet tip boxes close together as an etch support, and placing the slide upside down across the two boxes such that the area of the spots is facing down between the boxes and is thus untouched.
16. Move rapidly during the dunking in succinic anhydride solution. The half-life of succinic anhydride is very short, on the order of minutes. The dunking can be tricky, because this is the step where "comet tails" can be introduced. Also make sure that the blocking solution is completely rinsed off by water.
17. The T_m for a 9mer range from about 4–40°C. The average will likely be below room temperature; therefore, factors to consider with this protocol are temperature, salt concentration, and oligo concentration.
18. Ice will cause SDS to precipitate.
19. A good spot should be retained in a uniform and round shape.
20. The volume of the elution buffer should not be more than it was added in the column. Higher volume may reflect presence of residual alcohol in the column, which will not allow good hybridization.
21. Do not overdry.
22. To aliquot the dyes, resuspend the Cy3 or Cy5 dye in 50 μL high-purity dimethylsulfoxide. The aliquot dye should be stored in dark box and −20°C freezer, and should be used within 2–3 wk.
23. After addition of Cy3 and Cy5 dye, the preparation should not be exposed to light.
24. Make 5% BSA stock solution, stored in −20°C freezer.
25. Care should be exercised to avoid entrapment of air bubbles in between glass slide and the cover slip.

26. It is very important to maintain humidity.
27. Spots with confidence interval of 99 (more than threefold) with fluorescent intensity of at least 150-fluorescence intensity in either channels and with the size of 30 μm were only considered as good spots for analysis. These advanced filters prevent the potential effect of the poor quality spots in data analysis.
28. Human Universal Reference Total RNA is mixture of total RNAs from a collection of adult human tissues, chosen to represent a broad range of expressed genes. Both male and female donors are represented. Universal Reference Total RNA serves as a standard for comparing gene expression data and is useful as an internal control for microarray expression profiling experiments.

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18

Microarray Approach to Identify the Signaling Network Responsible for Self-Renewal of Human Embryonic Stem Cells

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Summary

This chapter introduces the representative method to culture human embryonic stem cells (hESCs) under the feeder and feeder-free conditions, the former of which is used to maintain or expand undifferentiated hESCs, and the latter can be used for preparation of pure hESCs RNA samples, or for screening factors influential on self-renewal of hESCs. Also described in this chapter: a protocol and tips for conducting gene chip analysis focusing on widely used Affymetrix microarrays. These techniques will provide an unprecedented scale of biological information to illuminate a key to decipher complex signaling networks controlling pluripotency.

Key Words: Human embryonic stem cells; microarray approach; pluripotency; mouse embryonic stem cells; signaling network.

1. Introduction

Stem cells can be defined by their two essential biological functions, namely eternal self-renew and generation of differentiated cell types *(1,2)*. Embryonic stem cells are pluripotent stem cells that have been derived from the inner cell mass of blastocysts *(3–5)*. It is unarguable that their differentiation potential is exceptionally versatile, as compared with other stem cell groups, in that they can give rise to all three germ-layer derivatives and germ cells *(6,7)*. Mouse embryonic stem cells (mESCs) have been extensively used for generating mutant mice as a powerful reverse-genetics approach to identify in vivo biological functions of genes of interest *(3–5,8)*. mESCs can be also used to understand molecular mechanisms that regulate early organogenesis by in vitro assay systems *(1)*. The recent derivation of human embryonic stem cells

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(hESCs) *(9,10)* has made enormous impact on stem cell research, as they could be a possible source for tissue replacement therapy to cure critical diseases such as Parkinson's disease, diabetes mellitus, and cardiac infarction. Despite their unique potential in understanding early human embryogenesis and generating human differentiated tissues, little is known about the molecular mechanisms that govern hESC identity.

There are a handful of genes, including *Oct-3/4*, *Rex-1*, and *Nanog*, that are known to be specifically regulated in pluripotent mESCs *(11–16)*. Although it is likely that most of the essential molecular mechanisms underlying pluripotency are conserved between mouse and human *(1,9,17)*, accumulating evidence suggests that there are unambiguous biological differences between mESCs and hESCs. The LIF/Stat3 pathway is sufficient to maintain mESCs in the undifferentiated state, whereas this pathway does not support self-renewal of hESCs *(1,9)*. Trophoblasts can be derived from hESCs through activation of the bone morphogenetic proteins signaling pathway, whereas mESCs are never differentiated into this lineage unless a specific gene function is disrupted *(18,19)*. Moreover, activation of the bone morphogenetic proteins signaling pathway can support self-renewal of mESCs in certain culture conditions rather than induction of differentiation *(20)*. Large-scale transcriptional profiling of hESCs using Affymetrix gene chips has recently been conducted to better understand the molecular identity that determines self-renewing hESCs. Approximately 900 genes are found significantly enriched in the undifferentiated state in hESCs *(21)*. Importantly, several molecular components integrated in major signal transduction pathways are specifically enriched in self-renewing hESCs indicating possible roles of specific signaling pathways in maintenance of pluripotency. Moreover, when compared with the reported mESC-enriched genes, roughly 200 genes overlap between hESCs and mESCs, suggesting that these common genes may be involved in core molecular networks regulating pluripotency conserved between the two species.

This chapter introduces the representative method to culture hESCs under the feeder and feeder-free conditions, the former of which is used to maintain or expand undifferentiated hESCs, and the latter can be used for preparation of pure hESCs RNA samples, or for screening factors influential on self-renewal of hESCs. Also described in this chapter: a protocol and tips for conducting gene chip analysis focusing on widely used Affymetrix microarrays. These techniques will provide an unprecedented scale of biological information to illuminate a key to decipher complex signaling networks controlling pluripotency.

2. Materials

2.1. Tissue Culture

1. Dulbecco's modified Eagle's medium (DMEM)/F-12 (Gibco, Carlsbad, CA; cat. no. 11320-032).

2. DMEM with D-glucose and L-glutamine, and pyridoxine hydrochloride, but no sodium pyruvate or sodium bicarbonate (Gibco; cat. no. 12100-046).
3. Dulbecco's phosphate-buffered saline (1X) without calcium/magnesium (Gibco; cat. no. 14190-14).
4. Knockout-DMEM (Gibco; cat. no. 10892-018).
5. Knockout serum replacement (Gibco; cat. no. 10828-028).
6. Fetal bovine serum (FBS) (HyClone, Logan, UT; cat. no. SH30070.03).
7. 2.0 m*M* L-glutamine (Gibco; cat. no. 25030-081).
8. 0.1 m*M* nonessential amino acids (Gibco; cat. no. 11140-050).
9. Human recombinant basic fibroblast growth factor (Gibco; cat. no. 13256-029).
10. 0.05% trypsin-EDTA (Gibco; cat. no. 25300-054).
11. Dispase, lyophilized (Gibco; cat. no. 17105-041).
12. Cell dissociation buffer (Sigma, St. Louis, MO; cat. no. G5914).
13. 55 m*M* (1000X) 2-mercaptoethanol (Gibco; cat. no. 21985-023).
14. Penicillin/streptomycin (Gibco; cat. no. 15070-063).
15. Gelatin, Type A (Sigma; cat. no. G-1890).
16. Matrigel (BD Biosciences, San Jose, CA; cat. no. 354234).
17. NYL filter unit, 500-mL (Nalgene, Rochester, NY; cat. no. 151-4020).
18. 0.2-μm syringe filter (Gelman, East Hills, NY; cat. no. 4612).
19. Falcon Multiwell six-well tissue culture-treated plate (BD Biosciences; cat. no. 353046).
20. 10 × 20-mm cell culture dish (Corning, Corning, NY; cat. no. 430167).
21. Falcon 14-mL polypropylene round bottom tube (BD Biosciences; cat. no. 352059).
22. Falcon 50-mL polypropylene conical tube (BD Biosciences; cat. no. 352098).

2.1.1. hESC Lines

Nineteen hESCs lines are eligible for federal funding of hESCs research (*see* **Note 1**). Our research has primarily used the H1 (WiCell Research Institute, Madison, Wisconsin) and BGN01 or BGN02 (BresaGen, Inc., Thebarton, Australia) lines because they were one of the few available and suitable for the feeder-free culture system. The focus is therefore on the culture method for these hESCs lines.

2.1.2. Mouse Embryonic Fibroblasts

A large stock of mouse embryonic fibroblasts (MEFs) can be prepared from E13 or E14 mouse embryos according to the generally used protocol. MEFs must be mitotically inactivated by irradiation or mitomycin C treatment before use for feeder cells (*see* **Note 2**).

2.1.3. Media

1. For H1 cell line (WiCell Research Institute): DMEM/F-12 supplemented with 20% KSR, 1.0 m*M* L-glutamine, 0.1 m*M* nonessential amino acids, 50 U/mL penicillin/streptomycin (*see* **Note 3**), 0.1 m*M* 2-mercaptoethanol, and 4 ng/mL of

basic FGF. For 1 L: mix 775 mL DMEM/F-12, 200 mL KSR, 10 mL L-glutamine, 10 mL nonessential amino acids, 5 mL penicillin/streptomycin, 100 µL 2-mercapotethanol, and 4 µg basic FGF.
2. For BGN01 or 02 line (BresaGen, Inc.): use 5% KSR and 15% FBS instead of 20% KSR used for the media for H1 cell line. For 1 L: mix 775 mL DMEM/F-12, 50 mL KSR, 150 mL FBS, 10 mL L-glutamine, 10 mL nonessential amino acids, 5 mL penicillin/streptomycin, 100 µL 2-mercapotethanol, and 4 µg basic FGF.
3. For MEFs: use DMEM supplemented with 10% FBS, and 100 U/mL of penicillin/streptomycin. For 1 L: mix 890 mL DMEM, 100 mL FBS, and 10 mL penicillin/streptomycin.

2.1.4. Dispase Solution

We use Dispase for the passaging of H1 cells. Alternatively, collagenase can be also used as WiCell recommends in their protocol (*see* **Note 4**).

1. Weigh appropriate amount of Dispase and dissolve in hESCs (H1) medium to make a final concentration of 6 U/mL.
2. If necessary, warm up the solution in a 37°C water bath to fully dissolve Dispase.
3. Filter the Dispase solution through 0.2-µm syringe filter (*see* **Note 5**).

2.1.5. Equipment for Embryonic Stem Cell Culture

We use regular cell culture equipment for hESCs and mESCs culture and separate CO_2 incubators for maintenance of hESCs, mESCs, or other general cell cultures. All procedures should be performed following general sterile tissue culture techniques (*see* **Note 6**).

1. Tissue culture hood.
2. CO_2 incubator.
3. 37°C water bath.
4. –70°C freezer.
5. –20°C freezer.
6. 4°C refrigerator.
7. Liquid nitrogen tank.
8. Centrifuge.
9. Inverted microscope.
10. Freezing container.

2.2. GeneChip

There are many different types of high-throughput gene array systems. We use Affymetrix microarrays because of their highly organized and reliable basic structures and interchangeable flexible format. Most of the reagents are available from Affymetrix (*see* **Note 7**).

1. SuperScript II First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA; cat. no. 11904-018).

2. SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen; cat. no. 11917-020).
3. T7-Oligo(dT) Promoter Primer Kit (Affymetrix, Santa Clara, CA; cat. no. 900375).
4. Control Oligo B2 (3nM) (Affymetrix; cat. no. 900301).
5. Eukaryotic Hybridization Control Kit (Affymetrix; cat. no. 900454).
6. Enzo BioArray RNA Transcript Labeling Kit (Affymetrix; cat. no. 900182).
7. Qiagen RNeasy Mini Kit (50) (Qiagen, Valencia, CA; cat. no. 74104).
8. Human Genome U133 array (HG-U133 2.0 Plus; Affymetrix; cat. no. 900466).
9. Hybridization Oven 640 (Affymetrix; cat. no. 800138).
10. Fluidics Station 450 (Affymetrix; cat. no. 00-0079).
11. GeneChip Scanner 3000 System (Affymetrix; cat. no. 00-0087).
12. Microarray Suite 5.1 (Affymetrix; cat. no. 690025).

3. Methods

3.1. hESC Culture

hESCs can be grown on either conventional MEFs feeder cells or Matrigel under the feeder-free condition. When compared with hESCs grown on MEFs feeder cells, hESCs may not be easily kept undifferentiated under the feeder-free condition because several factors, including the condition of Matrigel coating and MEFs-conditioned medium, would substantially affect self-renewal of hESCs. We therefore recommend that a primary batch of hESCs (in a cryovial tube sent from the provider) should be grown on MEFs feeder cells during initial several passages until a large enough number of frozen stocks in lower passages is prepared (*see* **Note 8**).

3.1.1. Cultivation of MEFs as Feeder Cells for hESCs

1. Thaw an MMC-treated frozen vial of MEFs in a 37°C water bath by gently swirling.
2. Plate cells on 0.1% gelatin-coated dishes at 2×10^4 cells/cm^2 in MEFs medium.
3. Twenty-four hours later, rinse MEFs with hESCs medium once to remove residual MEFs medium before plating hESCs.

*3.1.2. Cultivation of hESCs on MEFs Feeder Cells (*see **Note 9***)*

1. Thaw a frozen vial of hESCs in a 37°C water bath by gentle agitation before the last trace of ice melts (*see* **Note 10**).
2. Wipe the outside of the vial with 70% ethanol and immediately transfer the content to a 15-mL of conical tube filled with 10 mL of hESCs medium.
3. Gently pipet the cells up and down no more than five times.
4. Centrifuge the collected cells at 200*g* for 5 min.
5. Aspirate the supernatant, and gently flick the bottom of the centrifuge tube to dissociate the cell pellet.
6. Add an appropriate amount of hESCs medium (feeder-layer culture) or conditioned media (CM) (feeder-free culture) and resuspend the cells by gently pipetting.
7. Plate hESCs into culture vessels at the optimal concentration.

3.1.3. Passaging

We passage hESCs at between 7 and 12 d after initiation of culture before they start reaching confluency or showing any signs of differentiation.

1. Aspirate culture medium.
2. Add 1 mL of Dispase solution (for H1 line) or cell dissociation buffer (for BGN line) to hESCs in one well of a six-well plate.
3. Incubate the culture plate at 37°C (incubator) for 5–10 min (Dispase) or 3–5 min (cell dissociation buffer) (*see* **Note 11**).
4. Carefully observe hESCs under the inverted microscope periodically to see if they start detaching from the surface.
5. Remove hESCs from the culture surface by pipetting them using P1000 pipetman (*see* **Note 12**) or scraping them using 5 mL plastic or glass pipet (*see* **Note 13**).
6. Transfer hESCs into hESCs medium (more than 6 times' volume of Dispase solution used at **step 2** (e.g., 18 mL of hESCs medium for 3 mL of Dispase solution) and briefly pipet them to fully dilute the Dispase solution.
7. Centrifuge at 800*g* for 5 min.
8. Aspirate supernatant.
9. Briefly tap the bottom of the tube to gently dissociate hESCs pellet.
10. Resuspend hESCs in appropriate amount of hESCs medium (e.g., 2–2.5 mL for one well of a six-well plate).
11. Aspirate hESCs medium from MEFs-coated plates.
12. Plate hESCs onto MEFs-coated plates.

3.1.4 Freezing hESCs

When hESCs reach subconfluency in the culture plates, cells can be harvested and stored at –80°C.

1. Harvest hESCs according to the protocol shown in the passaging section.
2. After washing cells with hESCs medium two times, resuspend the pellet with the freezing medium consisting of 10% DMSO, 30% FBS, and 60% hESCs medium.
3. Add 1 mL of cell suspension to each cryovial tube.
4. Freeze cryovial tubes in a freezing container at –70°C overnight.
5. The next day, place the frozen cryovial tubes in the liquid nitrogen tank.

3.1.5. Cultivation of hESCs in the Feeder-Free Condition

The recently reported feeder-free system has provided a novel way to culture hESCs in the absence of direct contact with MEFs *(22)*. In this system, hESCs are maintained in the undifferentiated state by growing on a Matrigel-coated plate in medium-conditioned from MEFs (CM). This method is characterized by two major advantages as compared to the conventional MEFs-feeder method. First, this method provides pure, undifferentiated hESCs RNA or protein samples without any contaminated MEFs-derived material. This is particularly

critical when hESCs are subjected to gene chip analysis. This method is highly sensitive, potentially detecting any contaminated RNA samples whose sequences are similar to those in human.

The second advantage is that because the undifferentiated state of hESCs largely relies on CM, this system allows the identification of factors involved in maintenance of self-renewal of hESCs. Under the feeder-free condition, hESCs change their morphology from tight and compact undifferentiated shape to the flattened differentiated shape as early as 1 d after switching from CM to non-CM (*see* **Fig. 1**). Thus soluble factors that support self-renewal of hESCs can be screened simply through addition of candidate factors to non-CM in this system. The drawback of this system is, however, that several variable factors such as the quality of Matrigel and CM could substantially influence the undifferentiated condition of hESCs. Moreover, recent reports and personal communications suggest that hESCs passaged under the feeder-free condition are prone to be accompanied with chromosomal abnormality at relatively lower passages than that of hESCs grown on MEFs feeders (*see* **Note 14**) ***(23)***. It is therefore recommended to use this system for evaluation of undifferentiated hESCs in the experiment within several passages, rather than for preparation of master stocks or routine maintenance of hESCs. If a longer passaging under the feeder-free condition is required, regular karyotyping is encouraged to ensure the quality of hESCs. An example of the culture plan of hESCs under the feeder-free condition is shown below (*see* **Note 15**).

1. Day 1: plate MEFs on gelatin-coated dishes. Thaw Matrigel on ice overnight.
2. Day 2: replace MEFs medium with hESCs medium. Dilute Matrigel with DMEM/F-12, coat dishes with diluted Matrigel, and leave them on ice overnight.
3. Day 3: collect CM from MEFs culture. Rinse Matrigel-coated plates with DMEM/F-12 once. Thaw a frozen vial of hESCs and plate them on Matrigel-coated plates along with CM.

3.1.6. Preparation of CM From MEFs

MEFs are cultured for up to 7–8 d for preparation of CM. Beyond this period, activity of CM seems to be decreased, as judged by the ability to keep hESCs undifferentiated. It could be variable, however, depending on the batch and cell density of MEFs plated on the dish.

1. Coat six-well plates or 10-cm dishes with 0.1% gelatin for more than 20 min followed by rinsing with phosphate-buffered saline two times (*see* **Note 16**).
2. Plate MMC-treated MEFs on the gelatin-coated dishes at 1×10^5 cells/cm^2 in MEFs medium.
3. After overnight incubation, rinse MEFs with hESCs medium once (8 mL/dish) to remove residual MEFs medium and feed MEFs with 10–12 mL of hESCs medium.

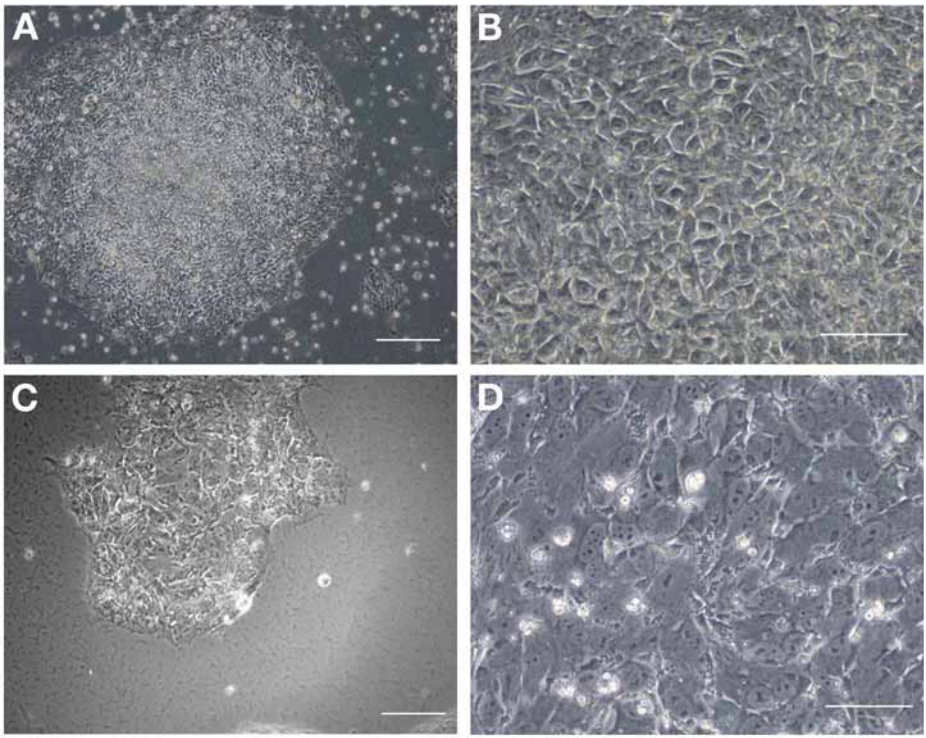

Fig. 1. Morphology of human embryonic stem cells (hESCs) (H1 line) cultured under the feeder-free condition. hESCs were grown in CM **(A**,**B)** or non-CM **(C**,**D)** for 2 d under the feeder-free condition and photographed by phase-contrast microscopy: **(A**,**C)** low magnification; **(B**,**D)** high magnification. Note compact and tight cell morphology representative of the undifferentiated hESCs **(A**,**B)** grown in CM. hESCs grown in non-CM show flattened and larger cytoplasm typical for differentiating hESCs **(C**,**D)**. Scale bars: **A**,**C**, 100 μm; **B**,**D**, 50 μm. (Please *see* the companion CD for the color version of this figure.)

4. Twenty-four hours later, recover the medium to use as MEF-conditioned medium (CM) (*see* **Note 17**).
5. Add additional 4 ng/mL of bFGF to CM before feeding hESCs.

3.1.7. Preparation of Matrigel-Coated Plates

This step is one of the most critical steps in preparation of the feeder-free culture of hESCs. As the condition of the coated-Matrigel significantly influences self-renewal of hESCs, one might need certain experience in growing hESCs on MEFs feeder cells to recognize if hESCs are in the good (undifferentiated) condition or not.

1. Thaw one vial (10 mL) of Matrigel stored at –20°C on ice overnight.
2. Aliquot 1 mL of Matrigel in each cryovial tube and store at –20°C (*see* **Note 18**).

3. One day before preparation of plates, thaw the frozen Matrigel aliquot on ice overnight.
4. Suspend 1 mL Matrigel in 20 mL of DMEM/F12 and plate on six-well plates at 1 mL per well (*see* **Note 19**).
5. Keep the Matrigel-coated plates on ice overnight while it is horizontally positioned so that each well is fully covered with Matrigel.
6. Before initiation of hESCs culture, incubate Matrigel-coated plates in the CO_2 incubator for more than 30 min to allow them to gelatinize.
7. Rinse the Matrigel-coated plates with DMEM/F-12 once to remove excessive Matrigel in each well (*see* **Note 20**).

3.1.8. Passaging of hESCs in the Feeder-Free Condition

hESCs are passaged at around 5–7 d after initiation of culture in the feeder-free condition. Beyond this period, hESCs at the margin or center of the colony start changing their morphology to form a flat differentiated shape.

All the passaging procedures are the same as that of hESCs grown on MEFs-feeder cells, with exceptions of using CM instead of hESCs medium when resuspending hESCs before plating, and using Matrigel-coated plates instead of MEFs-coated plates when plating hESCs (*see* **Note 21**).

3.1.9. Preparation of Frozen Stocks of hESCs in the Feeder-Free Condition

hESCs grown under the feeder-free condition can be stored in cryovials in the same way that is used for hESCs grown on MEFs-feeder cells.

3.2. GeneChip

Microarray approach has become one of the most powerful molecular techniques to comprehensively illuminate multiple transcriptional networks regulating complex biological phenomenon. Although most of the experimental processes are highly organized and even automated in the Affymetrix microarray system, failure to complete any given step (e.g., insufficient mixture of reagents) would substantially influence the final data quality. It is therefore quite essential to rigorously inspect the source samples (e.g., the initial sample RNA, cRNA after in vitro transcription) without any compromise before proceeding to the next step. This should be further emphasized to avoid wasting time on the data analysis step for low-quality, raw data. In-silico processes, including data extraction, filtration, and interpretation are generally far more time-consuming and labor-intensive work than wet-experimental steps (*see* **Note 22**). If one of the triplicate samples has less quality than the others, the entire resolution of the data analysis could be severely impinged through statistical filtration, resulting in the exclusion of many regulated genes. For these reasons, when the sample does not reach the highest standard, we repeat the experimental procedure until the sample quality meets the required level rather than proceeding to the next step.

3.2.1. Experimental Plan

Because the microarray project generally requires a considerable amount of time, effort, and budget, precise experimental planning is the primary key to succeed in the entire project. First, the number of conditions for comparison must be determined (e.g., hESCs vs adult-derived tissues, hESCs in the differentiation condition between different time points). The total number of arrays to be used can then be estimated by multiplying the number of conditions by the number of replicate per each condition. It should be noted that because array data are intrinsically accompanied with a certain level of signal variations between different samples even in the exact same condition, statistical filtration of the raw signal is inevitable to reliably select enriched genes while excluding potential noises. At minimum, triplicate samples in each condition are strongly recommended.

3.2.2. Preparation of Total RNA From hESCs

Extract RNA from hESCs grown in the triplicate condition using Qiagen RNeasy kit or other equivalent RNA extraction kits. As mentioned previously, the successful gene array experiment largely relies on the quality of the starting materials. The purity of the sample RNA should therefore not be compromised. Routine evaluation of the extracted RNA by ultraviolet spectrophotometer for protein contamination, and by the RNA Nano Lab chip for RNA degradation, as well as the use of only the highest quality of RNA sample is strongly recommended for the following procedure.

3.2.3 First-Strand cDNA Synthesis (Example: Total RNA 5.0–8.0 μg)

Use SuperScript II First-Strand Synthesis System for this step.

1. Mix X μL (5.0–8.0 μg, total volume (11 μL) of the sample RNA, 1 μL of T7-(dT)24 primer (100 pmol/μL), and (11-X) μL of DEPC-H_2O.
2. Incubate at 70°C for 10 min, and place on ice.
3. Add 1 μL of 10 m*M* dNTP mix, 2 μL of 0.1 *M* DTT, and 4 μL of 5X first-strand cDNA buffer to the mixture.
4. Incubate at 42°C for 2 min.
5. Add 1 μL of SuperScript II RT to the mixture, and incubate at 42°C for 1 h.

3.2.4. Second-Strand cDNA Synthesis

Use SuperScript Double-Stranded cDNA Synthesis Kit for this step.

1. Place the first-strand cDNA reaction on ice.
2. Add the following materials to the first-strand cDNA reaction: 91 μL DEPC-treated water; 30 μL 5X Second Strand Reaction Buffer; 3 μL 10 m*M* dNTP mix; 1 μL 10 U/μL *Escherichia coli* DNA ligase; 4 μL 10 U/μL *E. coli* DNA polymerase I; and 1 μL 2 U/ μL *E. coli* RNase H.

3. Mix the contents, and centrifuge the tube to spin down the condensation.
4. Incubate at 16°C for 2 h in a polymerase chain reaction machine or a cooling water bath.
5. Add 2 µL of T4 DNA polymerase to the mixture and incubate at 16°C for another 5 min.
6. Add 10 µL of 0.5 *M* EDTA to the tube and mix well to terminate the reaction. The second-strand cDNA reaction can be store at –20°C or subjected to the next step.

*3.2.5. Cleanup of Double-Stranded cDNA (*see ***Note 23****)*

1. Add 162 µL of Phenol:chloroform:isoamyl alcohol (25:24:1) to the second-strand cDNA reaction (162 µL), vortex quickly, and centrifuge at 12,000*g* for 5 min.
2. Transfer the aqueous phase to a fresh tube.
3. Add 0.5 vol of 7.5 *M* NH_4Ac and 2.5 vol of 100% cold (–20°C) ethanol to the sample, vortex roughly, and centrifuge at 12,000*g* for 20 min.
4. Wash the pellet with 500 µL of 80% ethanol at 12,000*g* for 5 min two times.
5. Air-dry the pellet or use speed vac for quick desiccation.
6. Resuspend the pellet in 12 µL of RNase-free water.

3.2.6. In Vitro Transcription

Use Enzo BioArray RNA Transcript Labeling Kit for this step.

1. Add the following reagents to 12 µL of the cleaned double-stranded cDNA sample in the indicated order: 10 µL of RNase-free water; 4 µL of HY Reaction Buffer (Vial 1); 4 µL of 10X Biotin Labeled Ribonucleotides (Vial 2); 4 µL of 10X DTT (Vial 3); 4 µL of 10X RNase Inhibitor Mix (Vial 4); and 2 µL of 20X T7 RNA polymerase (Vial 5) (total volume = 40 µL) (*see* **Note 24**).
2. Mix well and incubate at 37°C for 5 h while mixing the reaction every 30 min during the incubation period. The final reaction can be stored at –20°C or –70°C, or subjected to the next step.

3.2.7. Cleanup of In Vitro Transcription

Use Qiagen RNeasy kit for this step.

1. Add 60 µL of RNase-free water to the in vitro transcription (IVT) reaction (40 µL) in a 1.5-mL tube.
2. Add 350 µL of buffer RLT to the tube and mix thoroughly.
3. Add 250 µL of 100% ethanol to the sample and mix by pipetting.
4. Apply the entire content in the tube to an RNeasy mini column, and spin at 12,000*g* for 15 s.
5. Remove the column from the collection tube and place it on a new collection tube.
6. Add 500 µL of buffer RPE, and centrifuge at 12,000*g* for 15 s.
7. Discard flow through.
8. Add another 500 µL of buffer RPE, and centrifuge at 12,000*g* for 2 min.
9. Transfer the column to a new collection tube, and centrifuge at 12,000*g* for 1 min to remove any trace amount of buffer RPE.

10. Transfer the column to a new collection tube and add 40 µL of RNase-free water to the column.
11. Centrifuge the column at 12,000*g* for 1 min.
12. Take the filtered sample from the collection tube, and add it to the same column.
13. Centrifuge the column at 12,000*g* another 1 min to maximize the recovery of the sample.

3.2.8. Quantification of the IVT Sample (cRNA)

1. Quantify the amount of total RNA in the IVT sample by using spectrophotometer.
2. The quality of the RNA sample can be evaluated by RNA Nano Lab chip.
3. Determine the cRNA yield by subtracting the amount of total RNA used for the first-strand cDNA synthesis from the amount of RNA obtained after IVT using the following equation: $cRNA = RNA_{IVT} - RNA_{First} \times cDNA_{IVT/Total}$; where the amount of RNA in the IVT sample (RNA_{IVT}), the amount of total RNA used for first-strand cDNA synthesis (RNA_{First}), and the amount of cDNA used for IVT/amount of total double-stranded cDNA ($cDNA_{IVT/Total}$).

3.2.9. cRNA Fragmentation

1. Take 20–80 µg of cRNA (≤32 µL) from the purified IVT sample and put it in a 1.5-mL tube.
2. Add 8 µL of 5X Fragmentation buffer to the tube.
3. Add RNase-free water to make a final volume of 40 µL.
4. Incubate the reaction at 94°C for 35 min, and place it on ice.
5. The fragmented cRNA can be stored at –20°C.

3.2.10. Hybridization of the Probe Array

1. Prehybridize the probe array cartridge with 200 µL (for the standard cartridge) of 1X Hybridization buffer through a septa backside of the array by using a P200 pipetman. Avoid air contamination that may result in uneven prehybridization of the array.
2. Place the array cartridge in the hybridization oven at 45°C for 10 min with rotation.
3. Mix the following reagents in a 1.5-mL tube: 15 µg fragmented cRNA; 5 µL control oligonucleotide B2 (3 n*M*); 15 µL 20X Eukaryotic Hybridization Controls; 3 µL Herring sperm DNA (10 mg/mL); 3 µL acetylated BSA (50 mg/ mL); 150 µL 2X hybridization buffer; and X µL water to make a final volume of 300 µL.
4. Boil the hybridization mixture at 99°C for 5 min followed by incubation at 45°C for 5 min.
5. Centrifuge the hybridization cocktail at 12,000*g* for 5 min to separate insoluble materials at the bottom of the tube.
6. Remove the 1X hybridization buffer used for prehybridization from the array, and fill up with the hybridization cocktail, avoiding contamination of insoluble materials at the bottom of the tube.
7. Incubate the probe array in the hybridization oven at 45°C for 16 h with rotation at 60 rpm.

3.2.11. Array Data Analysis

1. After 16 h of hybridization, wash the probe array, stain, and scan to extract data from the array.
2. Analyze the raw data using Microarray Suite 5.0 (MAS5.0) (*see* **Note 25**).
3. To estimate the statistical significance of differential gene expression between two groups (e.g., hESCs in the undifferentiated state vs hESCs in the differentiated state) of three replicates each, we used a regularized t-statistics as described by Long and Baldi ***(24)***. All signal values were first log-transformed and the regularized variances were taken as $\sigma^2_{reg} = \frac{V_0\sigma_0^2 + (n-1)s^2}{V_0 + n - 2}$, independently for each group of replicates. We used s^2 is the sample variance ($n = 3$). σ_0^2 denotes the *a priori* expected variance for a gene, given its mean intensity, and is estimated from the data using an intensity-dependent regression of sample variance vs sample mean. The means and variances from both groups were used in the same regression.
4. For comparison of genes between different species (e.g., hESCs and mESCs), use NetAffx (www.netaffx.com) and TIGR database to search orthologous genes. It should be noted, however, that a certain number of genes in one species may not be able to correlate to the ortholog of other species due to still ongoing database construction.
5. After a group of genes that are distinctively expressed between two conditions are determined, the genes can be classified to subgroups by focusing on specific categories (e.g., gene function; Gene Ontology, protein domain, signaling pathways). In general, multiple different probes in the Affymetrix Gene Array can be corresponding to the same single gene. To determine how many distinct genes are included in the selected group, use NetAffx database to find out Unigene ID corresponding to each Probe ID so that overlapping genes are easily excluded. Validity of the selected group genes can be confirmed by evaluating whether known differentially regulated genes between the two conditions (e.g., *Oct-3/4* and *Nanog* in the undifferentiated state) are enriched in the selected gene pool (*see* **Note 26**) ***(21)***.

4. Notes

1. For more information regarding hESCs lines in the NIH Human Embryonic Stem Cell Registry, visit http://stemcells.nih.gov/registry.
2. Frozen vials of mitomycin C-treated or untreated MEFs can be available from several companies (e.g., Speciality Media).
3. The original recipe for H1 medium from the provider (WiCell Research Institute) does not contain any antibiotics. However, addition of penicillin/streptomycin to hESCs medium was not found to substantially affect any character of self-renewing H1 cells, and even beneficial to avoid risk of bacterial contamination. Because penicillin/streptomycin has no effect on fungi or mycoplasma, careful sterile culture procedure is always critical for each culture step.

4. Add collagenase to hESCs medium to make a final concentration of 1 mg/mL, and dissolve in a 37°C water bath. Filter the collagenase solution using 0.2-μm syringe filter.
5. It is recommended to prepare fresh Dispase solution for each passaging procedure. Alternatively, the solution can be kept at 4°C for several days.
6. It is quite important that the operator wear gloves and glasses during cell culture procedures to prevent any possible cell culture-related troubles (e.g., virus infection, explosion of a frozen vial after thawing).
7. Although we used Human Genome U133A and U133B chips that were separated, a combined gene array, Human Genome U133 2.0 Plus, that covers entire human genome in one array is currently available.
8. It is of note that, because chromosomal abnormality is reported to be occasionally found in high passaged hESCs in either culture method, regular karyotyping is recommended to ensure the condition of hESCs especially at higher passages *(23)*.
9. It is recommended that researchers working on hESCs for the first time start by growing hESCs on MEFs feeder cells rather than using the feeder-free system because of the latter's complexity. Experience in growing mESCs is beneficial but not absolutely required, because maintenance of hESCs is far quirkier than that of mESCs. Special attention must be paid at each process, and careful daily observation of hESCs through the inverted microscope is essential to get used to maintaining hESCs in the best condition.
10. It is particularly important to thaw the frozen vial as quickly as possible to minimize damage to the cells. Usually it is completed within 1–2 min.
11. Ten minutes can be considered the maximum incubation period for Dispase. Beyond this time, viability and quality (undifferentiated state) of hESCs after passaging is drastically decreased. For BGN cells, use cell dissociation buffer as the provider (BresaGen) recommends. Cell dissociation buffer's dissociation activity appears to be stronger than that of Dispase solution. Most of cases, cell dissociation is completed within 5 min. We also tested cell dissociation buffer or trypsin for passaging H1 cells. We found, however, that H1 cells dissociated by these reagents were not kept in as good condition as H1 cells treated with Dispase, although the exact reason remained unclear.
12. Although hESCs sometimes tightly adhere to the culture surface and are refractory to detach from the surface, the pipetting procedure should be completed in the shortest period to minimize the time in which hESCs are exposed to Dispase solution. If necessary, we even leave a part of hESCs colony unharvested to prioritize quality over quantity.
13. It is usually recommended that hESC colonies should be kept as large as possible when being passaged to maintain their viability and undifferentiated state.
14. Although the exact reason is unclear, it could be due to selected growth of certain cell populations that acquired growth advantage through the chromosomal rearrangement under relatively higher selective pressure driven by the feeder-free condition.
15. It usually takes two to three passages until hESCs become free from contaminated MEFs in the feeder-free condition.

16. The gelatin solution supports the attachment of MEFs on culture vessels. This treatment is especially important when MEFs are fed with hESCs (for H1) medium that induces a substantial morphological change in MEFs.
17. CM can be used immediately after collection or can be stored at –80°C for several weeks.
18. Cryotubes should be prefrozen at –20°C before making aliquots to avoid gelation of Matrigel. Pipetting and aliquoting procedures should be done as quickly as possible to keep Matrigel cold.
19. Property of Matrigel appears to be slightly different from batch to batch. It is therefore quite important to predetermine the optimal concentration of Matrigel for each batch.
20. Optimally coated Matrigel demonstrates a well-formed meshlike structure under the inverted light microscope. When the coated Matrigel is too diluted, the meshlike structure is less formed, resulting in flattening and differentiation of hESCs on the Matrigel even in the presence of CM. If the coated Matrigel is too dense, Matrigel tends to remain aggregated even after rinsing with DMEM/F-12, and prevents hESCs from attaching and spreading on the surface.
21. Importantly, when evaluating factors involved in the undifferentiated state in the screening process, it is beneficial to dissociate hESC colonies into small aggregates because smaller colonies are more sensitive to the differentiation environment than larger ones which are resistant to the differentiation signal.
22. There are several ways to computationally adjust the obtained data to fit representative profiling. There is, however, no way to make up for fundamental blemish at the raw data level.
23. A satisfying result can be obtained without using Phase Lock Gel (PLG) that Affymetrix recommends for the cleanup process. However, because PLG appears to help efficient recovery of the sample and avoid contamination, it would be beneficial for the cleanup step.
24. During the procedure, the reagents should be kept at room temperature to avoid precipitation of DTT.
25. The quality of the obtained data can be briefly evaluated by visual inspection of Chip intensity data or checking out the several parameters in the Chip Report generated by MAS5.0. Scaling factor is usually between 1.0 and 2.0. Internal positive and negative controls and 3′/5′ ratio also help to determine the condition of the array data.
26. It is also strongly recommended to examine expression of the selected genes by real-time reverse transcription polymerase chain reaction to confirm differential transcriptional regulation of the enriched genes between the two conditions.

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19

Massively Parallel Signature Sequencing

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Summary

Massively parallel signature sequencing is an ultra-high throughput sequencing technology. It can simultaneously sequence millions of sequence tags, and, therefore, is ideal for whole genome analysis. When applied to expression profiling, it reveals almost every transcript in the sample and provides its accurate expression level. This chapter describes the technology and its application in establishing stem cell transcriptome databases.

Key Words: Massively parallel signature sequencing; gene expression; stem cell; transcriptome.

1. Introduction

Stem cells and progenitor cells are present throughout development and persist in the adult *(1)*. Multiple classes of stem cells have been identified which differ from each other in their differentiation abilities, their cytokine responses, and their surface antigen characteristics *(2)*. Surprisingly, even well-characterized cells that initially appear to be identical change as they are maintained in culture. Similarly, their in vivo counterparts change when they mature and age. Thus the properties of stem cells at any given instant are a complex interplay between development, aging, and response to environmental conditions. These observations highlight the importance of carefully checking the properties of freshly harvested cells and monitoring them over time in culture. In addition, when analyzing cells, it is desirable to use a reliable and reproducible method that is cost-effective and sensitive enough to detect with high fidelity the global

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differences among the populations of cells, as well as the subtle differences introduced as they are propagated in culture or as they mature.

Analytical methods such as reverse transcription polymerase chain reaction (RT-PCR), immunocytochemistry and enzyme-linked immunosorbent assay offer both the sensitivity and reproducibility required, but unfortunately only a limited number of markers can be monitored routinely and it is difficult to determine *a priori* which markers will best serve to distinguish the states of cells. Large-scale methods such as focused microarrays, whole genome microarrays, expressed sequence tag (EST) sequencing, serial analysis of gene expression (SAGE), and massively parallel signature sequencing (MPSS) offer a range of advantages and weaknesses detailed next.

DNA microarrays allow simultaneous assessment of the expression of thousands of genes ***(3)***. However, one should be aware of the technical limitations associated with hybridization assays including the following:

1. Coverage: limited to RNA elements represented by DNA sequences on the array.
2. Reproducibility: data cannot be easily compared between microarray and reagent batches or different operators and laboratories.
3. Sensitivity: DNA array features always give a measurable signal whether or not RNA corresponding to that feature is present in the sample; thus the lower limit of detection is limited, typically to a few RNA copies per cell.
4. Dynamic range: any given array element or set of elements representing a transcript can provide a reasonably linear response between a few copies per cell and a few thousand; however, lower levels are obscured by the background signal and higher levels cannot be measured because of signal saturation. Note that functional RNAs are commonly present at low copy number, 50% of the transcripts in a typical cell are present at less than 10 transcripts per million (tpm), or around three copies per cell and variation (not measurable on microarrays because of saturation) in the tens to hundreds of thousands per million are also often biologically important.
5. Cross-hybridization: a confounding issue, even for short oligonucleotide array elements of 25 nucleotides, many researchers lump cross-hybridization together with background noise; however, such misleading signals often result from quite specific interactions with unintended (and frequently difficult to identify) transcripts.

These issues, coupled with the complexity of the data, limit the utility of microarrays for comprehensive surveys of gene expression.

EST analysis includes conventional capillary sequencing, SAGE, and MPSS. Sequences of as little as 14 bases are sufficient to uniquely identify 90% of human transcripts. Because EST analysis provides expression levels by counting the number of mRNA molecules in the sample for any given gene, the expression levels are inherently more quantitative than those obtained by hybridization assays. In general, EST analysis can be more reproducible,

sensitive, and accurate provided that a sufficiently large number of molecules are counted to achieve adequate statistical significance.

Wide adoption of EST analysis has been limited by cost and processing time. Recently, Lynx Therapeutics Inc., which developed MPSS for commercial production in 1999, has in the intervening years improved the methodology to obtain 20 basepair sequences from each mRNA molecule while reducing the cost and the amount of required RNA. These improvements have made it practical for individual laboratories to generate comprehensive, stable gene expression databases. MPSS has been applied to a wide range of species including many plants, oysters, fungal and protozoan pathogens, and humans and many of the common model organisms *(4–9)*. In this chapter, we describe the MPSS process and how it has been used to generate comprehensive, bankable gene expression data for stem cells from many sources in a variety of experimental conditions.

2. Materials

2.1. Cell Culture

2.1.1. Cell Culture Reagents

1. Dulbecco's modified Eagle's medium/Ham's (DMEM) (Invitrogen, Carlsbad, CA; cat. no. 1195073).
2. DMEM/F12 (Invitrogen; cat. no. 11330032).
3. Minimum essential medium alpha-medium (MEM) (Invitrogen; cat. no. 32571036).
4. 1X Phosphate-buffered saline (Invitrogen; cat. no. 14190144).
5. Fetal bovine serum (Hyclone, Logan, UT; cat. no. SH30071.03).
6. Knockout serum replacement (Invitrogen; cat. no. 10828028).
7. Penicillin-streptomycin (100X) (Invitrogen; cat. no. 15140122).
8. Non-essential amino acids (Invitrogen; cat. no. 11140050).
9. L-glutamine (Invitrogen; cat. no. 25030081).
10. β-mercaptoethanol (Specialty Media, Phillipsburg, NJ; cat. no. ES-007-E).
11. Basic fibroblast growth factor (bFGF) (Sigma, St. Louis, MO; cat. no. F0291).
12. Trypsin-ethylenediaminetetraacetic acid (EDTA): 0.05% trypsin, 0.53 m*M* EDTA (Invitrogen; cat. no. 25300054).
13. Collagenase IV (Invitrogen; cat. no. 17104-019).
14. Fibronectin, human (Invitrogen; cat. no. 33016-015).

2.1.2. Cells

1. hES cell line BG02 is obtained from BresaGen Inc. (Athens, GA).
2. H1, H7, H9, and H14 were obtained from Wicell (Madison, WI) and Geron (Menlo Park, CA).
3. MEF feeder cells from strain SVB are obtained from Specialty Media.

2.2. RNA Extraction

1. RNA STAT-60 (Tel-Test Inc., Friendswood, TX; cat. no. CS-110).
2. Chloroform (Sigma; cat. no. C-2432).
3. Isopropanol (Fisher, Hanover Park, IL; cat. no. A416-500).
4. Ethanol.
5. DEPC-treated water.

2.3. RNA Quality Assessment and mRNA Purification

1. Agilent Bioanalyzer (Agilent Technology, Palo Alto, CA; model no. 2100 Bioanalyzer).
2. DNA-free kit (Ambion, Austin, TX; cat. no. 1906).
3. Poly(A) Purist mRNA Purification Kit (Ambion; cat. no. 1916).
4. Ribogreen RNA Quantitation Kit (Molecular Probes, Eugene, OR; cat. no. R-11490).

2.4. MPSS Library Construction and Beadloading

1. Biotinylated oligodT primer (Lynx Therapeutics Inc., Hayward, CA).
2. SuperScript II reverse transcriptase (Invitrogen; cat. no. 18064-022).
3. dNTP mix (Amersham Pharmacia, Piscataway, NJ; cat. no. 27-2035-03).
4. DNA polymerase I (Invitrogen; cat. no. 18010-025).
5. Ribonuclease H (Invitrogen; cat. no. 18021-071).
6. Dpn II (New England Biolabs, Beverly, MA; cat. no. R0543B).
7. 3′ adapter (Lynx Therapeutics Inc.).
8. Klentaq Polymerase (Clontech, Palo Alto, CA; cat. no. 639108).
9. Pac I (New England Biolabs; cat. no. R0547L).
10. T7 Exonuclease (New England Biolabs; cat. no. M0263S).
11. DNA Klenow Polymerase (New England Biolabs; cat. no. M0212M).
12. Tween-20 (VWR, West Chester, PA; cat. no. MKH28501).
13. T4 DNA ligase (New England Biolabs; cat. no. M0202B-LX).
14. ATP (Amersham Pharmacia; cat. no. 27-2056-99).
15. Mme I (New England Biolabs; cat. no. R0637L).
16. MoFlo high-speed cell sorter (Dako-Cytomation, Inc., Fort Collins, CO).

2.5. MPSS

1. Dpn II (New England Biolabs; cat. no. R0543B).
2. Tween-20 (VWR; cat. no. MKH28501).
3. T4 DNA ligase (New England Biolabs; cat. no. M0202B-LX).
4. T4 Polynucleotide Kinase (New England Biolabs; cat. no. M0201B).
5. ATP (Amersham Pharmacia; cat. no. 27-2056-99).
6. Bbv I (New England Biolabs; cat. no. R0173B).
7. 1024 Encoder adaptors (Lynx Therapeutics Inc; part no. 330-101-003).
8. 16 Decoder probes (Lynx Therapeutics Inc; part no. 330-201).

3. Methods

3.1. Preparation for MPSS

3.1.1. Cell Culture

1. Cells are grown according to the feeder-free cell culture protocols described by Geron Inc. (*[10]* www.geron.com) to reduce the possibility of feeder cell contamination (*see* **Note 1** and Chapter 13).
2. On confluence, incubate cells in collagenase IV at 200 U/mL for 20 min while being monitored to assess the separation of the colonies from the substrate.
3. After the colonies appear to separate, gently agitate the dishes and aspirate the separated colonies into a separate container.
4. Fix a sister aliquot of cells to be used to assess the state of embryonic stem (ES) cells by immunocytochemistry with markers previously described *(11)*.

3.1.2. RNA Extraction

1. Collect undifferentiated cells as previously described.
2. Transfer cells to a 15-mL tube and centrifuge at 270*g* for 5 min.
3. Remove the supernatant and resuspend pellet in 1 mL of RNA-STAT 60 (for 10^6–10^7 cells).
4. Pipet up and down to lyse cells.
5. Transfer the lysates into a 1.5-mL tube and incubate 5 min on ice.
6. Add 200 µL of chloroform and gently mix by inversion six to eight times. Incubate 3 min on ice.
7. Centrifuge at full speed for 15 min.
8. Transfer aqueous phase to a new 1.5-mL tube.
9. Add 500 µL of isopropanol, vortex, and incubate 10 min on ice.
10. Centrifuge at full speed for 15 min.
11. Wash twice with 70% ETOH. Air-dry the pellet and resuspend in 50 µL DEPC-treated water (*see* **Note 2**).

3.1.3. RNA Quality Assessment and mRNA Purification

An aliquot of the RNA is used for test RT-PCR to assess expression of known markers, absence of markers of differentiation and absence of feeder contamination (*see* **Note 3**).

1. Run RNA on a gel to assess the quality.
2. After the quality of RNA and purity of the cells is considered acceptable, ship the RNA to Lynx for further processing.
3. The quality of total RNA is further assessed by an Agilent Bioanalyzer at Lynx Therapeutics, Inc., based on the ratio of 18S vs 28S rRNA and the distribution of RNA sizes.
4. The RNA that passes the quality assessment is treated by DNase to remove DNA using the DNA-free kit according to the protocol provided by the manufacturer.

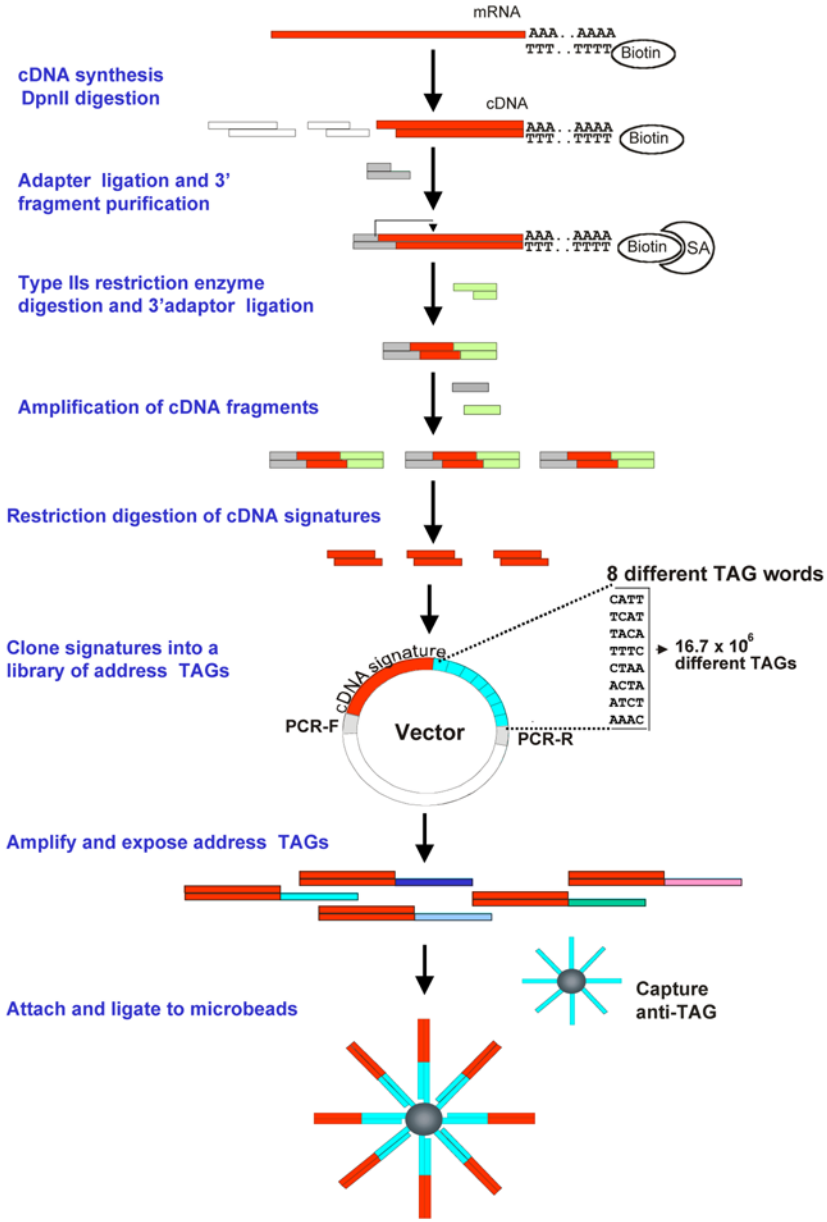

Fig. 1. Preparation of cDNA loaded microbeads. ES cell poly(A+) mRNA is converted into double-stranded cDNA, which is digested with Dpn II, followed by capture of the 3′-most DpnII fragments. These are converted to 20 base inserts flanked by the adapters to allow cloning into a plasmid containing a 32-basepair oligonucleotide tag. Each cDNA clone is associated with one of 16.7 million different 32 base tag sequences. The cDNA inserts, along with their associated tags, are amplified by poly-

5. Polyadenylated RNA is isolated from the DNase-treated RNA using the Poly(A) Purist mRNA Purification Kit according to the manufacturer's instructions. The mRNA is assessed qualitatively using an Agilent Bioanalyzer. Quantification of the mRNA is done either by an Agilent Bioanalyzer or by Ribogreen RNA Quantitation Kit.

3.1.4. MPSS Library Construction and Beadloading

1. The MPSS process at Lynx (**Fig. 1**) begins with the cloning of a fragment of every mRNA molecule in a sample onto the surfaces of 5-μm beads. Double-stranded cDNA is prepared from the 100 ng of the mRNA sample, using a standard protocol for reverse transcription (primed by biotinylated oligodT) followed by second-strand cDNA synthesis.
2. The cDNA is then digested with the restriction enzyme Dpn II (recognition sequence GATC) and purified on Streptavidin beads.
3. The 5′ termini of the affinity-purified 3′ end fragments (Dpn II to poly-A) are ligated to an adapter containing a type IIs restriction enzyme site. Subsequent cleavage with the type IIs restriction enzyme, Mme I generates a DNA fragment containing the adapter and a constant-length signature of 20 or 21 basepairs.
4. The 3′ ends of these fragments are then ligated to a second adapter and directionally cloned into the signature cloning vector, a complex mixture of vectors each containing one of the 16.7 million different Combi tags, to generate the signature library (*see* **Note 4**).
5. The resulting signature library is titered to determine the complexity.
6. From an aliquot of the library containing 1.28 million signatures, each unique tag-signature complex is PCR-amplified using a fluorescently labeled oligonucleotide. The Combi tag portion of each complex is rendered single stranded by treatment with T4 polymerase, and then hybridized to collection of microbeads that bear complementary Combi tags on their surface. Following hybridization, each loaded bead is decorated with approx 100,000 identical copies of a specific signature DNA fragments (*see* **Note 5**). Each cDNA clone is associated with one of 16.7 million different 32 base tag sequences.
7. The subset of microbeads that have captured complementary DNA fragments (i.e., beads that are loaded) are physically separated from the unloaded beads by fluorescence-activated cell sorting using a MoFlo high-speed cell sorter (*see* **Note 6**). The purified microbeads bearing signature DNA fragments are then loaded into flow cells for downstream MPSS analysis.

Fig. 1. (Continued)

merase chain reaction, and the resulting amplicons are treated with an exonuclease to render the tag portions single stranded. The tagged cDNAs are hybridized to 32-base complementary tags that are covalently linked to the microbeads. For every tag on a cDNA molecule, there is one bead with the complementary anti-tag. After loading, the tagged cDNAs are then ligated enzymatically to yield a microbead with approx 100,000 identical molecules covalently attached to the surface.

3.2. MPSS

1. MPSS technology enables the simultaneous sequencing of 20 base signatures from the cDNAs hybridized to every microbead. This is initiated by immobilizing one million or more microbeads in a monolayer array in a microfluidic flow cell for carrying out the sequencing biochemistry and imaging processes (*see* **Note 7**).
2. Sequencing is initiated by ligation of an adapter molecule to the GATC (*Dpn*II) single stranded overhang. The adaptor contains a *Bbv*I restriction site; *Bbv*I, a type IIs restriction enzyme, cuts the DNA asymmetrically at positions 13 (5′) and 9 (3′) nucleotides away from the recognition site. This produces DNA strands on the bead with four-base single-stranded overhangs immediately adjacent to the *Dpn*II site (*see* **Fig. 2**).
3. To determine which bases were revealed by the enzymatic cleavage, a set of encoded adapters are ligated to the overhang. Encoded adapters contain all possible combinations of a four-base single-stranded overhang at one end, a single-stranded decoding sequence at the other end and an internal *Bbv*I recognition site. Four types of encoded adapters are ligated to each bead. Each of them bears a common sequence complementary to the overhang but differs by the unique decoder sequence used to identify one base of the four-base overhang (*see* **Note 8**).
4. The identity of the ligated encoded adapter is then revealed by probing the decoding region sequentially with 16 fluorescently labeled decoder probes. Knowing the identity of the encoded adapter, thus, yields the identity of the four-base overhang in the signature.
5. To collect additional sequence information, the cycle is repeated by cleavage with *Bbv*I, which removes the first encoding adapter, and reveals the next four-base overhang for subsequent identification.
6. Samples are sequenced in two frames (called steppers) by the use of initiating adapters in which the restriction enzyme recognition site is offset by two bases (*see* **Note 9**).

3.3. MPSS Technology

3.3.1. MPSS Technology Overview

MPSS is a robust, high-throughput method for the cloning, identification, and quantification of all expressed transcripts in a cell. This unique and proprietary technology, invented by Sydney Brenner ***(12–14)***, has been established as one of the most powerful methods for identifying polyadenylated transcripts (and recently nonpolyadenylated RNAs such as micro and small interfering RNAs), and provides a complete, accurate, and permanent digital record of every mRNA molecule in the cell.

The performance characteristics of MPSS make it ideally suited for comprehensive genome-wide transcriptional profiling. MPSS captures and sequences a 20-basepair signature including and adjacent to the 3′-most DpnII restriction site in cDNA reverse-transcribed from its corresponding mRNA. It thus surveys

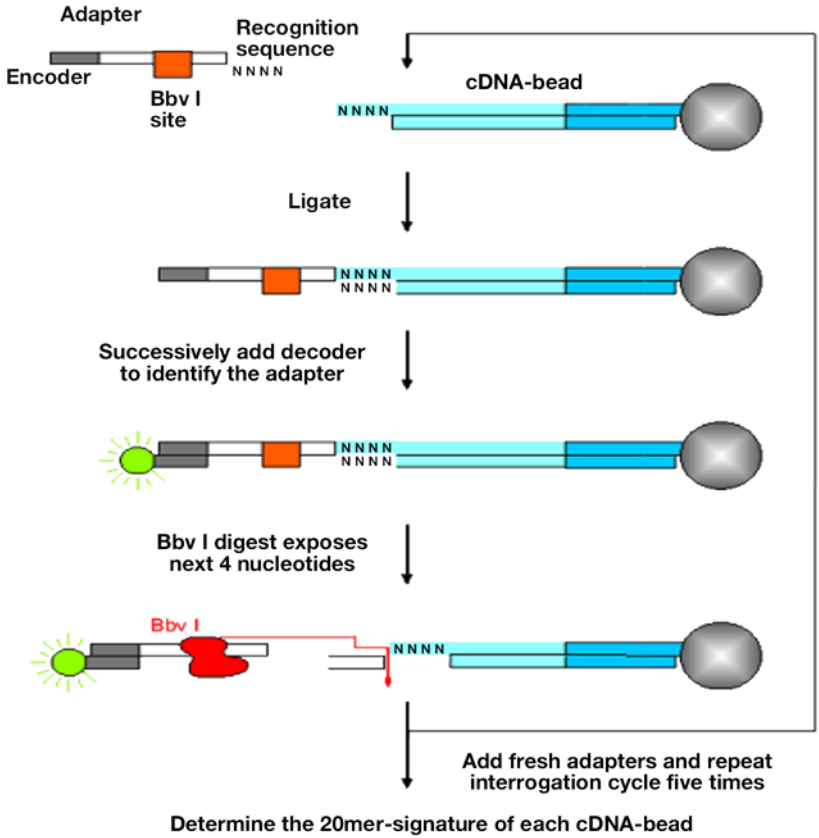

Fig. 2. Sequencing by adapter ligation cycling. Encoded adapters are ligated to the four base single-stranded overhangs at the end of the cDNAs attached to microbeads. Sixteen different fluorescently labeled decoder probes are sequentially hybridized to the ends of the encoded adapters to identify the sequence of the exposed four bases. The encoded adapter from the first round is then removed by digestion with BbvI, which exposes the next four nucleotides as a four-base single-stranded overhang. Repetition of the process yields up to 20 bases of sequence.

virtually all mRNAs in a sample, and provides direct sequence-based identification of transcripts. Because prior knowledge of sequence information is not required, the method is valuable for all organisms, including those with incompletely characterized genomes. MPSS determines the absolute level of every gene expressed in a sample by counting the number of individual mRNA molecules present. Transcript abundance measured in this digital manner provides an ideal format for comparing expression profiles across experiments and tissues.

A typical MPSS expression analysis counts more than one million transcripts per sample. As a result, the method is highly sensitive, with a large dynamic range, permitting the identification of high abundance transcripts, as well as transcripts present at levels below a single copy per cell. Accurate transcript measurement requires this depth of analysis because a typical mammalian cell contains more than 300,000 mRNA molecules with many, including critical regulatory molecules being expressed at only a few copies per cell. Heterogeneous samples such as ES cell cultures and complex tissues further exacerbate this problem necessitating the accurate measurement of transcripts that, on average, are present at less than one copy per cell.

The protocol for analysis involves obtaining a purified sample of cells, harvesting high-quality RNA, preparing messenger RNA, and subsequently a MPSS cDNA library that is loaded onto beads for sequencing. Sequencing results are then mapped to the transcriptome and the data are summarized for further use. The following sections describe the process used to generate MPSS data from human ES cells, including extracting mRNA from the cultured ES cells, capturing the signature of each mRNA molecule, and sequencing the signatures on the beads by MPSS.

3.3.2. MPSS Sequence Output

At the end of a MPSS run, the images are base-called to yield a signature sequence for each microbead, or clone. Clones of the same signature sequence are binned to generate the observed count of that signature. Therefore, the end result of the MPSS run is a table of signatures and their observed counts.

3.4. MPSS Data Analysis

3.4.1. MPSS Data Processing

For any given sample, Lynx typically sequences multiple flow cells in each of the two different reading frames (i.e., steppers), each starting with 1.2 million beads. This is done to obtain replicate information and to reduce potential reductions in sequencing yields due to the presence of palindromic four-base overhangs (*see* previous section and **Note 9**). Each run typically yields more than 600,000 "clones." These clones are then binned to distinct sequences (the signatures) to generate the frequency table of signatures and their observed counts from that run. For each signature, the observed counts of the same stepper runs are merged. The resulting count is divided by the total clone counts of the stepper to generate the "weighted average." Multiplication by 1 million yields the weighted average in tpm. The stepper with the higher tpm value is chosen to represent the signature. The outcome is 1 tpm value for one signature (*see* **Note 10**). The tpm value is the normalized absolute count of transcripts in the sample,

which is the basis for digital gene expression. The tpm value also provides the foundation for comparing gene expression across multiple samples.

Lynx has designed several filters to maximize data quality. For example, signatures that are only observed in one run with an observed count that is beyond the sampling distribution (current setting: >50 observed counts) are likely from errors and are removed from the datasets. Signatures that are more that 3 tpm in at least one of the surveyed samples are flagged as "significant."

The final signature count provides the first hand information about the number of transcripts in the sample. The number of signatures generated from a biological sample is both tissue and species dependent. A human cell line typically yields approx 20,000 distinct signatures, reflecting the amount of distinct transcripts in the sample ***(7)***. However, a wide range of signature numbers in different tissues has been observed. In general, MPSS identifies more signatures in more complex tissues. For example, human testis has approx 33,000 distinct signatures, whereas human salivary gland only has approx 18,000 distinct signatures.

3.4.2. Signature Annotation

After the signatures are identified and their tpm values are calculated, it is crucial to annotate the signatures with accurate genomic information. There are several methods to do so. One can map the signatures to genome or transcript sequences using alignment algorithms such as basic local alignment search tool (BLAST). For genomes that have little genomic sequences and few EST sequences, BLAST is an efficient method. For those genomes that have complete or nearly complete genome sequences and a wealth of ESTs, mapping by database is a better approach ***(15)***. We at Lynx have adopted the database approach to annotate MPSS signatures.

We first establish a signature database, or annotation tables, to include all signatures from every organism whose samples have been processed at Lynx. For any given organism, the following steps are taken to create this signature annotation table:

1. Extract all the possible signatures (i.e., 20-nucleotide virtual signatures, explained in the following section);
2. Rank the signatures by assigning the appropriate class (*see* discussion and class description in the following section);
3. Select the best possible annotation for each signature. The end result is one annotation per signature.

These virtual signatures are extracted from Unigene sequences, genomic sequence and mitochondrial genomic sequence. For every Unigene cluster, the EST sequences are first aligned to the genome to determine their orientations.

We then extract every signature, which is composed of GATC (the Dpn II recognition site) and the 3′-adjacent 16 bases, from each member sequence of the cluster (the source sequence [e.g., individual ESTs of the signature]). The extraction is performed on both strands of the source sequences. The signatures are also extracted from the genomic and mitochondrial sequences. Because each Unigene cluster usually contains many virtual signatures, some of which can be shared with other clusters or genomic sequences, we designed a classification scheme to organize the signatures while retaining the necessary source sequence information.

The extracted virtual signatures are classified according to their positions and the presence of polyA tail and polyA signal features in the source sequence. The polyA tail is defined as a stretch of As (at least 13 of 15 bases) that is no more than 50 bases away from the end of the source sequence. The polyA signal is either AATAAA or ATTAAA that has at least 1 base within the last 50 bases before the end of the source sequence or the polyA tail. Class 0 signatures, those that match more than 100 sites in the genome, are flagged as "repeat sequences." Class 1 comprises the sense-strand, 3′-most signatures of those source sequences that contain both a polyA tail and one or more polyA signals. Class 2 signatures are similar to class 1 but originate from source sequences that lack a polyA tail. Similarly, class 3 signatures are the sense strand 3′ most signatures of those source sequences that only contain a polyA tail. Class 4 signatures are the sense strand 3′ most signatures of those source sequences that contain neither polyA tail nor polyA signal(s). The entire class scheme is summarized in **Table 1**. All the virtual signatures extracted from the genomic sequences are classified as class 1000 signatures. If a signature appears in different source sequences of a Unigene cluster with different classes, the lowest class is selected. Therefore, each distinct signature within the Unigene cluster only has one class.

The rules for selecting the right class for a given signature are based on the molecular cloning process outlined in the technology section. If a signature only hits one Unigene cluster or a genome location, it is annotated to that Unigene cluster or the genome coordinates, respectively. If a signature hits to multiple Unigene clusters or genome locations, we designed the so-called "TopHit" algorithm to select the annotation. The origin of a signature with class 0 is deemed to be unidentifiable, therefore class 0 signatures are annotated with "repeats." Similarly, any signature that hits the mitochondrial genome is likely derived from mitochondria because of its high abundance in many biological samples. These signatures are annotated as mitochondrial genes or to mitochondrial genomic loci. Because of the cloning procedure, only the sense strand, 3′ most signatures are captured and sequenced. Therefore, classes 1, 2, and 3 are the favorable classes when a signature is shared among many Unigene

Table 1
Signature Classes

Virtual signature class	mRNA orientation	Poly-adenylation features	Position
0	Either; repeat warning	Not applicable	Not applicable
1		Poly-A signal, Poly-A tail	3 most
2		Poly-A signal	3′ most
3	Forward strand	Poly-A tail	3′ most
4		None	3′ most
5		None	Not 3′ most
6		Internal Poly-A	Not 3′ most
11		Poly-A signal, Poly-A tail	5 most
12		Poly-A signal	5′ most
13	Reverse strand	Poly-A tail	5′ most
14		None	5′ most
15		None	Not 5′ most
16		Internal Poly-A	Not 5′ most
22		Poly-A signal	Last before signal
23	Unknown	Poly-A tail	Last before tail
24		None	Last in sequence
25		None	Not last
26		Internal Poly-A	Not 3′ most
1000	Unknown; derived from genomic sequence	Not applicable	Not applicable

clusters of different classes. If a signature is shared among multiple Unigene clusters of the same classes, it is assigned with the annotation of the cluster containing the most EST sequences. When a signature is shared among multiple genomic loci, it is marked as "multiple genome hits." The TopHit algorithm guarantees each signature has a unique annotation in the virtual signature database. After the virtual annotation database is established, assigning the annotation to the MPSS signatures generated from a biological sample becomes straightforward. Studies conducted on human and mouse Unigene sequences have shown that the majority of 20 base signatures are unique enough to determine their origin. Roughly 95% of the sense strand 3′ most Dpn II signatures

derived from the human and mouse Unigene sequences are unique. Among the virtual 20 base signatures derived from human genome, 93% are unique.

3.4.3. Statistical Analysis for Digital Gene Expression

The digital nature of MPSS data derives from the fact that MPSS counts the absolute number of RNA copies in a sample. This makes the statistical analysis of MPSS data relatively straightforward. The deep sampling of transcripts in a given sample (>1 million sequences per sample) provides a high and measurable level of statistical confidence in the resulting data. Experimental tests run on many MPSS runs indicate that the observed count of a given signature show a binomial distribution. Therefore, statistical models based on binomial distribution are generally applicable to MPSS data.

For pairwise comparisons, we have adapted the Z-test model built by Man et al. *(16)* to analyze SAGE data (*see* **Note 11**). Although the Z-test model takes the sampling events into consideration, it does not consider the variations introduced in the steps from the RNA prep to the signatures on the beads. Unlike other expression profiling technologies, MPSS is sensitive enough to detect these variations. Recently, we at Lynx along with scientists from IBM and the Institute for Systems Biology have designed an alternative statistical model based on empirical data. This model, which is much more stringent than Z-test model, is described by Stolovitzky et al. *(17)*. The Z-test however is sufficient for most routine purposes.

The Z-test allows MPSS users to quickly identify differentially expressed genes between two samples. However, like many other statistical tests for pairwise comparison, the Z-test has limited analytical power when applied to multiple samples. Given of the digital nature of MPSS data, direct comparison of large numbers of samples is feasible. We have found that most commercial software packages that were originally designed for analyzing microarray data are equally useful in analyzing MPSS data sets.

3.4.4. Application of Commercial Gene Expression Analysis Tools to MPSS Data

There are many commercial software packages available for analyzing gene expression data, including DecisionSite from Spotfire Inc., Partek Pro from Partek Inc., GeneSpring from Silicon Genetics Inc., and Resolver from Rosetta Inpharmatics, We and other MPSS users have tested the four software packages listed here for analysis of MPSS data and have found that their tools are fully applicable to MPSS datasets. Other software packages that are not listed here are also likely to be useful. We routinely use Spotfire DecisionSite to analyze MPSS data. To load MPSS data, one simply drag-drops the data with the signatures and the annotation, along with the tpm values in different samples,

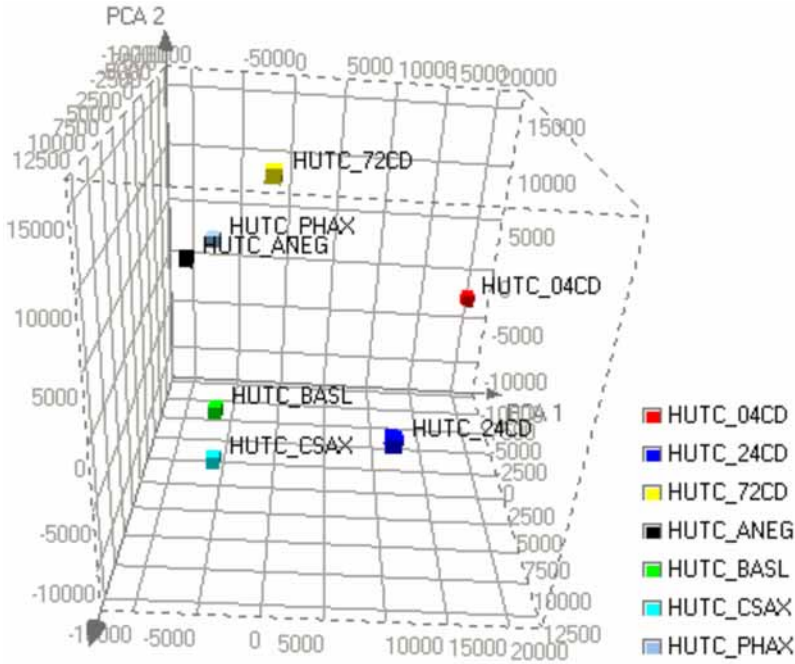

Fig. 3. Principle component analysis of MPSS data from human T cells under various conditions. Samples are: HUTC_BASL, unstimulated T cells; HUTC_04CD, T cells stimulated with anti-CD3 and anti-CD28 for 4 h; HUTC_24CD, stimulated with anti-CD3 and anti-CD28 for 24 h; HUTC_72CD, stimulated with anti-CD3 and anti-CD28 for 72 h; HUTC_ANEG, anergic T cells; HUTC_CSAX, treated with cyclosporin A (CsA); HUTC_PHAX, treated with phytohemagglutinin (PHA).

to DecisionSite. One example of application is shown in **Fig. 3**, where MPSS data from human T cells stimulated under various conditions were subjected to principle component analysis.

3.5. MPSS Data Integration

We developed a data integration tool called "Signome Browser" (short for signature on genome browser) for integrating MPSS data. An example of how the Signome Browser integrates MPSS data is shown in **Fig. 4**. In this case, through signature mapping, MPSS data of four human cell lines are integrated with the human genome (released in December 2003, version hg16), along with the other associated genomic information. The signatures identified by MPSS are depicted by the colored triangles as described in the legend. The browser provides a variety of navigation tools for the users to explore gene expression and gene structure across the genome. The tutorial for how to use the browser

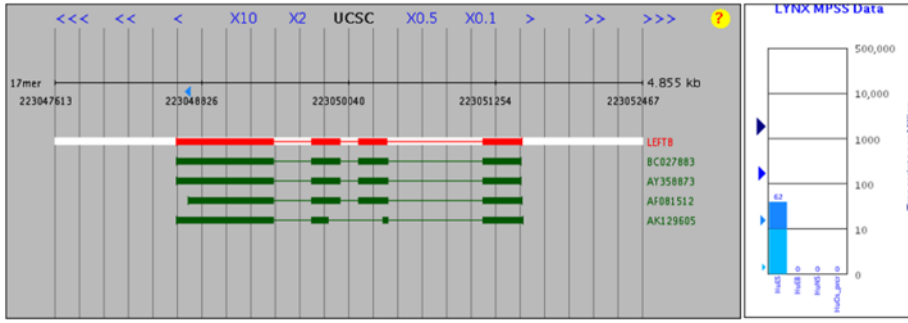

Fig. 4. Signome Browser, a bioinformatics tool built by Lynx to integrate MPSS data with other genomic information. (Left) The page of Signome Browser showing the structure of the LeftB gene (red bar: Refseq; green bars: mRNA) and its MPSS signature (blue triangle). The genome is depicted as the central line, along with the coordinates. (Right) A popup window, displayed by clicking on the blue triangle, showing the expression level of LeftB in four cell lines. HuES, human ES cells; HuEB, human embryoid bodies; HuNS, human neural stem cells; HuOS_prcr, human oligodendrocyte precursor. *See* sgbpub.lynxgen.com for more details.

is available on the Signome Browser web site (sgbpub.lynxgen.com). The publicly available mouse reference transcriptome database that is comprised of dataset of about 100 mouse tissues and cell lines has been integrated with other mouse genomic information. The dataset is also available at the same web site and at NCBI (www.ncbi.nlm.nih.gov/genome/guide/mouse/ MouseTranscriptome.htmL).

Through commercial software packages, MPSS data can also be integrated with the data generated from other platforms. MPSS data and microarray data can be compared when loaded to the commercial analysis packages listed previously. Data from both platforms, as well as EST and SAGE data, can be compared side by side in commercial softwares such as BLIS of Biotique System Inc.

3.6. MPSS Data for Systems Biology

Because of its digital nature, MPSS is an ideal technology for systems biology research. To systematically understand the molecular events underlying a biological phenotype, such as differentiation of stem cells, it is essential to have precise information on every molecule in the sample and be able to compare across multiple samples. MPSS is especially well suited for this type of analysis *(18)*. When combined with the carefully curated signal pathway information, MPSS provides unparalleled power to elucidate the underlying biological pathways.

3.7. MPSS Analysis of Stem Cell Studies

MPSS is especially well suited for the analysis of stem cells in general and embryonic stem cells in particular. Typically these cells have some defined set of characteristics that allow one to harvest comparable populations repeatedly. Similar populations have been identified from multiple species allowing cross-species comparisons and at least some markers defining the stem cell state exist. This information allows one to readily validate the samples, perform internal quality control tests of the MPSS process (checking expression of known genes for example), and allows selection of interesting subsets by cross species comparison. Cross-species comparisons in particular have been difficult with microarrays.

There are, however, caveats to using this technology, in part to the high sensitivity of the MPSS process, which should be kept in mind when planning a MPSS analysis. High-quality input RNA and accurate description of the state of cells or tissue are critical to obtain useful data. Many regulatory molecules and pathways are expressed at low levels and their levels may vary dramatically over short periods and MPSS will pick up these differences *(19)*. This sensitivity of MPSS must be kept in mind as it can be difficult to distinguish between important fundamental differences vs transient culture or isolation induced changes. On the other hand, this sensitivity can be used to identify trace of contaminants from the feeder cells, which normally compromises data from other technology platforms.

We have used MPSS to build a complete transcription profile of human ES cells, identify novel genes, determine critical active signaling pathways, identify the state of differentiation of ES cells, determine if the ES cells are contaminated with the feeder cells, and to map expressed genes to chromosomes to assess hot and cold spots of gene expression. By comparing the transcriptome of ES cells with that of embryoid bodies derived from them, we have been able to identify important regulatory pathways that initiate differentiation. Although it is impossible to discuss all of these in detail here, a few examples are highlighted in the sections following. In general, we have generated a database of genes expressed in ES cells of a rigorously defined state, mapped them to chromosomal locations, annotated them with Unigene Ids and Locus Ids, and selected the differentially expressed genes and pathways for further studies. We also confirmed the gene expression levels by comparing with existing databases generated by RT-PCR, EST scans, and microarrays *(11,20)*.

3.7.1. A Stem Cell Expression Database

One of the most powerful applications of MPSS has been to generate publicly accessible databases where, for instance, data from multiple ES cell

lines and their derivatives can be compared. For each cell line, we cataloged nearly every gene with their exact expression levels. The database is expandable by adding more MPSS datasets generated from any laboratory. This is a powerful and unique characteristic because the value of the database increases exponentially as more datasets are added and it is readily available to any researcher. With commercially available software tools, the database is particularly useful for analyzing expression levels across multiple samples even though an individual laboratory only generates datasets from a few samples. This makes a large-scale study a lot more affordable. In addition, the database can be integrated and made comparable with other public expression databases, including the SAGE and EST datasets maintained at NCBI. The initial ES cell datasets can be downloaded from Lynx ftp site (ftp.lynxgen.com).

3.7.2. Use of MPSS Data to Identify Cell-Specific Genes

Besides identifying differentially expressed genes, the MPSS database is useful for identifying cell or tissue specific genes. In the case of the human ES cell study, we compared the MPSS data from a pooled sample of undifferentiated human ES cell lines, H1, H7, and H9, grown in feeder-cell free conditions, to that of 36 human tissues and cell lines to look for genes that are unique to, or highly overexpressed in the ES cells. A list of 13 highly enriched genes of unknown function is shown in **Table 2**. Several of these genes are highly expressed in ES cells and absent in most other tissues tested. They are good candidate markers for undifferentiated human ES cells.

3.7.3. Confirmation of ES Cell Markers and Their Downregulation After Differentiation

MPSS not only detected the well-known ES cell markers, but also provide direct information about the level of expression. **Table 3** lists some of the well-known markers and their tpm values. DNMT3b and Sox-2 are known to be expressed at high levels in ES cells. Some of these markers are downregulated when ES cells differentiate into embryoid bodies.

3.7.4. Identification of Active Signaling Pathways

Because MPSS provides complete coverage of the transcriptome, it is ideal for identifying the active signaling pathways and revealing the interplay among them. Our analysis explains why human ES cells do not require LIF for long-term undifferentiated growth. Our data reveal little or no transcripts for the LIF pathway with high levels of suppressors of LIF-mediated signaling. Our results also confirm the importance of FGF signaling and further suggest that FGFR1 is the important signal transducer and that FGFs other than FGF4 are important (*see* below). These quantitative data provide a foundation that promises to

Table 2
Short List of Genes of Unknown Function That are Highly Enriched in Three ES Cell Lines Comparing to 36 Different Tissues and Cells[a]

Signature	GB:description	UniGene	ES cells	Other 36, TPM**
GATCTCCAG-TAGACTTA	CD250365:Homo-sapiens transcribed sequence	Hs.507833*	1627	NS-10
GATCTGTTA-ACAAAGGA	BC008934: claudin 6	Hs.247902	955	ND
GATCTAGAA-GTTGCAAC	NM_019079: hypothetical protein FLJ10884	Hs.135693	480	ND
GATCTTTTTT-TTTGCCC	NM_018189: hypothetical protein FLJ10713	Hs.317659	447	TH-47, HY-3, PG-3
GATCCCCAT-CCAAAAGA	AI636928:Homo sapiens transcribed sequences	Hs.197683	359	MCF7-2
GATCCACCT-AGGACCTC	CD174249:Homo sapiens transcribed sequence	Hs.522709	240	ND
GATCCGCCT-CCTTGGCC	AK092578:Sapiens cDNA FLJ35259 fis	Hs.105196	236	ND
GATCCTAGCC-AAGCCCC	BF223023:Homo sapiens transcribed sequences	Hs.67624	166	ND
GATCTGGCC-CGCCACCA	NM_032805: hypothetical protein FLJ14549 (ZNF206)	Hs.334515	148	ND
GATCGTTGT-GGTGGACT	XM_067369:similar to Heterochronic gene LIN-41	Hs.436515	143	ND
GATCCACCA-CATGGCGA	CD176172:Homo sapiens transcribed sequence	Hs.471439	91	ND
GATCCAACA-ATTCTACT	CD173198:Homo sapiens transcribed sequences	Hs.492000	77	TE-33
GATCTTCTAA-ACCCATC	BU608353:Homo sapiens transcribed sequence	Hs.507034	74	ND

*This gene (Hs.507833) is transcribed in the antisense direction to HDCMA18P (Hs.278635).

ND, not detected; NS, neural stem cells; TH, thymus; HY, hypothalamus; PG, pituitary gland; TE, testis.

Table 3
ES Cell Markers and Their tpm Values

Unigene ID	Class	Gene name	Signature	HuES_ TPM	HuEB_ TPM	P-Val: ES|EB
Hs.251673	1	DNMT3β	GATCGTTT-TTTCCCAGG	1080	51	0
Hs.816	4	Sox-2	GATCAGCAT-GTATCTCC	515	4	0
Hs.74471	1	Connexin 43	GATCATGTGT-TCTGGAG	392	359	0.324335542
Hs.106346	1	retinoic acid repressible	GATCTGAGA-GATTCAAG	217	82	1.12441E-10
Hs.278959	1	Galanin	GATCTCCCC-GCCGCAGC	199	26	0
Hs.278239	1	Lefty-B	GATCTGGGC-TAAAGTCC	62	0	2.54924E-10
Hs.120204	1	Fox-D3	GATCTCCTCT-TTCCTTG	61	0	1.97988E-11
Hs.25195	1	Lefty-A	GATCAGACA-GCCCCTAC	16	9	0.233019691

HuES_TPM, tpm value of human ES cells; HuEB_TPM, tpm value of human embryoid bodies; P-Val: ES|EB, *p* value for pariwsie comparison between ES cells and embryoid bodies.

untangle the complex interplay among the active signaling pathways in ES cells.

3.7.5. Cross-Species Comparison

Unlike other expression profiling technologies, MPSS allows one to compare data across species. This comparative transcriptome approach, when combined with comparative genomic studies, is very powerful in elucidating the underlying biology. To demonstrate this, we compared the MPSS data from human ES cells with that of murine ES cells. Although basic FGF2 is a critical ingredient for propagating human ES cells, it is not necessary for maintaining murine ES cells. Our data suggest that the expression pattern of FGF gene families is very different in human and murine ES cells. Although several FGF genes (FGF4 and FGF17) are expressed in murine ES cells at moderate levels, their receptors are not detectable, except for FGFR1, which is expressed at very low levels (<15 tpm). On the other hand, both FGF and FGFR are expressed in human ES cells. FGF2 and FGF17 are expressed at 52 tpm and 27 tpm, respectively. The main receptor for basic FGF2, FGFR1, is expressed at 730 tpm in human

ES cells, whereas several other FGF receptors (FGFR2, FGFR3, and FGFR4) are all much lower—17 tpm, 79 tpm, and 27 tpm, respectively. Furthermore, the FGF responsive substrate, FRS2, is expressed in human ES cells (12 tpm) but absent in murine ES cells. The MPSS expression profile therefore confirms previous phenotypic observations.

3.7.6. Detecting Subtle Differences

Because of its sensitivity, MPSS is able to detect subtle differences among cell lines grown under various conditions. In the pooled sample of ES cell lines H1, H7, and H9, gene expression from both X and Y chromosomes was observed, suggesting that expression differences between male and female ES cell lines are present and detectable. Likewise, MPSS detected expression of several major histocompatibility complex Class I and II genes, suggesting that MPSS can identify the composition of the ES cell samples. We also note that both H19 and Igf2 were expressed at detectable levels and that their ratio is likely reflecting the contribution of maternal and paternal chromosomes and may represent a simple assessment of the imprinting status of cells. In addition, we detect low level of mouse transcripts for those cells that are cocultured with mouse feeder cells and then grown for several passages in the feeder-free medium. These fine details of expression profiling are difficult to detect with other technologies.

3.8. Comparison of MPSS With Other Technologies

Many high-throughput technologies are available for conducting gene expression analysis. Like MPSS, some of them are digital in nature, they count RNA molecules in the sample. Others are based primarily on an analog format where the relative level of gene expression is established by quantifying the fluorescence intensities of labeled probes hybridized to targets on a solid support. Here we compare MPSS with EST data, SAGE, and microarray technologies.

Direct sequencing of cDNA clones was the first digital technology for measuring gene expression. The resulting EST database (dbEST) has been a valuable resource. Because of its shallow sampling depth, it is more commonly used for gene identity than gene expression measurement; however, because ESTs provide both sequence information and the rough estimate of the expression level, particularly for highly abundant genes, EST sequencing is still useful for cataloging and measuring activity for genes of less well-characterized organisms. On the other hand, EST sequencing is both time-consuming and costly to generate sufficient data for comprehensive gene expression profiling.

SAGE partially overcomes the shallow sampling depth of EST sequencing by concatenating tags of different RNA molecules together. It indeed gains sampling depth, but with less sequence information than provided by long EST

sequences. The length of the SAGE tags in the publicly available libraries (cgap.nci.nih.gov/SAGE) is 14 bases, which is too short to reliably map to the genome or transcriptome. The sampling depth of SAGE is usually less than 50,000 tags per library, which is better than EST sequencing, but too shallow for measuring gene expression with high statistical confidence. Recently developed long SAGE with 21 base tags provides better mapping *(21)*. However, at a fixed sequencing cost, the gain of information gained from longer tags is at the cost of sampling depth.

MPSS overcomes both shortcomings of SAGE by sequencing millions of longer tags at a lower overall cost than SAGE. A typical MPSS library contains about two million 20-base signatures. Among all the sequences included in the human Unigene collection, signatures of 14 nucleotides are less than 70% unique, whereas approx 95% of the 20-nucleotide MPSS signatures are unique. When restricted to the 3′ most restriction site (*Dpn*II for MPSS, *Nla*III for SAGE), signatures of 14 nucleotides are about 90% unique, whereas 20 base signatures are 97% unique. However, when mapping a signature of an uncharacterized gene to the human genome, signature length of 14 bases is only 9.5% unique, whereas 20 base signatures are about 94% unique. In addition, the sampling depth of MPSS provides unmatched resolution power.

It is also instructive to compare MPSS to microarrays for measuring gene expression. Notably, MPSS measures virtually all the genes expressed in a sample, not just those that have been preconfigured on the microarray. This is particularly advantageous for less well-characterized genomes or uncharacterized human genes. In addition, cross-hybridization of microarray probes makes it impossible to distinguish individual members of a gene family. With MPSS, the signature sequence, which is frequently located in the 3′ untranslated region, can usually permit measurement of each family member. Also, microarrays measure relative gene expression level, which complicates comparison of data from different laboratories. MPSS, on the other hand, measures the absolute copy number of transcripts. Therefore, it is ideal for establishing reference databases. However, MPSS is not as flexible as microarrays for profiling expression of a subset of genes. MPSS is best employed to build a comprehensive transcriptome database; after the transcripts are identified, microarrays can be constructed used to study a designated set of genes in a large number of biological samples.

Cross-platform comparisons conducted by many research groups show that detection sensitivity, dynamic range, and reproducibility vary between technology platforms. The detection limit of SAGE (about 50,000 tag data sets) is around approx 50 tpm. The detection sensitivity of microarray technologies is around 35 tpm, but it ranges widely. MPSS has by far the best quantitative detection sensitivity at approx 5 tpm (for 1 million signature datasets).

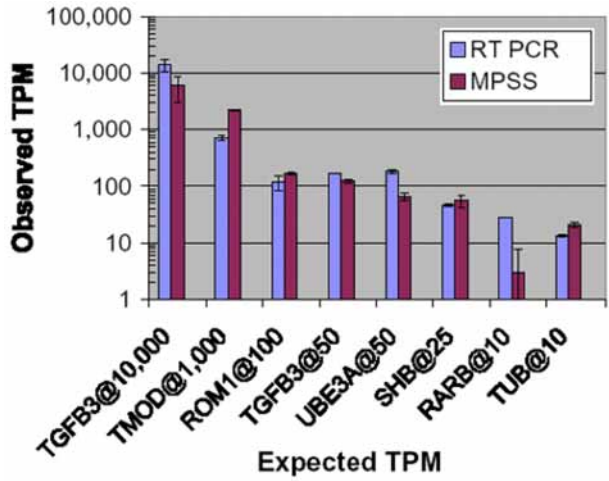

Fig. 5. Spiking experiments demonstrate that MPSS and real-time PCR generate comparable expression levels. Signatures of seven human genes (TGFB3, TMOD, ROM1, UBE3A, SHB, RARB, and TUB) were in vitro transcribed and spiked into yeast mRNA at various tpm levels. MPSS and real-time PCR were used to detect the "expression" levels. The results demonstrate that the detection accuracy and sensitivity by MPSS and real-time PCR match closely to the expected values.

Microarrays typically have a dynamic range of two to three logs *(22)*. MPSS measures gene expression as high as hundreds of thousands of tpm, as well as genes expressed at as low as 1 tpm, as shown in **Fig. 5**. The expression levels detected by MPSS at this wide range are closely comparable to what are detected by real-time PCR. The reproducibility is comparable between MPSS and microarrays.

3.9. Prospects

Being a high-throughput sequencing technology, MPSS is not only ideal for gene expression, but also a powerful tool for many other applications. With a slight modification to the procedure to allow the cloning of genomic fragments or non-polyA RNAs, MPSS is broadly applicable to many other assays. For example, chromatin immunoprecipitation (ChIP) to map the transcription factor binding sites is traditionally performed on microarray (ChIP-to-chip). Because it is cost-prohibitive to make a whole human genome tiling array, ChIP-to-chip is restricted to some narrow regions of human genome, such as CpG islands. By MPSS, the immunoprecipitated ChIP fragments can be directly sequenced and mapped to the genome to identify, at whole genome scale, the transcription factor binding sites. Similarly, MPSS can be useful in mapping

chromosome break points in cancer cells, identifying the DNase hypersensitive sites along the genome, and measuring small regulatory RNAs. Efforts are underway in Lynx to develop protocols for these assays. In addition, Lynx is in the process of developing an ultra high throughput sequencer, a "next-generation sequencer," in which tens of millions of sequence reads will be generated from a single run. We expect that these new instruments will enable sequencing of the human genome at a fraction of the current cost.

4. Notes

1. In cases in which cells have not been adapted to feeder-free conditions, we recommend passaging once in feeder-free conditions or using a collagenase isolation protocol, as described by Amit et al. *(23)* to remove ES cells but leave the feeder cells attached.
2. To remove the contaminants, RNA was precipitated by adding an equal volume of LiCl precipitation solution (Ambion, Austin, TX), washed with 70% ethanol, and resuspended in water.
3. Alternatively, an aliquot of the cells is plated and stained for mouse-specific markers to assess potential contamination. We have used cellular fibronectin as the marker for mouse fibroblasts.
4. Lynx's signature cloning vector, pMBS, is comprised of a mixture of 16.7 million different vectors. When cloned into the tagging vector, a unique DNA Combi tag sequence is attached to the signature fragment of cDNA derived from each mRNA.
5. After hybridization, each of the microbeads displays amplified copies of one starting mRNA molecule, with the Dpn II end distal to the bead, and available for sequencing. The amplified cDNA copies on each microbead originate from a single mRNA molecule. Thus, each microbead is conceptually equivalent to a bacterial clone, with each clone (microbead) harboring many copies of a single cDNA. Please see the previously mentioned article by Brenner et al. *(10)* for further details on the cloning process. The method described here differs in that only the 20 basepair signature sequence is captured on the beads rather than the entire DpnII-to-PolyA-tail fragment. This modification results in reliable and uniform representation of all transcripts, independent of the length of the DpnII to polyA fragment.
6. One of the primers used in PCR amplification is fluorescently labeled to enable the separation of loaded beads from the unloaded beads.
7. Reagents and buffers are washed over the microbeads in each cycle of the process. Sequencing is a fully automated procedure in which four nucleotides are sequentially exposed by enzymatic digestion, ligation of a family of encoded adapters, and decoding the sequence by sequential hybridization with fluorescent decoder probes (**Fig. 2**). The proprietary protocol elicits sequence-dependent fluorescent responses from the microbeads, which are recorded by a CCD camera after each cycle. Lynx has developed and implemented proprietary instrumentation and software to automate the delivery of reagents used in the sequencing process

and to compile, from the images obtained at each cycle, the signature sequences that result from each microbead. Please see the article by Brenner et al. ***(10)*** for a complete description of the sequencing process.

8. The sequences of encoded adaptors and decoders are listed in the article by Brenner et al. ***(10)***.
9. This ensures that signatures are not lost because of the presence of palindromes (four-base sequences that are palindromic in complementary fashion, such as GTAC) in one frame, which self-anneal on the surface of the microbead and prevent sequencing. Nevertheless, we still lose a small number of sequences with palindromes present in both frames (approx 3% of transcripts in human Unigene).
10. The equation to calculate the tpm value of a signature is, $tpm = \left[\frac{\sum_{i=1}^{n} x_i}{\sum_{i=1}^{n} c_i} \right] (10^6)$

    where x is observed count, n is the number of runs (per stepper), and c is total clone count.
11. If x_1 and x_2 represent the observed counts of a specific signature in samples 1 and 2, respectively, and n_1 and n_2 represent the total number of signatures generated from samples 1 and 2 before collapsing, the proportions $p_1 = \frac{x_1}{n_1}$ and $p_2 = \frac{x_2}{n_2}$ would each follow a binomial distribution. Because n_1 and n_2 are large in MPSS (typically more than 10^6), the difference $(p_1 - p_2)$ follows an approximate normal distribution defined by expression (**Eq. 1**),

$$N\left((p_1 - p_2), \sqrt{pq(\frac{1}{n_1} + \frac{1}{n_2})} \right) \tag{1}$$

where the unknown parameters p and q can be estimated as $p = \frac{x_1 + x_2}{n_1 + n_2}$ and, $q = 1 - p$, respectively.

The statistic test defined by **Eq. 2**,

$$= \frac{p_1 - p_2}{\sqrt{pq\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}} \tag{2}$$

follows a standard normal distribution and can be used to test whether the expression level of the signature, hence the gene represented, is significantly different between sample 1 and sample 2. With values of λ, standard statistical tables can be employed to determine the Z score and the p value. The resulting

p value allows one to more rationally identify genes whose expression levels have significantly changed from one sample to another. **Equation 2** implies an inverse relationship between the level of expression and the fold of change. For $p < 0.001$, it is possible to detect twofold changes for genes with expression level of 30–40 tpm. However, with the same confidence, it is possible to detect smaller changes for genes that are expressed at a higher abundance. For example, a 40% difference can be theoretically detected for genes that are expressed at 200 tpm. Similarly, smaller changes can be detected with lower confidence in more weakly expressed genes.

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Confrontation Cultures of Embryonic Stem Cells With Multicellular Tumor Spheroids to Study Tumor-Induced Angiogenesis

Maria Wartenberg, Andreas Finkensieper, Jürgen Hescheler, and Heinrich Sauer

Summary

Human embryonic stem cells efficiently differentiate blood vessels, which allows using this in vitro model to study the interaction of blood vessels with adjacent tissues. Herein, we introduce confrontation cultures of human embryonic stem cells with multicellular tumor spheroids to investigate molecular mechanisms of tumor-induced angiogenesis. Vascularization of tumor tissue by the host is a prerequisite for tumor growth, which has led to the development of antiangiogenic therapy. This promising anti-cancer therapy intends to reduce, halt, or even regress tumor growth by deprivation from blood, oxygen, and nutrient supply. Confrontation cultures of human embryonic stem cells with multicellular tumor spheroids allow the investigation of the time course of endothelial cell invasion into the tumor tissue, the concomitant analysis of changes in angiogenesis-related gene expression, and analysis of the cellular microenvironment (i.e., pericellular oxygen pressure, tissue pH, and levels of tissue reactive oxygen species). The in vitro model of confrontation cultures is suitable for routine screening of antiangiogenic agents in pre-clinical trials and may be used to replace animal experiments applied in antiangiogenesis research.

Key Words: Confrontation culture; tumor-induced angiogenesis; human embryonic stem cell; multicellular tumor spheroid.

1. Introduction

Avascular tumors in vivo or tumor spheroids in vitro will not grow beyond a size of a few millimeters, at which size passive diffusion can no longer provide nutrients for the cells in the depth of the tissue or waste products adequately diffuse out of the tumor tissue. The gradients in oxygen, pH, nutrients, and the absence of sufficient detoxification then result in growth arrest, development of central

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necrosis, and induction of a multidrug resistance (MDR) phenotype, which is correlated with the increased expression of the MDR transporter P-glycoprotein *(1,2)*. Such tumors usually remain in this state of equilibrium for decades unless they get access to the host circulation in the process of tumor-induced angiogenesis *(3)*. Compelling evidence shows that vascularization of the tumor by the host vasculature is a prerequisite for rapid tumor growth as well as for metastasis. Tumor-induced angiogenesis is mediated by tumor-secreted proangiogenic growth factors that interact with their surface receptors, which are expressed on the surface of endothelial cells. The most commonly found angiogenic factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor, bind to tyrosine kinase receptors on endothelial cell membranes which results in dimerization of the receptors and activation of autophosphorylation of tyrosines on the receptor surface thereby initiating several signalling proteins *(4)*. Binding of the SH-2 regions of these proteins to the phosphotyrosines on the receptor tyrosine kinases activates several pathways, which are crucial for triggering the cell cycle machinery. However, upregulation of an angiogenic factor is not sufficient in itself for a tumor to become angiogenic. Indeed certain negative regulators of blood vessel growth may need to be downregulated *(5,6)*. If there is a preponderance of proangiogenic factors in the local milieu, the neovasculature may persist. However, if the levels of angiostatic factors increase over the level of proangiogenic factors, the new tumor blood vessels may regress. Hence the switch to the angiogenic phenotype is regulated by a change in the local equilibrium between positive and negative regulators of the growth of microvessels.

Vasculogenesis, the *in situ* assembly of capillaries from undifferentiated endothelial precursor cells as well as angiogenesis, the sprouting of capillaries from preexisting blood vessels has been extensively studied in embryonic stem (ES) cells of mouse and human *(7,8)* origin (**Fig. 1**). It has been discussed that the vasculogenic potential of embryonic stem cells could be specifically of use in tissue engineering for the induction of tissue vascularization *(8)*. It has been conclusively demonstrated that ES cell-derived embryoid bodies represent a suitable in vitro model to study molecular events involved in vascular development. ES cells differentiate in vitro to endothelial cells through successive maturation steps with sequential expression of cell lineage-specific markers: platelet endothelial cell adhesion molecule (PECAM), Flk-1, tie-1, tie-2, vascular endothelial cadherin, MECA-32, and MEC-14.7 *(9)*. These endothelial cells differentiated from ES cells form functional capillary structures, which facilitate diffusion within the tissue and dissipate oxygen gradients within the tissue. We have recently introduced the embryoid body as a model system for in vitro testing of antiangiogenic agents *(10)*. Several agents that were already proven to be effective in clinical patient treatment were applied to embryoid bodies and displayed antiangiogenic capacity *(10)*. Furthermore, it was shown that the teratogenic agent thalidomide exerted antiangiogenic effects, which was related to generation of hydroxyl

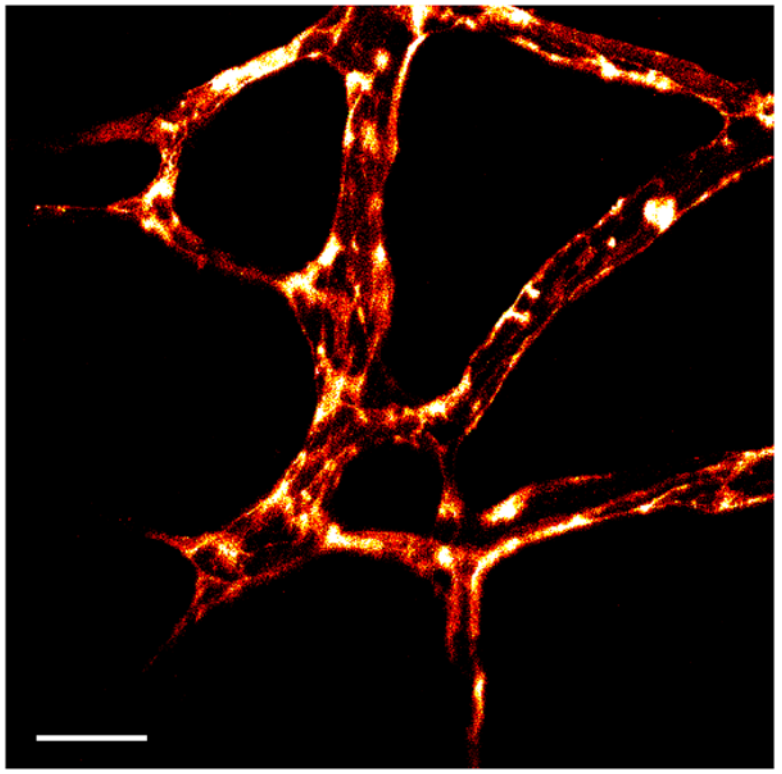

Fig. 1. Capillaries differentiated from human embryonic stem cells. Embryoid bodies were outgrown on cover slips. Endothelial cells were visualized by the use of an antibody directed against PECAM-1. The bar represents 50 µm. (Please *see* the companion CD for the color version of this figure.)

radicals by this compound ***(11)***. In a further study, we elaborated confrontation cultures consisting of embryoid bodies and multicellular tumor spheroids to study tumor-induced angiogenesis ***(12)***. Multicellular tumor spheroids are three-dimensional cell systems that have been used for more than 30 yr as model systems for avascular micrometastases or avascular microregions of solid tumors ***(13)***. By the use of the multicellular tumor spheroid model studies on mechanisms of cell cycle regulation, the tumor tissue micromilieu and the action of anticancer agents have been performed ***(13)***. Recently, we and others have demonstrated that upregulation of the MDR transporter P-glycoprotein in hypoxic regions of multicellular tumor spheroids is regulated by hypoxia-inducible factor (HIF)-1α ***(14)***. For generation of confrontation cultures of multicellular tumor spheroids and embryoid bodies, cells are initially cultured separately by a spinner flask technique and subsequently merged in hanging drops (**Fig. 2**). Invasion of endothelial cells into the tumor tissue was monitored by immunolabeling of cells

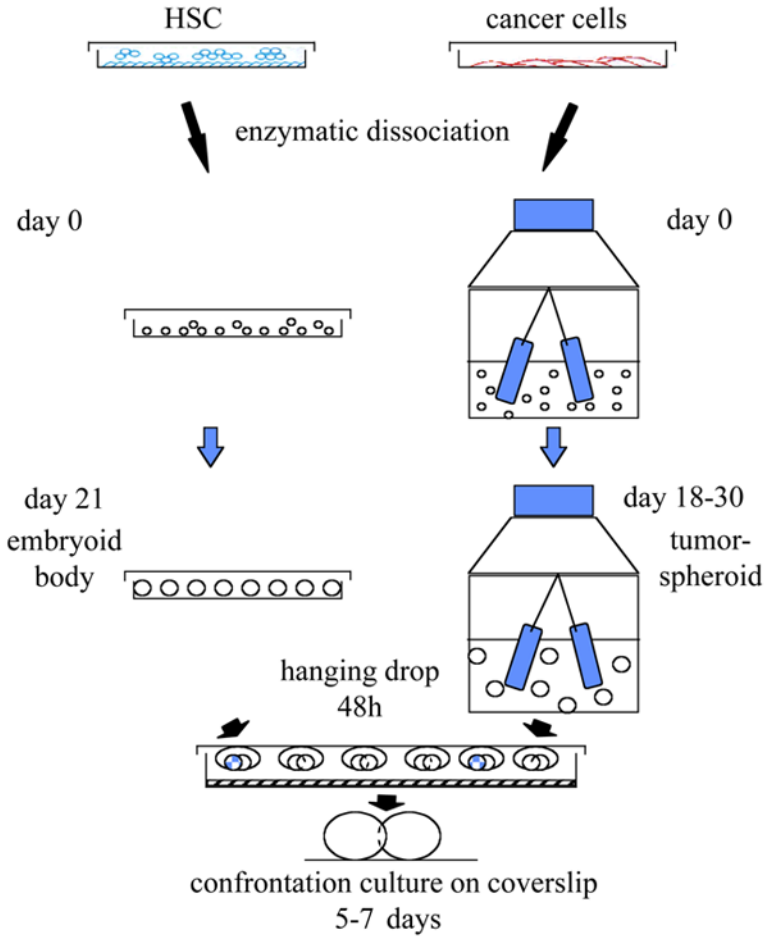

Fig. 2. Scheme of confrontation culture generation. Multicellular tumor spheroids are grown in spinner flasks whereas embryoid bodies are grown in liquid overlay culture. At a size of 350–450 µm single tumor spheroids and embryoid bodies are placed in hanging drops on the lid of a Petri dish. After 24 h, the tissues coalesce and form confrontation cultures, which allow to investigate the process of tumor-induced angiogenesis. (Please *see* the companion CD for the color version of this figure.)

with the endothelial cell-specific antibody PECAM-1. After 24–48 h in confrontation culture, first PECAM-1-positive cells appeared in the contact region between the embryoid body and the tumor spheroids. Within 5 d of confrontation cultures, endothelial cells migrated toward the tumor tissue, which resulted in vascularization of the tumor spheroid (**Fig. 3**). During confrontation culture, changes in the expression of genes involved in angiogenesis (e.g., HIF-1α, VEGF) as well as multidrug resistance can be monitored. We observed that

during tumor-induced angiogenesis, matrix metalloproteinases (MMPs) are upregulated. MMPs degrade the extracellular matrix and basal membrane thereby allowing the invasion of endothelial cells into the tumor tissue. This process involved the generation of reactive oxygen species, which are known to act as signalling molecules in a variety of signaling cascades including VEGF-signaling and MMP expression (*15*). Recently confrontation cultures consisting of multicellular tumor spheroids and human embryonic stem cells were established (**Fig. 4**). It was shown that capillary structures derived from embryonic stem cells readily invaded the tumor tissue. Hence this model may be ideally suited to study signal transduction events occurring during tumor-induced angiogenesis and may be useful in antiangiogenesis research.

2. Materials

2.1. Equipment

1. Confocal laser scanning microscope: LSM 410 (Carl Zeiss, Jena, Germany); Leica TCS SP2 (AOBS) (Leica, Bensheim, Germany).
2. Centrifuge: Biofuge Primo (Heraeus, Darmstadt, Germany).
3. CO_2 incubator: Heracell 240 (Heraeus).
4. Clean bench: HeraSafe (Heraeus).
5. Linear electron accelerator: Linac 5 (Varian Associates, Palo Alto, CA).
6. Stirrer system: CellSpin (Integra Biosciences, Fernwald, Germany; cat. no. 183001).
7. Gilson pipet P1000 (Gilson, Inc., Middleton, WI; cat. no. F123602).

2.2. Plasticware for Cell Culture

1. 75-cm^2 tissue culture flasks (BD Falcon, Heidelberg, Germany; cat. no. 353134).
2. 100 × 20-mm tissue culture Petri dishes (BD Falcon; cat. no. 3530003).
3. 50-mL polypropylene conical tubes (BD Falcon; cat. no. 352070).
4. Six-well tissue culture plates (BD Falcon; cat. no. 353046).
5. Cell scraper (Sigma/Greiner, Nürtingen, Germany; cat. no. C5981).
6. 60-mm bacteriological Petri dishes (VWR International GmbH, Darmstadt, Germany; cat. no. 391-3655).
7. 100-mm bacteriological Petri dishes (VWR International GmbH; cat. no. 391-3664).
8. In vitro fertilization dishes (BD Falcon; cat. no.353652).

2.3. Tumor Cells, ES Cells, and Feeder Cells

1. Tumor cells: for the generation of multicellular tumor spheroids, tumor cells of the androgen-independent cell line DU-145 were used (*see* **Note 1**).
2. ES cells: for the generation of embryoid bodies, the mouse ES cell line CCE as well as the human embryonic stem cell line H1 was used.
3. Feeder cells: human ES cells were grown on CF-1 embryonic fibroblasts (nomenclature Crl:CF-1 BR) origin obtained by Carworth from a Missouri laboratory (*see* **Note 2**).

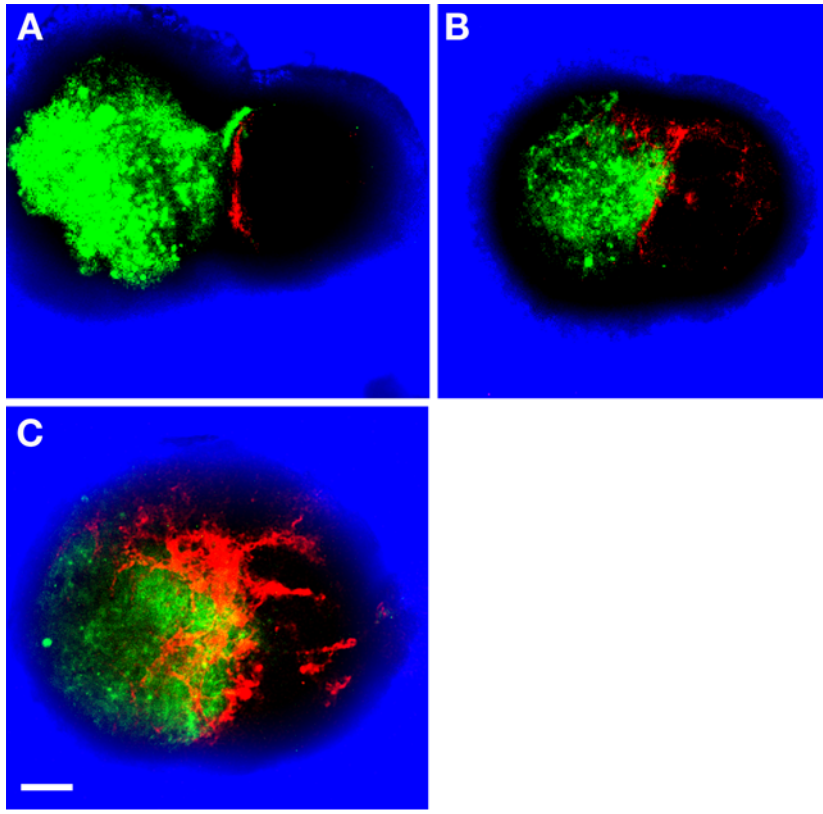

Fig. 3. Time course of tumor-induced angiogenesis in confrontation cultures of multicellular tumor spheroids and (mouse) embryoid bodies. After 24–48 h in confrontation culture, the first PECAM-1-positive cells appeared in the contact region between the embryoid body and the tumor spheroids (**A**). Within 5 d of confrontation culture, endothelial cells migrated toward the tumor tissue, which resulted in vascularization of the tumor spheroid (**B**,**C**). Shown are false color overlays of confocal fluorescence images (red, green) with a nonconfocal transmission image (blue). The tumor tissue was labeled with the long-term cell tracker dye CMFDA (green). Vascular structures were labeled with a monoclonal antibody directed against PECAM-1 (red). PECAM-1-positive structures were recorded within a tissue section of 100-µm thickness. The bar represents 100 µm. (Please *see* the companion CD for the color version of this figure.)

2.4. Media, Reagents, and Stock Solutions

2.4.1. For the Cultivation of H1 Human ES Cells

1. Feeder-cell medium: Dulbecco's modified Eagle's medium (DMEM), 4500 mg/L glucose, and L-glutamine (Gibco-Invitrogen GmbH, Karlsruhe, Germany; cat. no. 41965–039). For 500 mL: add 450 mL DMEM, 50 mL heat-inactivated fetal

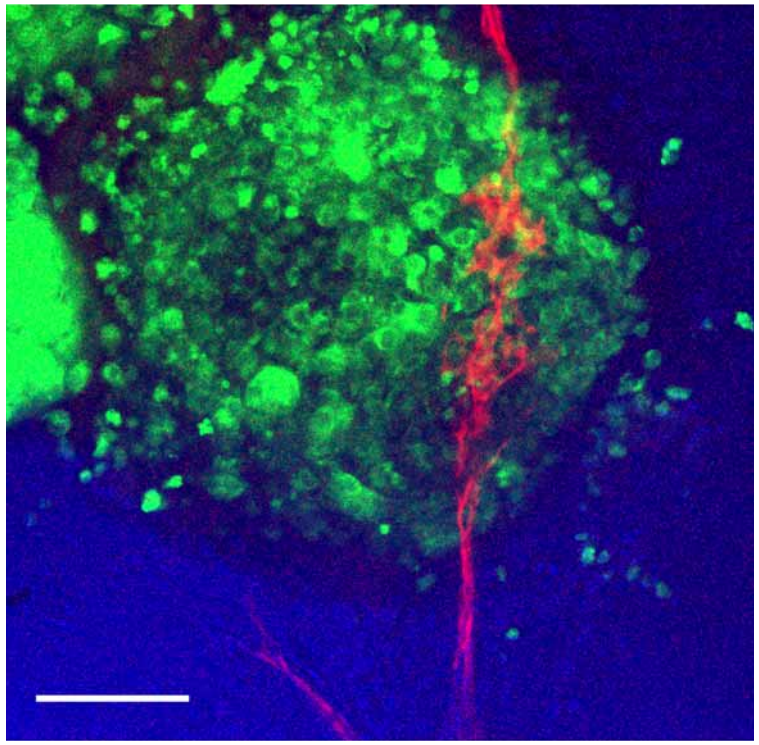

Fig. 4. Invasion of capillary-like structures in a confrontation culture grown from a prostate cancer (DU-145) multicellular tumor spheroid and an embryoid body grown from human embryonic stem cells (cell line H1). Confrontation cultures were outgrown for 7 d on cover slips. Shown are false color overlays of confocal fluorescence images (red, green) with a nonconfocal transmission image (blue). The tumor tissue was labeled with the long-term cell tracker dye CMFDA (green). Vascular structures were labeled with a monoclonal antibody directed against PECAM-1 (red). The bar represents 100 μm. (Please *see* the companion CD for the color version of this figure.)

bovine serum (Gibco-Invitrogen GmbH; cat. no. 16000-044), 2.5 mL penicillin/streptomycin (Biochrom AG, Berlin, Germany; cat. no. A2213), and 5 mL nonessential amino acids (NAA) (Biochrom AG; cat. no. K0293).

2. Human stem cell growth medium: knockout DMEM, high glucose, with sodium pyruvate, without glutamine (Gibco-Invitrogen GmbH, cat. no. 10829-018). For 500 mL: add 400 mL knockout DMEM, 100 mL knockout serum replacement medium (Gibco-Invitrogen GmbH; cat. no. 10828-028), 2.5 mL penicillin/streptomycin (Biochrom AG; cat. no. A2213), 5 mL nonessential amino acids (NAA) (Biochrom AG; cat. no. K 0293), 5 mL L-glutamine (PAA Laboratories, Cölbe, Germany; cat. no. M11-004), 3.92 μL β-mercaptoethanol (Sigma-Aldrich Taufkirchen, Germany; cat. no. M-7522), and 1 mL human basic fibroblast growth factor (Sigma-Aldrich; cat. no. F0291).

3. Human ES cell differentiation medium: knockout DMEM high glucose, with sodium pyruvate, without glutamine (Gibco-Invitrogen GmbH, Karlsruhe, Germany, cat. no. 10829-018). For 500 mL: add 400 mL knockout DMEM, 100 mL inactivated fetal bovine serum (Sigma-Aldrich; cat. no. F7524), 2.5 mL penicillin/streptomycin (Biochrom AG; cat. no. A2213), 5 mL nonessential amino acids (NAA) (Biochrom AG; cat. no. K 0293), 5 mL L-glutamine (PAA Laboratories, Cölbe, Germany; cat. no. M11-004), 3.92 µL β-mercaptoethanol (Sigma-Aldrich Taufkirchen, Germany, cat. no. M-7522).

2.4.2. For the Cultivation of DU-145 Prostate Cancer Cells

1. Ham's F-10 medium, with glutamine (PAA Laboratories; cat. no. E15-815). For 500 mL: add 450 mL Ham's F10 medium, 50 mL fetal calf serum (Sigma-Aldrich; cat. no. F7524), 2.5 mL penicillin/streptomycin (Biochrom AG; cat. no. A2213), 5 mL nonessential amino acids (NAA) (Biochrom AG; cat. no. K0293), 5 mL L-glutamine (PAA Laboratories, Cölbe, Germany; cat. no. M11–004), 3.92 µL β-mercaptoethanol (Sigma-Aldrich Taufkirchen, Germany, cat. no. M-7522).

2.4.3. For Enzymatic Dissociation

1. 0.2% trypsin and 0.05% ethylenediaminetetraacetic acid (EDTA) in phosphate buffered saline (PBS) (Gibco-Invitrogen GmbH; cat. no. 25200072).
2. Collagenase type 2 (PAA Laboratories GmbH; cat. no. Ko1-040-2).

2.4.4. For Immunohistochemistry

1. Monoclonal anti-PECAM-1 antibody (Research Diagnostics, Flanders, NJ; cat. no. RDI-CBL 473).
2. Cy5-conjugated sheep anti-mouse antibody (Jackson ImmunoResearch Laboratories, West Grove, PA; cat. no. 800-367-5296).
3. Triton X-100 (Sigma-Aldrich; cat. no. x-100).
4. Fat-free milk powder (Naturaflor, Töpfer GmbH, Dietmannsried, Germany).

2.4.5. For Long-Term Labeling of Cells

1. Long-term cell tracker dye 5-chloromethylfluorescein diacetate (CMFDA) (Molecular Probes, Eugene, OR; cat. no. C2925).
2. Orange-fluorescent tetramethylrhodamine CellTracker Orange CMTMR (Molecular Probes; cat. no. C-2927).
3. Red-fluorescent CellTracker Red CMTPX (Molecular Probes; cat. no. c-34552).

2.4.6. Stirrer System

1. Cellspin stirrer system equipped with 250-mL spinner flasks (Integra Biosciences; cat. no. 182026).
2. Sigmacote solution (Sigma, Deisenhofen; cat. no. SL-2), stored at 4°C.
3. 5 *N* NaOH solution.

3. Methods

3.1. Spinner Flask Culture of Multicellular Tumor Spheroids

3.1.1. Preparation of Spinner Flasks

1. Wash clean spinner flasks with excessive Milli-Q-plus water and dry for 1 h at 60°C.
2. Siliconize spinner flasks by moistening the interior as well as the mallets with Sigmacote.
3. Dry the silicon coat in an oven for 1 h at 120°C.
4. Rinse spinner flasks three times with 250 mL Milli-Q-plus water and autoclave subsequently.
5. Moisten the interior of the flasks with 20 mL of complete Ham's F-10 medium before the addition of tumor cells; exchange the medium with 125 mL of complete Ham's F-10 medium.

3.1.2. Procedure for the Cleaning of the Spinner Flask at the End of the Experiment

After the end of the experiment, the spinner flasks have to be cleaned before the inoculation with fresh tumor cells.

1. Remove the old medium with residual tumor cells.
2. Wash the flasks with 70% ethanol and subsequently with 1 L water.
3. Remove the silicon coat by adding 250 mL 5 *N* NaOH to the spinner flasks for a maximum of 12 h.
4. Remove NaOH and wash the flasks with at least 5 L water.
5. Clean the interior of the flasks thoroughly with a brush.
6. Subsequently, rinse the flasks with 1 L Milli-Q-plus water.

3.1.3. Inoculation of Spinner Flasks With Tumor Cells

1. Grow tumor cells to confluency in 75-cm^2 tissue culture flasks.
2. Remove cell culture medium and wash once with 0.2% trypsin and 0.05% EDTA in PBS.
3. Remove the trypsin solution and incubate cells for 5 min with 2 mL 0.2% trypsin and 0.05% EDTA.
4. Triturate the cells with a 2-mL glass pipet until a single-cell suspension is achieved.
5. Seed tumor cells in 125 mL complete Ham's F-10 cell culture medium.
6. Stir at a speed of 20 rotations per min. The stirring direction is reversed every 1440°.
7. Add 125 mL complete Ham's F-10 medium after 24 h to yield a final volume of 250 mL.
8. Exchange 125 mL of the cell culture medium every day.
9. Grow multicellular tumor spheroids for 15–20 d until they have reached a diameter of 250–350 μm.

3.1.4. Staining of Multicellular Tumor Spheroids With Long-Term Cell Tracker Dye

To discriminate multicellular tumor spheroids grown in confrontation culture from embryoid bodies, tumor spheroids are labeled with the long-term cell tracker dye 5-chloromethylfluorescein diacetate (CMFDA).

1. Transfer 50–100 tumor spheroids from spinner flasks to 60-mm bacteriological Petri dishes, and incubate them for 60 min in F-10 complete cell culture medium supplemented with 10 μ*M* CMFDA (stock solution 10 m*M* dissolved in DMSO).
2. Wash tumor spheroids in fresh cell culture medium and postincubate for further 24 h in 60-mm bacteriological Petri dishes.
3. Use the 488-nm band of a confocal laser scanning microscope for fluorescence excitation. For emission use a 515–525 nm bandpass filter set.

3.2. Cultivation of Human Embryonic Stem Cells and Feeder Cells

3.2.1. Cultivation of Feeder-Layer Cells

1. Isolate CF-1 embryonic fibroblast feeder cells from 16-d-old mouse embryos.
2. Cultivate CF-1 feeder cells at 37°C and 6% CO_2 on 100 × 20-mm tissue culture Petri dishes in complete DMEM cell culture medium.
3. Dilute cells 1:3 during the first three passages (cells from one confluent Petri dish are distributed to three Petri dishes).
4. Treat cells with 4 mL trypsin-EDTA at 37°C for passaging.
5. After 3 min, triturate cells and transfer the cell suspension to a 50-mL polypropylene conical tube.
6. To stop enzymatic digestion, a fourfold amount of complete cell culture medium is added to the cell suspension.
7. Subsequently, centrifuge the cell suspension for 5 min at 120*g*.
8. After discarding the supernatant the cell pellet is resuspended in fresh cell culture medium.
9. For inactivation, suspended CF-1 cells of passage 2 are treated by a linear electron accelerator at 60 gy for 5 min.
10. To achieve an optimum cell density CF-1 cells are seeded at a concentration of 7.5×10^5 cells/mL in six-well tissue culture plates. Cells are cultivated for 2 d before they are seeded with human embryonic stem cells.

3.2.2. Growth of Undifferentiated Human Embryonic Stem Cells

1. Cultivate H1 cells on CT-1 feeder cells in knockout DMEM medium.
2. Replace cell culture medium daily.
3. Dissociate cells enzymatically every 7 d, by treatment with collagenase type 2 at a concentration of 1 mg/mL, wherein the cells were first left undisturbed for 10 min in the incubator at 37°C.
4. Remove cells from the bottom of the Petri dishes with a cell scraper.

5. Aspirate cells up and down for several times by the use of a 1000 µL Gilson pipet, and transfer to a 50-mL polypropylene conical tube.
6. Centrifuge cells at 67*g* for 5 min. The supernatant is discarded and cells are seeded on new feeder cell layers.

3.2.3. Differentiation of Human Embryonic Stem Cells

1. To achieve differentiation of embryonic stem cells, cells are enzymatically dissociated from feeder layers and grown in the absence of feeder cells for approx 14 d in knockout DMEM. Cell culture medium is changed daily.
2. After confluency, cells are enzymatically dissociated by collagenase digestion and transferred into 60-mm bacteriological Petri dishes.
3. During 7–10 d, embryoid bodies are formed, which are either plated to cover slips for further differentiation or transferred to hanging drops for the generation of confrontation cultures.

3.3. Generation of Confrontation Cultures From Embryoid Bodies and Multicellular Tumor Spheroids

Confrontation cultures of embryoid bodies and multicellular tumor spheroids are ideally suited to investigate tumor-induced angiogenesis. To discriminate the tumor tissue from the embryoid body-derived tissue, tumor spheroids have to be stained either by reporter gene transfection based on fluorescent reporters (GFP, YFP, RFP) or incubation with long term cell tracker dyes (e.g., CellTracker Green CMFDA, Orange-fluorescent tetramethylrhodamine CellTracker Orange CMTMR, Red-fluorescent CellTracker Red CMTPX). On cultivation in hanging drops tumor spheroids and embryoid bodies come into intimate contact and coalesce. This allows the subsequent invasion of capillary structures from the embryoid body (host tissue) to the tumor spheroid (cancer tissue). The penetration of capillaries from the embryoid body towards the tumor spheroid is accompanied by dramatic changes in the pericellular oxygen tension of the tumor spheroid. The improved supply with nutrients results in excessive growth of the tumor spheroids with concomitant increased oxygen consumption *(12)*. Furthermore, during tumor-induced angiogenesis, changes in angiogenesis-related genes can be investigated. In this respect, we have demonstrated that VEGF protein is upregulated in the tumor tissue. We also demonstrated that tumor-induced angiogenesis resulted in increased P-glycoprotein-mediated drug resistance, which may be related to the decreased pericellular oxygen tension in the tumor tissue *(12)*. The latter finding is of primordial interest for investigations on the capacity of antiangiogenic therapy in cancer treatment since our data suggest that this promising cancer therapy may add to circumvention of a MDR phenotype.

3.3.1. Preparation of Hanging Drops

For the differentiation of embryoid bodies in distinct cell phenotypes, cells must be cultivated in three-dimensional embryoid bodies that allows for the generation of confrontation cultures with equal-sized multicellular tumor spheroids.

1. Grow embryoid bodies as well as multicellular tumor spheroids to a size of 350–450 µm, which is achieved in human embryoid bodies after approx 21 d of culture (14 d when using in vitro fertilization dishes, 7 d in 60-mm bacteriological Petri dishes, and multicellular tumor spheroids after 18 d of culture).
2. Add 15 mL PBS to a 100-mm bacteriological Petri dish.
3. Place 20 µL drops (N = 30–50) of stem cell differentiation medium on the lid of the bacteriological Petri dish prepared in **step 2**.
4. Transfer one embryoid body and one tumor spheroid into each of the drops by using a 1000 µL (blue tip) Gilson microliter pipet (*see* **Note 3**).
5. Cover the Petri dish prepared in **step 2** with the lid prepared in **step 3**; the embryoid body and the tumor spheroid will come into intimate contact at the tip of the hanging drop and will coalesce.
6. After 48 h, remove the confrontation cultures from the hanging drops by using a 1000-µL (blue tip) Gilson microliter pipette (*see* **Note 3**). Transfer confrontation cultures to a 100-mm bacteriological Petri dish filled with 15 mL of stem cell differentiation medium.
7. Plate confrontation cultures to cover slips coated with 0.1% gelatin in PBS.
8. Cultivate confrontation cultures for different times up to 10 d with medium change every day.

3.4. Immunohistochemistry and Analysis of Tumor-Induced Angiogenesis by Confocal Laser Scanning Microscopy

3.4.1. PECAM-1 Staining of Endothelial Cells

Capillary areas in embryoid bodies and in tumor tissue following tumor-induced angiogenesis are visualized by immunostaining against PECAM-1 (CD31) and confocal laser scanning microscopy.

1. Remove the cell culture medium from confrontation cultures, wash once with PBS.
2. Add 4% ice-cold paraformaldehyde and incubate for 45 min.
3. Remove paraformaldehyde; permeabilize cells with PBS supplemented with 1% Triton X-100 (PBST).
4. Block unspecific binding with 10% fat-free milk powder dissolved in 0.01% PBST for 1 h.
5. Incubate cells with primary antibody (dilution 1:100) dissolved in 10% milk powder either for 90 min at room temperature or at 4°C overnight.
6. Wash samples four or five times with 0.01% PBST.
7. Incubate cells with secondary antibody (Cy5-conjugated sheep anti-mouse) for 60 min at room temperature in the dark.
8. Wash samples four or five times in 0.01% PBST.

3.4.2. Analysis of Tumor-Induced Angiogenesis by Confocal Laser Scanning Microscopy

Capillary structures are investigated by the depth-discriminating properties of a confocal laser scanning microscope. Because fluorescence has to be recorded in the depth of the tissue, it is advisable to label capillary structures by the use of Cy5 dye, which is excited by the 633-nm band of a red helium/neon laser (*see* **Note 4**). The tumor tissue is discriminated by recording CMFDA fluorescence by the 488-nm band of an argon ion laser. The overall tissue is visualized by recording transmission images. To achieve this, the used confocal laser scanning microscope should be equipped with a transmission channel. Preferably, measurements should be performed by an inverted confocal microscope.

1. Place immunohistochemically stained confrontation cultures in an incubation chamber the bottom of which is formed by a cover slip. Select a ×16 oil immersion objective.
2. Choose the 488-nm band (blue) of an argon ion laser of the confocal setup (for visualization of tumor tissue stained with CMFDA [*see* **Subheading 3.1.4.**]) as well as the 633-nm band (red) of a helium-neon laser. Use band-pass emission filters 515–525 nm for CMFDA fluorescence recording and long-pass LP655-nm filters for recording of Cy5-PECAM-1 immunofluorescence. Focus on the periphery of confrontation cultures.
3. Set confocal software settings to perform five full-frame (512 × 512 pixel) images separated by a distance of 20 μm. Perform a series of five z-scans, which results in information on the extension of capillary areas and their spatial organization in a tissue section of 100-μm thickness.
4. Record a transmission image of the confrontation culture by using the 488-nm band of an argon ion laser.
5. Reconstruct a pseudo-three-dimensional overlay image (blue = transmission image; green = CMFDA fluorescence; red = PECAM-1 immunofluorescence) by using the extended depth of focus software option of the confocal setup (*see* **Note 5**).
6. Calculate the mean area in square millimeters of PECAM-1-positive cell areas as the integrated sum of pixel values above background level in the overlay image by use of the image analysis software of the confocal laser scanning microscope.

3.4.3. Analysis of Protein Expression in Confrontation Cultures During Tumor-Induced Angiogenesis

Tumor-induced angiogenesis is accompanied by changes in a variety of genes, which may be directly related to angiogenesis (e.g., *HIF-1α, VEGF, Ang1,2, MMPs*) or are associated to cell cycle regulation (e.g., cyclins, CDK-inhibitors) and multidrug resistance (e.g., P-glycoprotein, multidrug resistance-associated protein). Changes in protein expression can be easily recorded by semiquantitative immunohistochemistry and confocal laser scanning microscopy. This method allows the investigation of the specific expression

patterns of protein in the embryoid body part, the tumor spheroid part of the confrontation culture as well as in the confrontation area of both tissues. By the use of semiquantitative immunochemistry we have recently assessed changes in HIF-1α VEGF, P-glycoprotein, HSP27 ***(12)*** as well as of MMPs in different regions of confrontation cultures ***(15)***.

1. Incubate confrontation cultures with antibodies directed against the protein under investigation. Label with appropriate secondary antibodies linked to the Cy5 dye, which allows discrimination against the CMFDA dye used to counterstain the tumor tissue.
2. Because the immunofluorescence gray level values are not necessarily proportional to the amounts of expressed antigen, calibration measurements have to be performed to correlate the immunofluorescence values to the protein levels of antigen. Prepare solutions of Cy5-labeled antibody in PBS in a concentration range of 0.175–5.8 μ*M*.
3. Place 5 μL of fluorescent antibody solution under a cover slip and record images from increasing concentrations of antibody solution.
4. Quantify fluorescence counts (gray level values) from images by using the image analysis facilities of the confocal set up.
5. Prepare graphs from the analyzed data sets. This should result in a linear relation between increasing concentrations of fluorescence dye and Cy5 fluorescence.
6. Calculate the amount of expressed antigen as follows: the number of emitted photons and the concentration of the antibody follow a linear relationship up to a maximum antibody concentration C_{max}, which is defined by $C_{max} = 0.05/2.303 \times x \times \varepsilon$, where x is the thickness of the optical section penetrated by the laser beam and ε represents the molar extinction coefficient (250,000 M^{-1}/cm^{-1} for Cy5). Under the experimental conditions described previously, C_{max} was calculated to be 8.7 μ*M*. The relation between the emitted photons and the concentration of the fluorochrome can be estimated according to Parker's law: $I_F/I_0 = 2.303 \times \phi_r \times x \times C \times \varepsilon$, where I_F is the emitted fluorescence, I_0 is the excitation light intensity, ϕ_r is the fluorochrome-specific fluorescence yield (i.e., $\phi_r = 0.28$ for Cy5), and C is the concentration of the secondary antibody. The slope of this linear relation is dependent on the settings of the confocal microscope: the excitation light intensity, the pinhole settings, the settings of the photomultiplier, as well as the specific properties of the fluorochrome (i.e., the quantum yield and the molar extinction coefficient). Because tumor spheroids, embryoid bodies, and confrontation cultures are incubated during the immunolabeling in a surplus of antibody, the amount of bound primary and secondary antibody directly correlates to the amount of expressed antigen.

3.5. Conclusions

Herein the use of embryonic stem cells of mouse and human origin for studying tumor-induced angiogenesis is demonstrated. Confrontation cultures of embryoid bodies and multicellular tumor spheroids represent the only in vitro model for the investigation of blood vessel and tumor tissue interaction. Previous attempts to study endothelial cell growth into tumor tissue by using

multicellular tumor spheroids in confrontation culture with endothelial cells failed due to a lack of endothelial cell invasion (***16***). In confrontation cultures of embryoid bodies with multicellular tumor spheroids, the time course of endothelial cell invasion into the tumor tissue can be monitored as well as changes in physiological parameters (e.g., intracellular pH, calcium, and reactive oxygen species). The confrontation culture allows changes in protein expression during tumor-induced angiogenesis to be monitored, and may be exploited as in vitro model to study the effects of antiangiogenic agents.

4. Notes

1. It should be possible to study tumor angiogenesis with different types of multicellular tumor spheroids. Apparently the species origin of the tumor cells is not important. In our hands, tumor-induced angiogenesis could be studied with DU-145 (human) cancer cells and mouse ES cells as well as with DU-145 cells and human ES cells. Experiments were furthermore performed by the use of mouse ES cells and breast cancer cells of mouse origin (4T1).
2. Not descended from "Swiss" mice from Rockefeller Institute (probably of wild albino origin). Intensively inbred by Carworth for more than 20 generations. This line was then reduced to a single pair and progeny outbred from that point forward to form a new stock. Caesarean rederived in 1974 from a representative cross section of the Carworth CF-1 colony. Coat color Albino; carries brown behind its albino gene.
3. For this use a 1000 µL (blue tip) Gilson microliter pipet; cut the end of the blue tip with a scissor and flame it to avoid injury of the tissues.
4. Red laser light allows recording of fluorescence in deeper cells layers as compared with blue laser light because of reduced light absorption of the red wavelength.
5. The extended depth of focus option of the confocal set up generates pseudo three-dimensional image of a stack of optical sections. This pseudo three-dimensional image contains all fluorescence information of a tissue block of selected thickness (in our hands 100 µm) and can be used to calculate the area covered by immunolabeled cell areas.

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21

A Novel Experimental Platform for Investigating Cancer Growth and Anti-Cancer Therapy in a Human Tissue Microenvironment Derived From Human Embryonic Stem Cells

Maty Tzukerman and Karl L. Skorecki

Summary

There is no available experimental system wherein human cancer cells can be grown in the context of a mixed population of normal differentiated human cells for testing biological aspects of cancer cell growth (tumor cell invasion, angiogenesis) or response to anti-cancer therapies. Human embryonic stem cells when implanted into immunocompromised mice develop teratomas containing complex structures, comprising differentiated cell types representing the major germline-derived lineages. We sought to determine whether human cancer cells would grow within such teratomas and display properties associated with malignancy such as invasiveness and recruitment of blood vessels. Ovarian cancer cells (HEY), stably expressing an H2A-GFP fusion protein, which allows tracking of tumor cells, were injected into mature teratomas and developed into tumors. The growth, proliferation capacity, invasion, and induction of blood vessel formation were examined. We propose using the novel experimental platform we have described, consisting of human tumor cells growing within a human cellular microenvironment derived from human embryonic stem cells, to develop a preclinical model for investigating and manipulating the stromal response in tumor cell growth, as an additional tool in cancer research.

Key Words: Human embryonic stem cells; tumor microenvironment; tumorigenesis; angiogenesis; cancer cells.

1. Introduction

Human embryonic stem cells (hESC) have the potential to differentiate into different cell types representing derivatives of all three major embryonic lineages *(1,2)*. Teratomas generated after injection of hESC into immunocompromised

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mice, contain a wide variety of differentiated tissues and structures. Using these human differentiated cellular microenvironments, we have been able to demonstrate and investigate the growth and tumorigenic properties of a variety of human cancer cell types.

In recent years, evidence has accumulated that tumorigenesis properties are markedly affected by the surrounding tissue microenvironment at both primary and metastatic sites. It has been demonstrated that tumor progression is associated with extensive remodeling of adjacent tissues to provide a supportive microenvironment for cancer cell proliferation, migration, invasion, and neoangiogenesis required for supporting cancer growth. As an example, human prostate carcinoma-associated fibroblasts can promote tumorigenic transformation in initiated human prostate epithelial cells *(3)*. In more recent studies, it was shown that the connective tissue stroma surrounding neoplastic breast tissue was induced into a state that "remembered" and favored subsequent neoplastic growth in naive breast epithelium *(4,5)*. Also, in recent studies it was shown that the genomic rearrangements characteristic of a given tumor type (e.g., lymphoma), were also evident in the endothelial cells of adjacent tumor-induced blood vessels *(6)*.

The recent appreciation of the important role for the tissue microenvironment has led to the development of novel anti-cancer therapeutic strategies targeted to frustrate stromal response factors, which support tumor growth. Targets for investigation have included proteases, heparinase, and other enzymes expressed by cancer cells or by adjacent stromal cells, which degrade extracellular matrix components and facilitate the release of cytokines and growth factors that stimulate angiogenesis, or support the growth and invasion of cancer cells *(7–9)*. A great deal of attention has been directed at the development of antiangiogenic molecules in particular, some of which have reached clinical trials *(10,11)*.

Truly in vitro models such as focus formation and anchorage-independent growth are not particularly well suited for the investigation of interactions of tumor cells with the surrounding microenvironment of adjacent differentiated human cells and tissue structures or for testing the potential efficacy of therapies directed at the tumor microenvironment. Accordingly, many preclinical studies have been conducted using tumor xenograft growth in immunocompromised mice. However, in the numerous published studies using this model, it has been the murine, rather than the human stromal response, which has been the target of investigation or experimental therapeutic intervention. Therefore, we have used hESC to study the interactions of tumor cells with the surrounding micro environment of differentiated normal human cell tissues and structures.

In a recently published study *(12)*, we injected HEY ovarian cancer cells into hESC-derived teratomas (**Fig. 1**). The HEY ovarian cancer cells had been transfected so as to stably express a histone H_2A-GFP fusion protein to facilitate

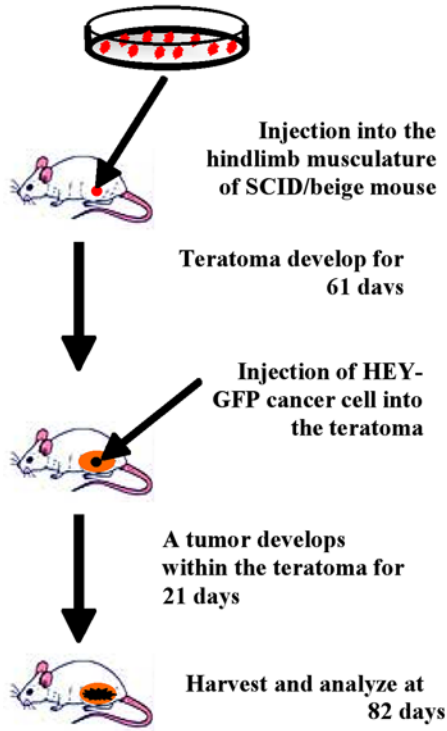

Fig. 1. Schematic representation of the experimental protocol. (Copyright [2003] National Academy of Sciences, USA. Reproduced with permission from **ref.** ***12***.) (Please *see* the companion CD for the color version of this figure.)

the tracking of tumor cell growth and invasion. These HEY-GFP cells were injected into teratomas derived from hESC that had been allowed to mature in SCID/beige mice. After various periods of growth, immunohistochemical analysis of the mixed teratoma-tumor structures confirmed the feasibility of using this approach to examine human tumorigenesis properties in a human cellular microenvironment. The salient findings were: (1) the development of a homogeneous mass of tumor cells with high proliferative capacity within the differentiated microenvironment of the teratoma (**Fig. 2**); (2) the identification of invasion by tumor cells into surrounding differentiated teratoma structures (**Fig. 3**); and (3) the existence of blood vessels of human origin-derived form hESC differentiation, growing adjacent to and within the HEY-GFP derived tumor (**Fig. 4**). Such blood vessels can be identified by using antibodies to human-specific endothelial cell surface antigenic epitopes such as CD31 or CD34. In this regard, we have demonstrated specific staining in teratomas bearing tumors ***(12)***. This contrasts with one previous report that failed to detect human endothelial

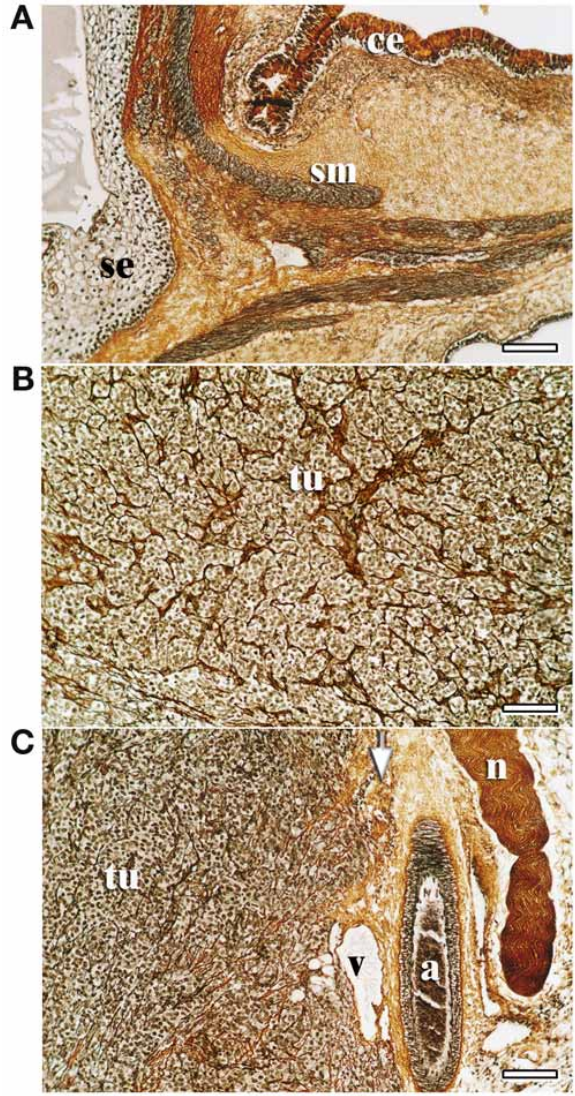

Fig. 2. Morphological appearance of teratoma bearing tumor. Staining of paraffin sections with Gomori technique for reticulin fibers reveals: **(A)** Typical appearance of teratoma derived structure in SCID/beige mice, with derivatives of three embryonic germ layers. se, stratified epithelium of ectodermal origin; m, smooth muscle of mesodermal origin; ce, columnar epithelium with goblet cells of endodermal origin. **(B)** Homogeneous mass of HEY ovarian carcinoma tumor cells (tu). **(C)** Boundary region of tumor cells (tu) adjacent to a differentiated teratoma structure consisting of a neurovascular bundle with a venule (v) arteriole (a) and a nerve (n). Bar = 200 μm in all three panels. (Copyright [2003] National Academy of Sciences, USA. Reproduced with permission from **ref.** ***12***.) (Please *see* the companion CD for the color version of this figure.)

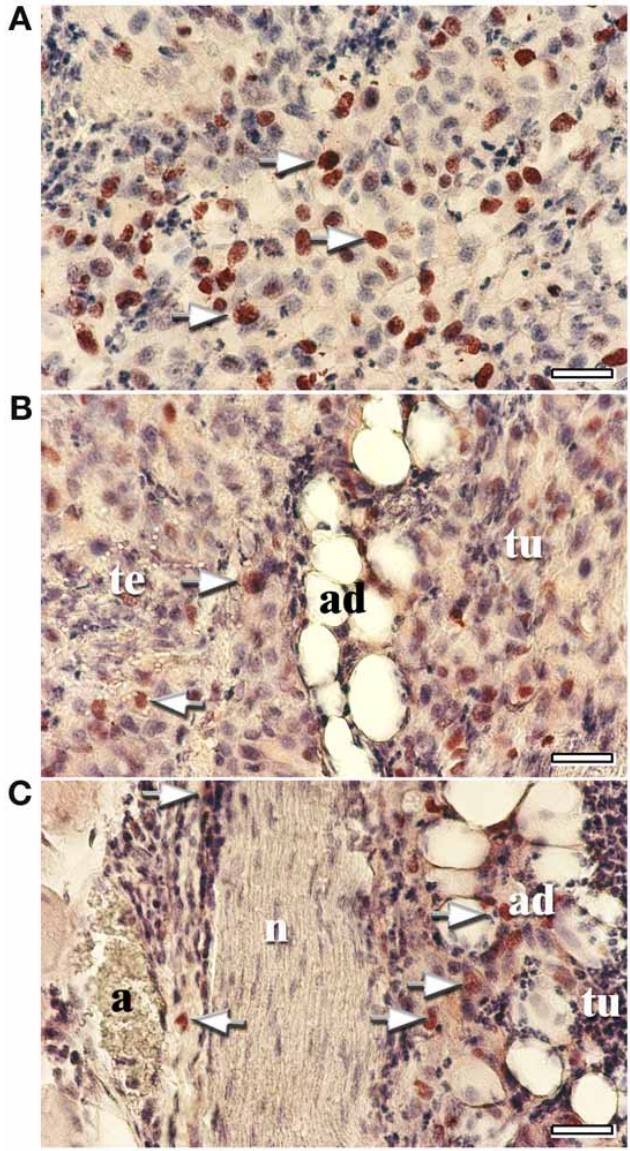

Fig. 3. Infiltration of HEY-GFP cells into human teratoma derived tissue. **(A)** Arrows indicate HEY-GFP positive nuclear immunostaining of HEY-GFP cells in a field of tumor cells (bar = 50 µm). **(B,C)** Arrows show migration of tumor (tu)-derived GFP positive cells into the teratoma (te) derived adipocytes (ad) and crossing adjacent nerve tissue (n). (Bar = 50 µm). (Copyright (2003) National Academy of Sciences, USA. Reproduced with permission from **ref.** *12*.) (Please *see* the companion CD for the color version of this figure.)

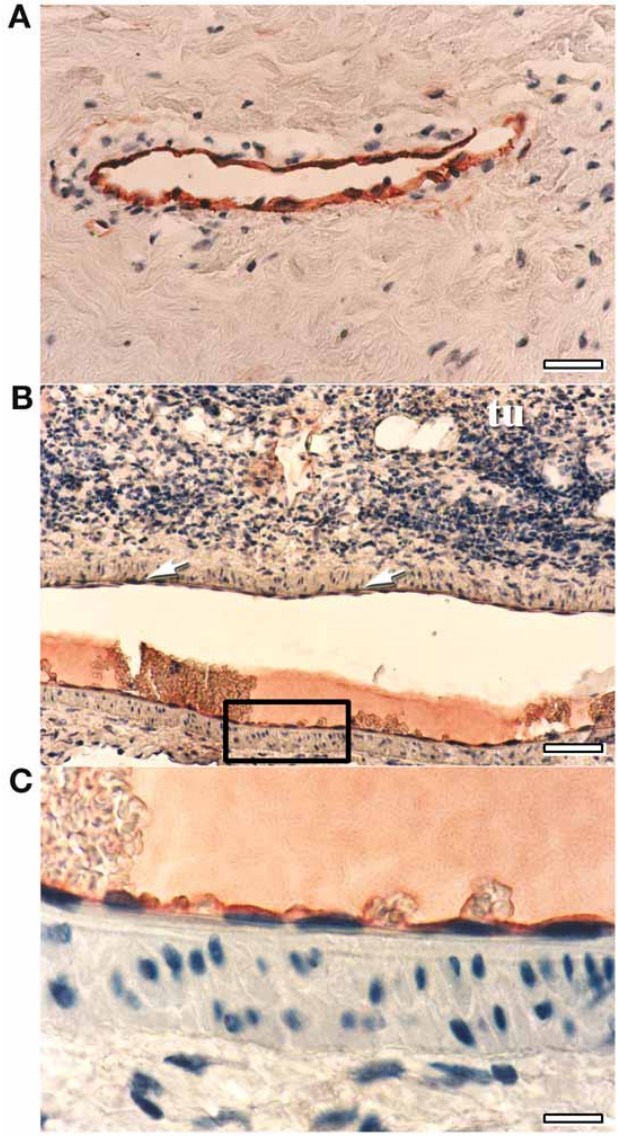

Fig. 4. Identification of blood vessels of human origin adjacent and within the tumor. **(A)** Positive control: a specimen of human breast carcinoma stained with anti CD34 human specific antibody (bar = 50 μm). **(B)** Photomicrographs showing low power magnifications of CD34 positive immunostaining of an arteriole adjacent to a mass of HEY tumor cells growing within a human teratoma (bar = 100 μ*M*). **(C)** Higher power magnification of the inset from **B** demonstrating specificity of staining of the endothelial cell layer (bar = 20 μ*M*). a, arteriole; v, venule; bv, blood vessel; tu, tumor. (Copyright [2003] National Academy of Sciences, USA. Reproduced with permission from **ref.** *12*.) (Please *see* the companion CD for the color version of this figure.)

markers in hESC-derived teratomas ***(13)***, though a more recent report has also detected blood vessels of human origin ***(14)***. Therefore, it will be important to rigorously quantitate differences in blood vessel development between teratomas without and with tumor growth using these immunoreagents. This immunostaining approach can be supplemented with quantitative molecular analysis of the known neovasculogenesis gene expression profile.

Accordingly, in a recent set of studies, we successfully extended this novel experimental approach using human stem cell technology to generate a novel experimental platform in which human tumor cells can be grown in a preclinical setting in a tissue microenvironment consisting of differentiated human cells for investigating cancer growth and anti-cancer therapy. We have examined and compared multiple different human tumor cell types, with varying tumorigenesis properties, and correspondingly found differences in growth, invasion, and angiogenic responses in this new model. We also found significant differences in the response to anticancer therapy in tumors growing in the human cell microenvironment of a teratoma in immunocompromised mice in comparison to the same tumors growing after direct injection into the hind limb muscle tissue of these mice. It remains to be seen whether such differences may be predictive of expected responses in clinical trials. It will also be possible to extend the applications of this model in a number of basic and applied directions. Among others these include the following.

1. Pharmacologic or genetic manipulation of either the tumor cells or the hES-derived stromal cells. These could include stable transfection of the hESC with constructs targeted to modify the levels of expression of key enzymes and growth factors involved in the stromal response to tumor growth and progression.
2. Molecular analysis of hES-derived surrounding stromal cells, isolated from the tumor growths. Isolation of stromal cells of different histologic specificity can be achieved by laser micro dissection and subjected to gene or protein expression analysis on relevant microchip arrays.
3. Vital and functional monitoring of the tumor growth in response to therapeutic intervention, using modern imaging techniques applied to appropriately labeled or marked hES or tumor cells, including hybrid imaging with micro-positron emission tomography/computed tomography, or with luminescence or fluorescence detection.

In the current chapter, we describe the experimental protocols we are using to investigate tumorigenesis properties related to the surrounding human cellular microenvironment derived from hESC. Although the current chapter focuses on the teratoma model illustrated in **Fig. 5**, the principle of growing a human tumor in an hESC-derived human microenvironment can also be extended to in vitro models, such as microinjection of tumor cells into embryoid bodies (*see* **Fig. 5**) ***(15)***.

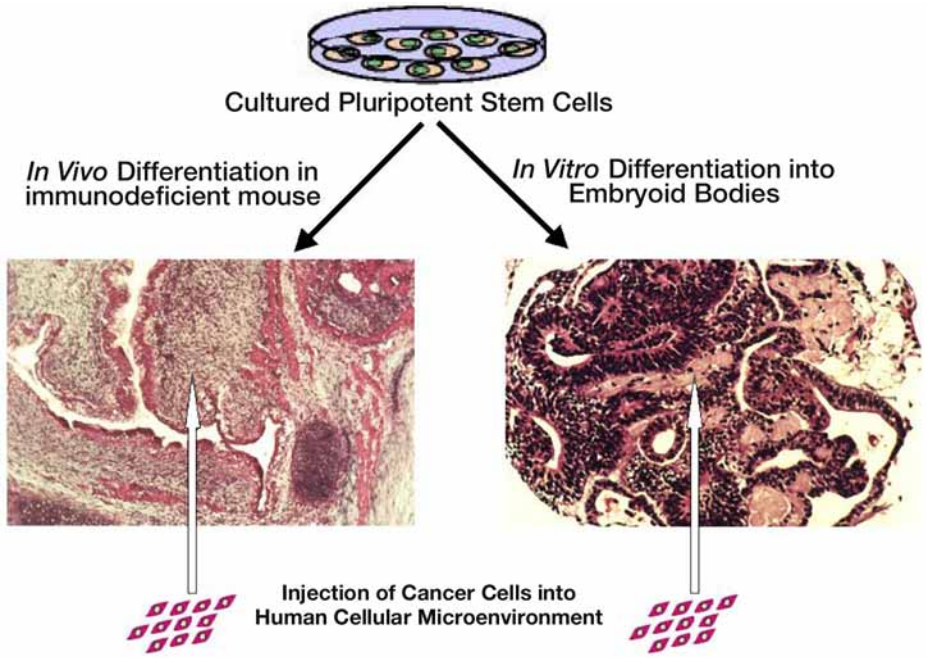

Fig. 5. An experimental platform for growing human tumor cells within a human cellular microenvironment derived from differentiation of human embryonic stem cells in vitro and in vivo. (Copyright [2003] Discovery Medicine. Reproduced with permission from **ref.** *15*.) (Please *see* the companion CD for the color version of this figure.)

2. Materials

2.1. Cancer Cell Line, Reporter Plasmid, and Stable Transfection

1. HEY ovarian cancer cell line (obtained from Ontario Cancer Institute, University of Toronto; Toronto, Ontario, Canada). Grown in the presence of 5% CO_2 and 95% humidity at 37°C.
2. Growth media for HEY cells. RPMI 1640 (Biological Industries, Bet Haemek, Israel; cat. no. 01-100) supplemented with 10% fetal calf serum (FCS), 1% L-glutamine (Biological Industries; cat. no. 03-020) and 1% pen-strep (Biological Industries; cat. no. 03-031-1B). For 500 mL medium: combine 450 mL RPMI 1640, 50 mL FCS, 5 mL 200 m*M* L-glutamine, and 5 mL of 10 mg/mL pen-strep solution.
3. 10-cm culture dish (NUNC, Roskilde, Denmark; cat. no. 179231).
4. Phosphate-buffered saline (PBS) Dulbecco's solution (Invitrogen Corporation, Paisley, UK; cat. no. 14190-094).
5. Trypsin-EDTA (Biological Industries; cat. no. 03-050-1B).
6. The cDNA coding region for the EGFP fused downstream to the histone H2A (kindly provided by M. Brandeis, Hebrew University, Jerusalem, Israel) was

inserted into *Age*I and *Not*I restriction sites of the pEGFP-N1 expression vector (Clontech, Palo Alto, CA; cat. no. 632318).
7. QIAGEN plasmid purification kit (QIAGEN Inc., Santa Clarita, CA; cat. no. 12163).
8. FuGENE 6 transfection reagent (Roche, Indianapolis, IN; cat. no. 1814443).
9. Geneticin-G418 (Life Technologies, Grand Island, NY; cat. no. 11811-098).
10. Inverse fluorescence microscope TS100 (Nikon, Tokyo, Japan).

2.2. Propagation of Undifferentiated hES Cells

2.2.1. Preparation of Mitotically Inactivated Mouse Embryonic Fibroblasts Feeder Layer

1. Mitomycin C (two bottles of 2 mg; Sigma-Aldrich, Rechovot, Israel; cat. no. M-4287): dissolve 4 mg in 500 mL DMEM with L-glutamine medium (Invitrogen Corporation; cat. no. 41965-039) and filter (0.22-μm; Millipore, Billerica, MA; cat. no. SCGPU05 RE). Aliquots of 8 mL are kept at –20°C.
2. 250-mL flasks (NUNC; cat. no. 156472).
3. Sterile pipets, individually wrapped (Greiner, Bio-One, Frickenhausen, Germany; cat. no. 606180).
4. 50-mL sterile tubes (Greiner, Bio-One; cat. no. 227270).
5. Six-well tissue culture plates (NUNC; cat. no. 152795).
6. 10X Trypsin for splitting MEFs (Invitrogen Corporation; cat. no. 15400–054).
7. Medium C: combine 450 mL (90%) DMEM w/ L-glutamine (Invitrogen corporation; cat. no. 41965-039), 50 mL (10%) FCS (Biological Industries; cat. no. 04-121-1A), 2.5 mL (0.5%) pen-strep solution (Biological Industries; cat. no. 03-031-1B).
8. Tabletop Multifuge3s centrifuge (Herause, Hanau, Germany).
9. Medium A: combine 400 mL (80%) DMEM with glutamine (Invitrogen Corporation; cat. no. 41965-039), 100 mL (20%) fetal bovine serum defined (Hyclone, Logan, UT; cat. no. SH30070,03), 5 mL (1%) nonessential amino acids (Invitrogen Corporation; cat. no. 11140-035), 1 mL (0.1 mmol/L) 2-mercaptoethanol (Invitrogen Corporation; cat. no. 31350-010).
10. Gelatin type A (Sigma-Aldrich; cat. no. G-1890): dissolve 500 mg in 500 mL sterile double distilled H_2O (1 mg/mL = 0.1%).
11. Medium B: combine 400 mL (80%) knockout DMEM (Invitrogen Corporation; cat. no. 10829-018), 100 mL (20%) knockout SR serum replacement (Invitrogen Corporation; cat. no. 10828-028), 5 mL (1%) nonessential amino acids (Invitrogen Corporation; cat. no. 11140-035), 1 mL (1 mmol/L) L-glutamine (Biological Industries; cat. no. 03-020), 1 mL (0.1 mmol/L) 2-mercaptoethanol (Invitrogen Corporation; cat. no. 31350-010), 1 mL (4 ng/mL) human recombinant basic fibroblast growth factor (Invitrogen Corporation, cat. no.13256-029).
12. Basic fibroblast growth factor. Dissolve a 10-μg vial of lyophilized basic fibroblast growth factor in 5 mL 0.1% BSA solution (0.1 g bovine serum albumin-fraction V, Roche Diagnostics; cat. no. 735078) in 100 mL PBS (Invitrogen Corporation; cat. no. 14190-094). Store 1-mL aliquots at –20°C.

2.2.2. Propagation of Undifferentiated hES Cells

1. Human embryonic stem cells clone 9.1 (kindly provided by J. Itskovitz-Eldor, Technion and Rambam Medical Center, Haifa, Israel).
2. Collagenase type IV (Invitrogen Corporation; cat. no. 17104-019). For a 1 mg/mL solution: dissolve 50 mg collagenase in 50 mL knockout DMEM (Invitrogen Corporation; cat. no. 10829-018), filter through a 0.45-µm membrane filter (Millipore; cat. no. SCHVU01 RE). Aliquots of 10-mL are kept at 4°C.
3. Sterile cell scraper (Greiner, Bio-One; cat. no. 541070).
4. 50-mL sterile tubes (Greiner, Bio-One; cat. no. 227270).
5. Sterile pipets individually wrapped (Greiner, Bio-One; cat. no. 606180).
6. Tabletop Multifuge3s centrifuge (Herause).
7. Medium B (*see* **Subheading 2.2.1., item 11**).

2.3. In Vivo Differentiation of hES Cells

1. Sterile cell scraper (Greiner, Bio-One; cat. no. 541070).
2. 50-mL sterile tubes (Greiner, Bio-One; cat. no. 227270).
3. Sterile pipets, individually wrapped (Greiner, Bio-One; cat. no. 606180).
4. Tabletop Multifuge3s centrifuge (Herause).
5. Medium B (*see* **Subheading 2.2.1., item 11**).
6. 70% Ethanol (dilute from absolute ethanol; FRUTAROM, Haifa, Israel; cat. no. 5551640). Mix 70 mL absolute ethanol with 30 mL double distilled H_2O.
7. 1-mL syringe (B.D. Plastipak, Becton Dickinson, Madrid, Spain; cat. no. 300013).
8. 25-G sterile needle (B.D. Microlance, Becton Dickinson; cat. no. 300600).

2.4. Injection of Tumor Cells Into the Teratoma

1. 50-mL sterile tubes (Greiner, Bio-One; cat. no. 227270).
2. Sterile pipets, individually wrapped (Greiner, Bio-One; cat. no. 606180).
3. Tabletop Multifuge3s centrifuge (Herause).
4. RPMI 1640 (Biological Industries; cat. no. 01-100) supplemented with 10% FCS, 1% L-glutamine (Biological Industries; cat. no. 03-020) and 1% pen-strep solution (Biological Industries; cat. no. 03-031).
5. Trypsin-EDTA (NUNC; cat. no. 152795).
6. 1-mL syringe (B.D. Plastipak, Becton Dickinson; cat. no. 300013).
7. 21-G sterile needle (B.D. Microlance, Becton Dickinson; cat. no. 300600).

2.5. Histological Analysis

2.5.1. Processing Tissue for Histological Analysis

1. Dissecting tools: fine-tipped Dumont forceps (Fine Science tools, Vancouver, Canada; cat. no. 11251-30), straight fine scissors 10.5 cm (Fine Science tools; cat. no. 14094-11).
2. 50-mL sterile tubes (Greiner, Bio-One; cat. no. 227270).
3. Neutral buffered formalin (NBF): 40% formaldehyde (Bio-Lab, Jerusalem, Israel; cat. no. 72026), 46 m*M* Na_2HPO_4 (Merck, Whitehouse Station, NJ; cat. no. 10686),

33 m*M* NaH_2PO_4 (CARLO ERBA Reagenti, Milano, Italy; cat. no. 480087). For 3 L of NBF: combine 300 mL of 40% formalin, 12 g $Na_2HPO_4 \cdot 2H_2O$, 19.5 g Ma_2HPO_4 anhydrous and 2700 mL of H_2O.
4. 70, 95, and 100% ethanol (*see* **Subheading 2.3., item 6**); solutions prepared with double distilled H_2O.
5. Chloroform (Bio-Lab; cat. no. 71061).
6. Embedding wax: Paraplast Plus Wax (Kendall Tyco Health Care Group, Mansfilled MA; cat. no. 502004).
7. Microtome (Leica, Bensheim, Germany; model no. LeicaRM 2135).
8. Poly-L-lysine-coated slides, Super FrostPlus (Manzel-Glaser, Braunschweig, Germany; cat. no. MG051101).

2.5.2. Hematoxylin and Eosin Staining of Paraffin Sections

1. Coplin Jar (Finkelman, Petach Tikva, Israel; cat. no. FNK 25146).
2. Ethanol (*see* **Subheading 2.3., item 6**).
3. 3% Acetic acid (Merck; cat. no. 100063).
4. Hematoxylin solution (Merck; cat. no. 4305).
5. 1% Eosin-Y (Harleco, Kansas City, MO; cat. no. Na 45380) in 95% ethanol.
6. Upright Microscope CX31 equipped with DP70 camera (Olympus, Lake Success, NY).

2.6. Immunohistochemistry

1. Coplin jar (Finkelman; cat. no. FNK 25146).
2. Xylene (Gadot, Haifa, Israel; cat. no. 1330-20-71).
3. Absolute ethanol (*see* **Subheading 2.3., item 6**).
4. Microwave (Electrolux supplemented with probe).
5. 3% citrate buffer (pH 6.1). Prepare 0.1 *M* citrate acid solution: 1.921 g citric acid (FRUTAROM; cat. no. 55110) in 100 mL double-distilled H_2O. Mix 9.1 mL with 40.9 mL 0.2 *M* Na_2HPO_4 (Merck; cat. no. 10686), add double-distilled H_2O to 100 mL.
6. 3% hydrogen peroxide (H_2O_2) (Merck, cat. no. 107209): dilute 3 mL in 97 mL methanol (FRUTAROM; cat. no. 55562380).
7. PBS Dulbecco's solution (Invitrogen Corporation; cat. no. 14190-094).
8. Non-immune goat serum blocking solution (Histostain-SP kit; Zymed Lab Inc., San Francisco, CA; cat. no. 95-6543B).
9. Antibodies: rabbit polyclonal anti-GFP (1:2000) (Molecular Probes, Eugene, OR; cat. no. A-6455), mouse monoclonal anti-human CD34 (1:50) (DakoCytomation, Glostrup, Denmark; M-7165), rabbit polyclonal anti-mouse CD31 (1:2000) (kindly provided by J. Mardi, Yale University, New Haven, CT).
10. Secondary antibodies: goat anti-rabbit (Zymed Lab Inc.; cat. no. 50-235), anti-mouse biotinylated secondary antibody (Histostain-SP kit; Zymed Lab Inc.; cat. no. 95-6543B).
11. Pre-immune rabbit (Zymed, Lab Inc.; cat. no. 50-061) or mouse (Zymed, Lab Inc.; cat. no. 50-235) sera (for negative controls).
12. AEC-substrate chromogen kit (Zymed, Lab Inc.; cat. no. 00-2007).
13. Hematoxylin solution (Merck; cat. no. 4305).

2.7. Extraction of RNA From Teratomas, Teratomas Bearing Tumors, and From Tumors

1. Dissecting tools (*see* **Subheading 2.5.1., item 1**).
2. Scalpel (size 10; Albion Surgical Ltd., Sheffield, England; cat. no. 1531746).
3. 50-mL sterile tubes (Greiner, Bio-One; cat. no. 227270).
4. Sterile pipets, individually wrapped (Greiner, Bio-One; cat. no. 606180).
5. Tri-reagent (Molecular Research Center Inc., Cincinnati, OH; cat. no. TR-118).
6. Homogenizer POLY TRON 2100 (Kinematica, Lucerne, Switzerland).
7. 70% ethanol (*see* **Subheading 2.3., item 6**).
8. DEPC-H_2O (Biological Industries; cat. no. 01-852-1A).
9. 1-bromo-3-chloro-propane (Sigma-Aldrich; cat. no. B-9673).
10. Centrifuge (Sorval, Asheville, NC; model no. RC5C).
11. Isopropanol (FRUTAROM; cat. no. 5553120).
12. 75% ethanol (*see* **Subheading 2.3., item 6**).
13. RNAsin (Promega, Madison, WI; cat. no. N-211A).

2.8. Removal of Genomic DNA From RNA Samples

1. DNase I RNase-free (Roche; cat. no. 776-785).
2. H_2O saturated phenol solution (Biological Industries; cat. no. 01-852-1A).
3. Chloroform: isoamyl alcohol (24:1) (Bio-Lab; cat. no. 03072301).
4. 3 *M* NaAc pH 5.5 (Merck; cat. no. 106268).
5. Micro-Centrifuge 5417R (Eppendorf, Hamburg, Germany).
6. 70% ethanol (*see* **Subheading 2.3., item 6**).
7. DEPC-H_2O (Biological Industries; cat. no. 01-852-1A).
8. RNAsin (Merck; cat. no. 4305).
9. Spectrophotometer UV/Visible Ultraspec 200 (Pharmacia Biotech, Piscataway, NJ).

2.9. Molecular Analysis of Gene Expression Pattern in Teratomas Bearing Tumors

1. QuantiTect SYBR Green Reverse Transcription Polymerase Chain Reaction (RT-PCR) kit (QIAGEN Inc.; cat. no. 204243).
2. Rotor-gene 2000 (Corbett Research, Sidney, Australia).

3. Methods

3.1. Establishing Cancer Cells Stably Expressing EGFP

1. Seed 10^4–5×10^5 cells per 10-cm tissue culture dish (depending on the size and the proliferation rate) in the appropriate culture media.
2. Grow for 24 h.
3. Remove culture media and add fresh media.
4. Mix 95 µL media with 5 µL FuGENE6 transfection reagent.
5. Incubate for 5 min at room temperature.
6. Add 1 µg pEGFP-N1 vector DNA (transfection grade) dissolved in H_2O, mix and incubate for an additional 15 min at room temperature.

7. Add mixture to the media in the culture dish and mix well.
8. Incubate for 48 h in the appropriate cell growing conditions.
9. Examine the transfection efficiency in the culture dish using an inverse fluorescence microscope.
10. Remove the culture media, wash cells once with PBS, add trypsin, incubate at 37°C for 5 min, and collect the cells.
11. Split the cells into 10–15 × 10-cm dishes (depend on the transfection efficiency).
12. Add 300 μg/mL G418 into the growing media for selection of stably Neo-resistance gene expressing cells (that also express the EGFP).
13. Transfer single resistant colonies that express EGFP into a 24-well plate and continue propagating the cells.

3.2. Propagation of Undifferentiated hESC

3.2.1. Preparation of Mitotically Inactivated Mouse Embryonic Fibroblasts Feeder Layer

1. Remove mouse embryonic fibroblasts (MEFs) growing media from the 250-mL flask.
2. Add 10 mL of mitomycin C solution to cover the cells in the flask.
3. Incubate for at least 2 h in the presence of 5% CO_2 at 37°C.
4. Remove mitomycin C solution and add 2 mL of 10X Trypsin solution to the cells.
5. Incubate for 10 min.
6. Add 5 mL medium C and dissociate the cells by mixing up and down with a 5-mL sterile pipet.
7. Transfer cells into 50-mL tube and centrifuge for 5 min at 680*g* using a tabletop centrifuge.
8. Remove media and resuspend cells in 10 mL medium A.
9. Count the cells and dilute them to get 1.8×10^6 cells per 3 mL medium A.
10. Use six-well plates pre-coated with 0.1% gelatin for 24 h.
11. Remove gelatin solution and add 2 mL medium B.
12. Into each well, seed 0.5 mL of the cell suspension from **step 9**.
13. The plates are ready for seeding hES cells after 24 h.

3.2.2. Propagation of Undifferentiated hES Cells

1. Remove medium A from MEFs feeder layer and seed hES cells in 2 mL medium B per well. Cultures are grown in 5% CO_2, 95% humidity at 37°C.
2. Feed hES cells with fresh medium B every day until colonies are formed.
3. Passage cultures routinely every 4–5 d (*see* **Note 1**).
4. For passaging the cells, remove medium from the cells.
5. Add 0.5 mL 0.1% collagenase type IV.
6. Incubate cells for 30 min at 37°C.
7. Collect the cells using a cell scraper into a 50-mL tube and centrifuge for 5 min at 540*g* using a tabletop centrifuge at room temperature.

8. Resuspend cells in medium B and seed on a fresh plate containing MEFs feeder layer.

3.3. In Vivo Differentiation of hESC

1. Collect colonies of undifferentiated hESC using cell scraper into a 50-mL tube in medium B (*see* **Note 2**).
2. Centrifuge for 5 min at 540*g* using a tabletop centrifuge at room temperature.
3. Remove media as to have the pellet of cells in 100 µL media.
4. Swab the hind leg of a 4-wk-old SCID/beige male mouse with 70% ethanol.
5. Inject cells in 100 µL media into the hind limb musculature using a 23-G needle (*see* **Note 3**).

3.4. Injection of Tumor Cells Into the Teratoma

1. Collect cancer cells expressing EGFP using Trypsin into a 50-mL tube.
2. Count the cells and resuspend to have 10^6 cells in 100 µL growing medium.
3. Inject cancer cells directly into a well-established (*see* **Note 4**) teratoma using a 21-G needle.
4. Allow tumors to develop within the teratomas (*see* **Note 5**).
5. Harvest teratomas bearing tumors and control teratomas for analyses.

3.5. Histological Analysis

3.5.1. Processing Tissue for Histological Analysis

1. Fix harvested teratomas and teratomas bearing tumors in 10% NBF for 48 h in 50-mL sterile tubes.
2. Remove NBF and dehydrate the tissues in graduated ethanol: 70% ethanol for overnight at 4°C, 95% ethanol; three times for 20 min and 100% ethanol; three times for 30 min at room temperature.
3. Clear tissues in chloroform; two times for 20 min.
4. Embed tissue in paraffin; three times for 1 h at 60°C.
5. Mount 6-µm paraffin sections on Super FrostPlus microscope slides.

3.5.2. Hematoxylin and Eosin Staining of Paraffin Sections

1. Deparaffinize paraffin sections in a Coplin jar containing xylene for 20 min at room temperature.
2. Hydrate slides in graduated ethanol solutions as follow: 100% ethanol; two times for 5 min and 95% ethanol; two times for 5 min. Wash with water until the solution is clear.
3. Soak the slides in 3% acetic acid for 3 min.
4. Stain sections with hematoxylin for 8 min (*see* **Note 6**).
5. Rinse slides with tap water.
6. Stain sections with eosin for 2 min (*see* **Note 6**).
7. Examine histological performance of teratomas and teratomas bearing tumors using a microscope.

3.6. Immunohistochemistry

1. Deparaffinize slides by soaking in xylene in a Coplin jar for 20 min at room temperature.
2. Dehydrate slides in graduated ethanol solutions as follow: 100% ethanol; two times for 5 min and 95% ethanol; two times for 5 min. Wash with water until the solution is clear.
3. Retrieve antigens using a microwave exposure at 90°C for 8 min in 3% citrate buffer (pH 6.1).
4. Inactivate endogenous peroxidases with 3% hydrogen peroxide (H_2O_2) in methanol for 30 min at room temperature.
5. Wash slides in distilled water and in PBS three times for 2 min.
6. Block nonspecific binding by incubating sections in 10% nonimmune goat serum for 1 h (for GFP) and for 24 h (for CD31 and CD34) at 4°C.
7. Incubate slides for 24 h at 4°C with the primary antibodies: rabbit polyclonal anti-GFP (1:2000), mouse monoclonal anti-human CD34 (1:50), rabbit polyclonal anti-mouse CD31 (1:2000). Use preimmune rabbit or mouse sera as negative controls.
8. Incubate slides with goat anti-rabbit or anti-mouse biotinylated secondary antibody for 10 min at room temperature.
9. Incubate with streptavidin-peroxidase conjugate for 10 min at room temperature.
10. Perform detection of immunostaining using Histostain-SP kit (AEC; 3-amino-9-ethylcarbazole), according to the manufacturer's instructions.
11. Counterstain with hematoxylin (*see* **Note 7**).

3.7. Extraction of RNA From Teratomas, Teratomas Bearing Tumors, and From Tumors

1. Harvest teratomas, teratomas bearing tumors, and tumors (*see* **Note 8**).
2. Wash tissues with PBS and cut into small pieces using a scalpel (*see* **Notes 9** and **10**).
3. Transfer tissues into premarked 50-mL sterile tubes and snap freeze in liquid nitrogen. Maintain frozen tissues at –80°C until RNA extraction.
4. Homogenize frozen tissue in Tri-reagent: 1 mL Tri-reagent per 50–100 mg tissue, using a homogenizer (precleaned with 70% ethanol and then with DEPC-H_2O).
5. Homogenize at medium speed until the tissue is completely homogenized. Clean homogenizer in between different tissue samples.
6. Incubate homogenate at room temperature for 5 min.
7. Add 0.1 mL 1-bromo-3-chloro-propan per 1-mL homogenate.
8. Incubate at room temperature for 10 min.
9. Transfer homogenate solution into 2-mL Eppendorf tubes and centrifuge at 15,500*g* for 15 min at 4°C using an Eppendorf Micro-Centrifuge.
10. Carefully transfer the upper phase into a clean sterile tube.
11. Add 0.5 vol of isopropanol (according to the initial volume of Tri-reagent), mix carefully and incubate for 5 min at room temperature.
12. Centrifuge at 15,500*g* for 15 min at 4°C using an Eppendorf Micro-Centrifuge.

13. Carefully aspirate the supernatant and wash with 75% cold ethanol solution (–20°C).
14. Centrifuge at 15,500*g* for 15 min at 4°C using an Eppendorf Micro-Centrifuge.
15. Aspirate the supernatant and invert the tube to air-dry the pellet for 20–30 min.
16. Dissolve the pellet in DEPC-H_2O supplemented with RNAsin (RNase inhibitor) as required for the size of the pellet.

3.8. Removal of Genomic DNA From RNA Samples

1. Digest 50–100 µg total RNA with 50 µL of DNaseI (RNase-free) at 37°C for 1 h.
2. Add 0.5 vol of H_2O saturated phenol (according to the RNA solution) and mix well.
3. Add 0.5 vol of chloroform:isoamyl alcohol (24:1) and mix.
4. Centrifuge at 15,500*g* for 15 min at room temperature using an Eppendorf Micro-Centrifuge.
5. Transfer the aqueous phase (the top phase) to a fresh microtube.
6. Precipitate RNA by adding 0.1 vol of 3 *M* NaAc pH 5.5.
7. Add 2 vol of 100% ethanol, mix and incubate at –80°C for 1 h.
8. Centrifuge at 15,500*g* for 20 min at 4°C using an Eppendorf Micro-Centrifuge.
9. Wash the pellet with 200 µL cold 70% ethanol (RNase-free, –20°C).
10. Spin again for 10 min at 4°C.
11. Discard the supernatant, air-dry the pellet and resuspend in DEPC-H_2O supplemented with RNAsin.
12. Quantify RNA by reading the absorption at 260 nm and calculate the concentration (*see* **Note 11**).
13. Dilute RNA to 1 µg/µL with DEPC-H_2O in order to be used for RT-PCR and store samples at –80°C.

3.9. Molecular Analysis of Gene Expression Pattern in Teratomas Bearing Tumors: One-Step RT-PCR Using a Real-Time Thermal Cycler

1. Use 40 ng RNA from each sample for one-step real-time RT-PCR.
2. Examine each sample in quadruplicate using the QuantiTect SYBR Green RT-PCR kit.
3. For each reaction mix the following: 2.5 µL RNA (40 ng), 1 µL primer 1 (100 ng), 1 µL primer 2 (100 ng), 12.5 µL 2X PCR buffer, 0.5 µL RT-mix, and DEPC-H_2O to 25 µL.
4. Use the following conditions: for RT, 30 min at 50°C. For PCR, initial activation step, 15 min at 95°C (*see* **Note 12**).
5. Use the following conditions for amplification: denaturation, 15 s at 94°C; annealing, 30 s at 50–60°C (*see* **Note 13**); extension, 30 s at 72°C; data acquisition, 15 s at X°C (*see* **Note 14**).
6. Use 35–45 cycles depending on the abundance of the target gene.
7. Perform a melting curve analysis of the RT-PCR products to verify specificity and identity of the PCR products.

8. Check the specificity of the RT-PCR products by agarose gel electrophoresis.
9. For each sample perform Real-Time RT-PCR analysis for a reference gene as an internal control (e.g., *β-actin* gene, *GAPDH* gene).

4. Notes

1. Undifferentiated cells are routinely passaged every 4–5 d to avoid cell differentiation.
2. Usually 1 × 6-well plate overloaded with colonies (approx 5×10^6 cells) is sufficient for developing a teratoma.
3. Teratomas formed are palpable at 6–7 wk after injection of hESC.
4. Teratomas should be allowed to develop for an additional 3 wk before the injection of cancer cells.
5. The period for developing tumor within the teratomas depends on the aggressiveness of the cancer cell-derived tumor.
6. Hematoxylin is a basic dye that stains the basophilic components (cell nucleus and acidic structures). Eosin is an acidic dye that stains acidophilic components (cytoplasmic proteins).
7. All stages are performed in a humid atmosphere ("humid chamber") and at room temperature. Washing with PBS buffer pH 7.4 was performed between stages.
8. Plain teratomas and tumors derived from direct injection of cancer cells into the hind limb musculature are used as controls for teratomas bearing tumors.
9. Because the teratoma tissue is heterogeneous, it is important to extract RNA from all of it for molecular analysis of human origin vasculogenesis.
10. Each teratoma generated from in vivo differentiation of hESC represents a unique pattern of differentiation events regarding the relative proportion and type of different cells and structures. Therefore it is important to use a group of at least four or five different injected mice for each case.
11. The RNA concentration is calculated according to the equation: 1 OD_{260} = 40 μg/mL.
12. This heating step is activating HotStarTaq DNA Polymerase, deactivating reverse transcriptase, and denaturing the cDNA template.
13. Annealing temperature should be determined for each pair of primers, approx 5–8°C below T_m of primers.
14. To suppress fluorescence reading caused by the generation of primer-dimers, an additional step can be added to the three-step cycling protocol. The temperature should be above the T_m of primer-dimers but approx 3°C below the T_m of the specific PCR product.

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The Analysis of Mitochondria and Mitochondrial DNA in Human Embryonic Stem Cells

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Summary

As human embryonic stem cells (hESCs) undergo differentiation, they express genes characteristic of the lineage for which they are destined. However, fully differentiated individual cell types can be characterized by the number of mitochondria they possess and the copies of the mitochondrial genome per mitochondrion. These characteristics are indicative of a specific cell's requirement for adenosine triphosphate (ATP) and therefore cellular viability and function. Consequently, failure for an ESC to possess the full complement of mitochondria and mitochondrial DNA (mtDNA) could limit its final commitment to a particular fate. We describe a series of protocols that analyze the process of cellular mitochondrial and mtDNA differentiation during hESC differentiation. In addition, mtDNA transcription and replication are key events in cellular differentiation that require interaction between the nucleus and the mitochondrion. To this extent, we describe a series of protocols that analyze the initiation of these key events as hESCs progress from their undifferentiated state to the fully committed cell. Last, we describe real-time polymerase chain reaction protocols that allow both the identification of mtDNA copy number and determine whether mtDNA copy is uniform (homoplasmy) in its transmission or heterogeneous (heteroplasmy).

Key Words: Mitochondria; mitochondrial DNA; human embryonic stem cells; differentiation; cardiomyocytes; homoplasmy; heteroplasmy; transcription and replication.

1. Introduction

Human embryonic stem cell (ESC) commitment and successful differentiation necessitates differential expression of nuclear genes. For example, ESC-derived

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cardiomyocytes are marked by the specific expression of α-MHC, ANF, and Nkx2.5 (*1*). However, different cell types will have very distinctive metabolic requirements, suggesting that cellular differentiation will also incorporate mitochondrial and mitochondrial DNA (mtDNA) differentiation (*2*). Loss of mitochondrial function can trigger the onset of apoptosis (*see* **ref.** *3* for review) and clinically can result in compromised cellular function and the onset of severely debilitating or lethal mtDNA diseases arising specifically through failure of the electron transfer chain (ETC) to generate sufficient levels of adenosine triphosphate (ATP) (*4*).

The ETC is encoded for by both chromosomal and mtDNA. Within each mitochondrion, there are one or more copies of mtDNA. This genome encodes 13 of the subunits of the ETC complexes, associated with the process of oxidative phosphorylation (OXPHOS), along with 22 tRNAs and 2 rRNAs. Commonly, mutation or depletion of this genome can result in the onset of mitochondrial disease, such as cardiomyopathy *(4)*. The mixing of two or more mtDNA populations results in heteroplasmy, consisting of wild type and mutated/deleted molecules, and the clinical onset of a mtDNA specific disease is governed by the degree of heteroplasmy *(5,6)*. However, embryo reconstruction—for example, cytoplasmic transfer *(7)* and nuclear transfer *(8)*—can also result in heteroplasmy, in which donor mtDNA can supplement the recipient oocyte's mtDNA population giving rise to the presence of two or more distinct mtDNA molecules being present *(9)* reviewed in **ref.** *2*).

Each cell type can be characterized by its number of mitochondria. This has been especially highlighted in muscle (*see* **ref.** *10* for review). Furthermore, the number of mtDNA genomes per mitochondrion is also specific to each cell type. For example, there is a significant difference between skeletal and cardiac muscle, with 3650 ± 620 and 6790 ± 920 mtDNA copies per diploid nuclear genome, respectively ($p = 0.006$; *11*). Similar differences have been observed in, for example, peripheral blood mononuclear cells and subcutaneous fat *(12)*, cultured fibroblasts *(13)*, and bovine oocytes and bovine fetal heart fibroblasts *(14)*. Consequently, the final commitment of an ESC to a cellular phenotype would have to include this subtle distinction.

1.1. mtDNA Transcription and Replication

mtDNA copy number is regulated through a variety of mtDNA transcription and replication factors. Transcription and replication of the mtDNA genome are semiautonomous and require interaction between the nucleus and the mitochondria. The nuclear-encoded mitochondrial transcription factor A (TFAM) *(15)*, regulates the number of mtDNA transcripts *(16)*. Low levels of TFAM are associated with mtDNA depletion and several severe mtDNA diseases, for example, infantile mitochondrial myopathy *(17)*. Regulation of TFAM expression

is a precise phenomenon associated with key developmental markers. For example, murine heterozygous TFAM knockout embryos have reduced mtDNA copy number and phenotypically the offspring have respiratory chain deficiency of the heart. Tissue-specific knockout mice also have reduced respiratory chain enzyme activities accompanied by decreased mitochondrial ATP production coupled to mtDNA depletion *(18)*. In addition, homozygous TFAM knockout embryos suffer from severe mtDNA depletion and abolished OXPHOS. Most interestingly, these $TFAM^{-/-}$ embryos proceed through implantation and gastrulation, but die prior to embryonic d 10.5 *(19,20)*. This arises because mtDNA copy number remains unchanged until at least the blastocyst stage *(19,21–23)*, with each newly divided blastomere possessing fewer copies of the genome following each stage of embryonic division *(24)* because there is no active mtDNA replication. Those blastomeres giving rise to the inner cell mass are those that generate fetal tissue and are, most likely, harvested to generate undifferentiated human ESC (hESC) lines. Consequently, our ability to determine the exact timing of the initiation of transcription and replication is vital to the maintenance of the undifferentiated ESC.

Those ESCs generated through, for example, NT or cross-species NT *(25)* or interspecific crossing could have compromised OXPHOS function. For example, cybrid technology, the transfer of a donor nucleus into an enucleated recipient cell by cellular fusion, demonstrates that rat mtDNA against a mouse nuclear background results in compromised ATP production though the mtDNA is replicated, transcribed, and translated efficiently *(26,27)*. Additionally, primate foreign mtDNA will only be amplified in the host cell when its own mtDNA has been eliminated *(28)*. Furthermore, with somatic donor cell NT, the likelihood of the donor cell being completely reprogrammed is variable. As a result, the reconstructed embryo may not clearly generate differentiated cell types reflecting the appropriate mitochondrial characteristics of that cell type (*see* **ref.** *2* for discussion).

TFAM function necessitates interaction with other factors such as mitochondrial transcription factor B1 (TFB1M) and B2 (TFB2M), which regulate basal transcription of mammalian mtDNA, especially in the human ovary *(29)*, nuclear respiratory factor (NRF)-1, and polymerase γ (PolG). Antiretroviral drugs used to reduce HIV load in HIV-positive patients produce a steady decline in mtDNA wild type *(30,31)* through the inhibition of γ (PolG) *(32)*. Indeed, loss of γ (PolG) function can result in cells being depleted of mtDNA and dying in culture *(33)*. Furthermore, disruption to NRF-1 leads to reduced expression of TFAM and decreased levels of mtDNA *(34)*.

MtDNA-depleted cells *(35)* provide an excellent baseline model for determining the levels of expression of mtDNA-encoded genes and the nuclear-encoded mtDNA-specific transcription and replication factors. In this respect, hESCs

can be treated with ethidium bromide, a known mtDNA depletion agent that also reduces mtDNA transcription factor activity in some cell types *(36)*.

1.2. MtDNA Transmission

MtDNA does not follow the normal Mendelian mechanism of inheritance and is thus transmitted through the female germ line *(37,38)*. An oocyte contains far more cytoplasm than the fertilizing sperm cell. This results in the mother contributing approximately 100,000 mitochondria to the embryonic cytoplasm and greatly diluting the 100 mtDNA copies *(39)* or so provided by the father. Sperm mitochondria are also thought to be degraded and actively destroyed by ubiquitination in the oocyte cytoplasm and subsequent proteolysis *(40)*. However, recent reports suggest that paternal mtDNA can avoid destruction during human embryonic development to the blastocyst stage *(41)*. Furthermore, sperm mtDNA has been identified in a male patient presenting with a mitochondrial disease arising from a mutation present in his father's mtDNA *(42)*. Additionally, nonhuman primates generated through blastomere nuclear transfer possess sperm mtDNA *(9)*. This raises concerns for the generation of ESC lines using such technology and such cell lines should be screened for heteroplasmy.

Here, we define a series of protocols that can be used to describe the process of cellular mitochondrial and mtDNA differentiation during hESC differentiation. Furthermore, we describe polymerase chain reaction (PCR) protocols that will define the initiation of mtDNA transcription and replication as hESCs progress through migration to the fully committed cell. Last, we describe real-time PCR protocols that allow both the identification of mtDNA homoplasmy or heteroplasmy and mtDNA copy number.

2. Materials

2.1. Culturing of hESCs for Mitochondrial Analysis

1. Dulbecco's modified Eagle's medium (DMEM) (1X) (4.5 g/L D-glucose) (Invitrogen Life Technologies, Paisley, UK; cat. no. 31053-028).
2. Knockout serum replacement (Invitrogen Life Technologies; cat. no. 10828-028).
3. Human recombinant bFGF-2 (10 μg) (Invitrogen Life Technologies; cat. no. 13256-029).
4. 200 m*M* L-Glutamine (Invitrogen Life Technologies; cat. no. 25030-024).
5. MEM nonessential amino acids (100X) (Invitrogen Life Technologies; cat. no. 11140-122).
6. 100 U/mL penicillin/0.1 mg/mL streptomycin (Sigma, Gillingham, UK; cat. no. P-0781).
7. 0.1 m*M* mercaptoethanol β-(ME) (Sigma; cat. no. M-7522). 0.1 *M* stock solution (1000X): add 0.1 mL β-ME to 14.1 mL DMEM.
8. Dulbecco's phosphate-buffered saline (PBS) (Sigma; cat. no. D-8537).

9. Trypsin/EDTA (1X) (Sigma; cat. no. T-3924).
10. Collagenase IV (160 U/mg) (Worthington Biochemicals, Lakewood, NJ; cat. no. LS004188).
11. 0.1% gelatin in sterile water (Sigma; cat. no. G9391-500G).
12. Stem cell media. For 1 L: to 784 mL DMEM high glucose, add 200 mL knockout serum replacement in a flow cabinet with sterile serological pipets. Add 5 mL 1 m*M* L-glutamine, 10 mL 1% MEM nonessential amino acids, 1.0 mL β-ME, and 4 μg human recombinant bFGF-2.

2.1.1. Tissue Culture Plastic and Glassware

1. 22-mm diameter cover glass (BDH, Poole, UK; cat. no. 406/189/44).
2. 100-mm tissue culture dish (Falcon, Oxford, UK; cat. no. 1029).
3. 15-mL conical tube (Falcon; cat. no. 2095).
4. Six-well multiwell tissue culture dish (Nunc, Loughborough, UK; cat. no. 140675).

2.2. MtDNA Depletion

1. DMEM with 4500 mg glucose (500 mL; Sigma; cat. no. D6546).
2. L-glutamine (200 μ*M*; 100 mL; Sigma; cat. no. G7513).
3. 10% v/v FBS (500 mL; Sigma; cat. no. F9665).
4. 50 μg/mL uridine (5 g; Sigma; cat. no. U-3003).
5. Penicillin-streptomycin solution (100 mL; Sigma; cat. no. P0781).
6. 5 μg/mL stock ethidium bromide solution (ethidium bromide powder; Sigma; cat. no. E7637-5G dissolved in ddH_2O).
7. 1X mtDNA depletion media. For 1 L: prepare media containing DMEM at 4500 mg/L glucose containing 1 m*M* pyruvate and supplemented with 100 mL FBS (v/v), 20 mL 200 μ*M* glutamine, 5 mL 10 mg/mL of uridine, and 10 mL penicillin-streptomycin solution.
8. 1X mtDNA aerobic media. For 1 L: prepare DMEM containing 1000 mg/L glucose supplemented with 100 mL dialyzed FBS, and 10 mL penicillin-streptomycin solution.

2.3. Mitochondrial hESC-Specific Transient Transfection

1. Six-well multiwell tissue culture dish (Apogent, Loughborough, UK; cat. no. 140675).
2. DMEM with 4500 mg glucose (500 mL; Sigma; cat. no. D6546).
3. L-glutamine (200 μ*M*; 100 mL; Sigma; cat. no. G7513).
4. 10% v/v FBS (500 mL; Sigma; cat. no. F9665).
5. 50 μg/mL uridine (5 g; Sigma, cat. no. U-3003).
6. Lipofectamine 2000 (1 mg/mL; Invitrogen Life Technologies; cat. no. 11668-027).
7. OptiMEM 1 medium (OptiMEM 1 with GlutaMAX 1; 500 mL; Invitrogen Life Technologies; cat. no. 51985-026).
8. PBS (500-mL; Sigma; cat. no. D8537).
9. 1X trypsin-EDTA solution (100 mL; Sigma, cat. no. T3924).

2.3.1. siRNA Transfection

1. Ambion Silencer siRNA Construction Kit (Ambion, Austin, TX; cat. no. 1620). Composed of:
   a. DNA Hyb buffer.
   b. T7 Promoter primer.
   c. 10X Klenow reaction buffer.
   d. 10X dNTP mix.
   e. Exo-Klenow.
   f. T7 enzyme mix.
   g. 10X T7 reaction buffer.
   h. 2X NTP mix.
   i. Digestion buffer.
   j. DNAse.
   k. RNase.
   l. Sense control DNA.
   m. Antisense control DNA.
   n. siRNA binding buffer. Add 5.3 mL 100% ethanol before use.
   o. siRNA wash buffer. Add 11 mL 100% ethanol before use.
   p. Nuclease-free water.
   q. Filter cartridges.
   r. 2-mL tubes.
2. TE buffer containing 10 m*M* Tris-HCl pH 8.0 (Sigma; cat. no. T-6066) and 1 m*M* EDTA (Sigma; cat. no. E-5513) made up in ddH_2O.

2.3.2. Vector Transfection

DNA to be transfected, for example pDSRed2-Mito Vector (BD Biosciences, Oxford, UK; cat. no. 6975-1).

2.4. DNA Extraction

1. Puregene DNA Isolation Kit (Flowgen, Ashby de la Zouch, UK; cat. no. D-5000A). Composed of:
   a. Cell lysis solution.
   b. RNase A solution.
   c. Protein precipitation solution.
   d. DNA rehydration solution.
2. 100% isopropanol.
3. 70% ethanol.
4. Autoclaved sterile double-distilled water (ddH_2O); replaces the DNA rehydration solution.
5. All solutions and reagents are stored at room temperature.

2.5. RNA Extraction

1. RNA extraction kit (Ambion; cat. no. 1914). Composed of:
   a. Lysis/binding solution, stored at 4°C.

b. Autoclaved sterilized ddH_2O to make 64% ethanol. For 35 mL 64% ethanol: add 22.4 mL of ethanol to 12.6 mL of ddH_2O. Store at 4°C.
c. Wash solution no. 1. Store at 4°C.
d. Wash solution no. 2/3 concentrate. Store at 4°C. The final solution is obtained after adding 28 mL of 100% ethanol to 7 mL of wash solution no. 2/3 concentrate.
e. Elution solution. Store at 4°C.
f. 10X DNase buffer I. Store at –20°C.
g. DNase I. Store at –20°C.
h. DNase inactivation reagent. Store at 4°C.
i. PBS. PBS tablets (Sigma; cat. no. P-4417). For a 0.01 *M* solution: dissolve 1 PBS tablet in 200 mL of sterile ddH_2O.

2.6. RT-PCR

2.6.1. Generation of cDNA

1. Reverse transcription system kit (Promega, Madison, WI). Composed of:
   a. 25 m*M* $MgCl_2$.
   b. 10X reverse transcription buffer.
   c. 10 m*M* dNTP mixture.
   d. Recombinant RNasin inhibitor (40 U/µL).
   e. Oligo-dT primer (500 µg/mL).
   f. AMV-reverse transcriptase (RT) (25 U/µL).
   g. 800 ng/µL of DNase-treated total RNA is required.
2. 0.2-mL sterile thin-walled PCR tubes (Sarstedt, Nümbrecht, Germany; cat. no. 72.737.002).

2.6.2. PCR

1. Diluted cDNA.
2. 10X NH_4 (no Mg^{2+}) buffer (Bioline, London, UK; cat. no. BIO-21040).
3. 50 m*M* $MgCl_2$ solution (Bioline; cat. no. BIO-21040).
4. 100 m*M* total dNTPs (Bioline; cat. no. BIO-39028).
5. Forward primer (50 pmol/µL, Alta Bioscience; Birmingham, UK).
6. Reverse primer (50 pmol/µL; Alta Bioscience).
7. Bio*Taq* polymerase (5 U/µL, Bioline; cat. no. BIO-21040).
8. 0.2-mL sterile thin-walled PCR tubes (Sarstedt; cat. no. 72.737.002).

2.7. DNA Sequencing

2.7.1. Isolation of PCR Products From Agarose Gel

1. QIAquick Gel Extraction Kit Protocol (DNA) (Qiagen, London, UK; cat. no. 28704). Composed of:
   a. Buffer QG.
   b. Buffer PE (add ethanol according to kit protocol instructions).
   c. QIAquick spin column.
2. 100% isopropanol.

3. Ethanol (96–100%).
4. Autoclaved sterilized ddH_2O used instead of buffer EB.

All solutions and reagents are stored at room temperature.

2.7.2. Cycle Sequencing: Preparing the Reaction

1. 3.2 µmol of forward/reverse primer.
2. 20 ng of purified DNA, obtained from PCR product.
3. 96-well reaction plate (Abgene Epsom, UK; cat. no. AB-0600).
4. Terminator Ready Reaction Mix (ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems, Foster City, CA; cat. no. 4390244). Consists of:
   a. A–BigDye Terminator.
   b. C–BigDye Terminator.
   c. G–BigDye Terminator.
   d. T–BigDye Terminator.
   e. Deoxynucleoside triphosphates (dATP, dCTP, dITP, dUTP).
   f. Amplitaq DNA polymerase, FS.
   g. $MgCl_2$.
   h. Tris-HCl buffer, pH 9.0.

2.8. Allele-Specific PCR

1. Amplifluor Single Nucleotide Polymorphisms (SNP) HT Genotyping System FAM-JOE (Chemicon International, Temecula, CA; cat. no. S7909).
   a. Red Tag (20X Amplifluor JOE SNPs Primer, 5.0 µ*M* stock).
   b. Green Tag (20X Amplifluor FAM SNPs Primer, 5.0 µ*M* stock).
2. Red or green tagged allele-specific primers (5′-end) (AltaBioscience).
3. Other reagents:
   a. 10X NH_4 (no Mg^{2+}) buffer (Bioline; cat. no. BIO-21040).
   b. 100 m*M* total dNTPs (Bioline; cat. no. BIO-39028).
   c. 50 m*M* $MgCl_2$ solution (Bioline; cat. no. BIO-21040).
   d. Diamond DNA polymerase, high specificity (5 U/µL) (Bioline; cat. no. BIO-21058).
4. Prepare primer mixes. For 20X solution: add 2.5 µL green primer (100 µ*M*); 2.5 µL red primer (100 µ*M*); 25 µL common primer (100 µ*M*) and 470 µL (ddH_2O).
5. Prepare master mix. For 1X solution: add 13.35 µL ddH_2O; 2 µL buffer; 0.4 µL dNTPs; 0.8 µL $MgCl_2$; 1 µL 20X Primer Mix (*see* **item 4**); 0.75 µL Red Tag (20X Amplifluor JOE SNPs Primer, 5.0 µ*M* stock); 0.5 µL Green Tag (20X Amplifluor FAM SNPs Primer, 5.0 µ*M* stock); and 0.2 µL Diamond DNA polymerase, high specificity (5 U/µL).
6. CAS-1200 precision liquid handling system and software (Corbett Research, Mortlake, NSW Australia; version 4.07).
7. 72-well Rotorgene-3000 4 Channel Multiplexing System Real Time PCR machine and Rotorgene (version 6) software (Corbett Research).

2.9. Real-Time PCR to Determine mtDNA Copy Number and MtDNA- and Cardiac-Specific Transcript Levels

Prepare 1X master mix: add 10 µL ABsolute QPCR SYBR Green Mix (Abgene); 0.5 µL forward primer (50 µ*M*); 0.5 µL reverse primer (50 µ*M*); and ddH_2O (to 20 µL depending on template volume).

2.10. Determination of Mitochondrial Membrane Potential and Functionality With JC-1 Staining and Fluorescent Microscopy

1. 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolylcarbocyanine iodide dye (JC-1, Molecular Probes, Eugene, OR; cat. no. T3168).
2. Sterile double-well slides (Fisher Scientific, Loughborough, UK; cat. no. MNK-140-020U); or sterile circular cover slips (BDH; cat. no. 406/0189/44) placed in six-well plates (Nunc; cat. no. 140675).
3. hESC medium, as described in **Subheading 2.1., item 12**, except a phenol-free DMEM (Sigma; cat. no. D1145) should be used, so as not to interfere with fluorescence microscopy.
4. 1X PBS (*see* **Subheading 2.1., item 8**).
5. 2 ng/mL Hoechst 33342 (Sigma; cat. no. B2261).
6. Nikon E1000 fluorescence microscope and/or Leica TCSSP2 confocal microscope.
7. Prepare a 1-mg/mL stock solution of JC-1 by dissolving 5 mg of JC-1 (as supplied by the manufacturer) in 5 mL DMSO. Divide the JC-1 stock solution into 200-µL aliquots and freeze at –20°C until required.

2.11. Immunocytochemistry of Proteins Encoded by the Mitochondrial Genome in hESCs

1. Mouse monoclonal antibody against COX-I (Molecular Probes; cat. no. A-6403).
2. Mouse monoclonal antibody against COX-III (Molecular Probes; cat. no. A-6401).
3. Chicken Alexa Fluor 488 anti-mouse immunoglobulin G conjugate (Molecular Probes; cat. no. A-21200).
4. 1X PBS (*see* **Subheading 2.1., item 8**).
5. Fixation solution: PBS with 2% (v/v) formaldehyde (*see* **Note 1**).
6. Permeabilization solution: PBS with 1% (v/v) Triton X-100.
7. Blocking solution: PBS with 100 m*M* glycine and 2 mg/mL bovine serum albumin.
8. Washing solution: PBS with 0.1% (v/v) Triton X-100. Washing solution may also consist of PBS without detergent.
9. Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA; cat. no. H-1200).
10. Incubator set at 37°C.
11. Staining chamber (*see* **Note 2**).
12. Microscope slides.
13. Nail polish.
14. Epifluorescence microscope with an appropriate filter (in this case, for 488-nm emission), or confocal microscope.

3. Methods

3.1. Culturing of hESCs for Mitochondrial Analysis

1. Undifferentiated hESC HSF-6 cells are cultured on irradiated-γ CF-1 murine embryonic fibroblasts.
2. Once hECSs have reached confluence, they are disassociated with either 2 mL of trypsin/EDTA (for 100-mm dish or 0.5 mL for six-well dish) or collagenase IV.
3. Cells are classified as undifferentiated or migratory for subsequent analysis. Migratory cells are those that have begun to migrate from the central colony. Differentiated cells are classified to their appropriate cell type according to relevant markers of differentiation.

3.1.1. Preparation of Cover Slips for hESC Plating

1. Heat the 0.1% gelatin solution in the 37°C water bath. Place one cover slip and add 1 mL gelatin to each well of six-well dish. Incubate overnight at 37°C to allow the gelatin to polymerize.
2. Remove the remaining liquid and return the dishes to the incubator until the coated cover slips are dry (1 h).
3. Alternative protocol:
   Place the cover slips in 0.1% gelatin overnight.
   Place one cover slip in each well of a six-well dish and return the dish to the incubator until the coated cover slips became dry (1 h).

3.1.2. Plate Stem Cells on Cover Slips for Immunocytochemistry

1. Change the medium in the 100-mm tissue culture dish 2 h before trypsinization and return to the incubator. Remove the medium from the tissue culture dish and wash once or twice with PBS (12 mL or with 2 mL for each six-well dish).
2. Add 2 mL of trypsin/EDTA to the tissue culture dish (for 100-mm or 0.5 mL for six-well dish) and incubate at 37°C until the colonies become detached (check the dish every 2 min under a microscope).
3. Transfer the trypsin cell suspension to a sterile conical tube and incubate at 37°C for a few more minutes, pipet the cell suspension up and down with a micropipet to dissociate the cells.
4. Add several milliliters of stem cell media, spin the suspension at low speed and remove the supernatant to ensure that the cells are no longer exposed to trypsin.
5. Resuspend the cells with several milliliters of media and split this volume in the gelatin-coated wells prepared the day before. The number of cells per well depends on how long the cells will be cultured before immunocytochemistry is carried out.
6. Make up the media volume to 2 mL and return the dishes to the incubator.
7. Feed the cells every day until they reach the optimal density for immunocytochemistry.

3.2. MtDNA Depletion

hESC cultures are depleted of mtDNA according to the protocol of King and Attardi (1996).

1. Culture the hESCs in mtDNA depletion media for 5 d before treatment and change the media every day.
2. After 5 d, the hESCs are then grown in the presence of 50 ng/mL ethidium bromide for 2–4 wk to isolate cells completely lacking mtDNA. Again the media is changed every day and supplemented with fresh ethidium bromide.
3. After culture is depleted, a batch of cells is cultured in mtDNA depletion media. Transfer to aerobic media will test for depletion as cells devoid of mtDNA cannot respire aerobically.

3.3. Mitochondrial hESC-Specific Transient Transfection

Transfection, the introduction of foreign DNA material into the genome of target cells, can result in stable integration and expression of the foreign protein *(43)*. This technique can therefore be used to increase expression of existing genes or induce the expression of additional genes. We have also successfully transfected a mammalian expression vector (*see* **Note 3**) designed for fluorescent labeling of mitochondria, because it has a mitochondrial targeting sequence. This allows us to analyze nuclear-mitochondrial interactions and communication and to identify populations of mitochondria. Transfected cells, and the mitochondria within them, can be detected by fluorescence microscopy. Alternatively, gene expression can be reduced or eliminated through, for example, the use of siRNAs *(44)*. The process, termed RNA interference, involves the transfection of target-specific double-stranded RNA which causes posttranscriptional gene silencing of that target.

3.3.1. siRNA Design and Preparation

1. Identify a target gene for RNA interference and design sense and antisense oligonucleotides for an appropriate region (*see* **Note 4**).
2. Resuspend the template oligonucleotides to 200 µ*M* in nuclease-free water. Determine the actual final concentration in µg/mL using a spectrophotometer by diluting the oligonucleotides in TE buffer (*see* **Note 5**).
3. Prepare 100 µ*M* solutions of each oligonucleotide by diluting in TE buffer.
4. Hybridize each template oligonucleotide to the T7 promoter primer by mixing the following in a tube. Add: 2 µL T7 promoter primer, 6 µL DNA Hyb buffer, 2 µL either sense or antisense template oligonucleotide. Heat the mixture to 70°C for 5 min then leave at room temperature for 5 min.
5. To the hybridized oligonucleotides, add the following: 2 µL 10X Klenow reaction buffer, 2 µL 10X dNTP mix, 4 µL nuclease-free water, 2 µL Exo-Klenow. Gently mix then transfer to a 37°C incubator for 30 min.

6. Assemble transcription reactions for each oligonucleotide containing: 2 μL sense or antisense siRNA template from **step 5**, 4 μL nuclease-free water, 10 μL 2X NTP mix, 2 μL 10X T7 reaction buffer. Incubate at 37°C for 2 h. Combine the sense and antisense reaction mixes into a single tube and continue incubation at 37°C overnight.
7. Digest the siRNA with RNase and DNase in the following reaction mix: 6 μL digestion buffer, 48.5 μL nuclease-free water, 3 μL RNase, and 2.5 μL DNase. Mix gently and incubate at 37°C for 2 h.
8. Bind and wash the siRNA: add 400 μL siRNA binding buffer to the nuclease digestion reaction from **step 7** and incubate at room temp for 2–5 min. Apply 100 μL of siRNA wash buffer to a filter cartridge placed in a 2-mL tube. Add the siRNA in binding buffer and centrifuge at 10*g* for 1 min. Discard the flow-through and replace the filter. Wash the filter cartridge with 2 × 500 (μL siRNA wash buffer again centrifuging at 10*g* for 1 min.
9. Transfer the filter cartridge to a new collection tube and elute the siRNA by adding 100 μL nuclease-free water preheated to 75°C. Incubate at room temperature for 2 min. Centrifuge at 12,000*g* for 2 min to collect the purified siRNA in the tube.
10. Finally determine the concentration of the siRNA using a spectrophotometer (*see* **Notes 5** and **6**). Store siRNAs at –20°C until transfection.

3.3.2. Transfection

1. Twenty-four hours before transfection, plate cells in a six-well multiwell culture dish in media without antibiotics. Incubate at 37°C in air with 5% CO_2.
2. For each well, dilute the DNA or siRNA to be transfected in 250 μL OptiMEM 1 medium and incubate at room temperature for 5 min (*see* **Note 7**).
3. Mix 10 μL Lipofectamine 2000 with 240 μL OptiMEM 1 medium, combine with the DNA-OptiMEM 1 mix from **step 2** and incubate at room temperature for 20 min to allow complexes to form.
4. Remove media from cells and add 500 μL of the final complexes from **step 3** to each well. Mix gently by rocking back and forth.
5. Incubate cells at 37°C for between 24 and 72 h.
6. Additional media may be added after 4 h to prevent cells from drying out.
7. After the desired incubation time, gene expression analysis can be performed including DNA PCR to look at gene levels, reverse transcriptase PCR to analyze transcript levels, and immunocytochemistry to determine protein levels. Overexpression studies would lead to increases in gene expression and siRNA experiments should result in a knock-down of the target gene.

3.4. DNA Extraction

Total DNA is required for real-time PCR amplification to determine mtDNA copy number and to analyze variants by allele-specific PCR.

Total DNA is extracted using the Puregene DNA Isolation Kit, (Flowgen, UK).

1. Pellet hESCs at 13,000g for 5 s.
2. Remove the supernatant and resuspend the hESCs in 10–20 μL of residual supernatant and vortex vigorously to ensure thorough resuspension.

3. Extract the DNA by resuspending the cells in 300 μL of cell lysis solution.
4. Add 1.5 μL of RNase A solution, invert multiple times and incubate at 37°C for 5–20 min.
5. Precipitate the proteins by cooling the sample on ice for 1 min. Add 100 μL of protein precipitation solution and vortex at high speed for 20 s. Centrifuge the precipitated proteins at 13,000*g* for 1 min.
6. Precipitate the DNA by pouring the supernatant containing the DNA into a sterile 1.5-mL microcentrifuge tube already containing 300 μL 100% isopropanol and mix by repeated inversion. Centrifuge the mixture at 13,000*g* for 1 min to form a small, white pellet.
7. Pour off the supernatant and add 300 μL 70% ethanol. Repeat the centrifugation at 13,000*g* for 1 min. The ethanol is then poured off and the tube inverted and placed on absorbent paper for 10–15 min before rehydration.
8. Recover the DNA in 50 μL of autoclaved sterile ddH_2O.

3.5. RNA Extraction

RNA is required for RT-PCR to determine whether the transcripts of a gene are expressed. For mtDNA-encoded genes it is essential that pure populations of RNA, free of contaminating DNA, are extracted as the mtDNA genome has no introns. Consequently, primers overlapping introns cannot be designed to overcome this shortcoming and it would therefore be impossible to distinguish between genomic DNA and cDNA.

1. Wash the ESCs sample with 125 μL of PBS; re-pellet the cells, centrifuging at 13,000*g* for 1 min and discard the supernatant.
2. Add 500 μL of lysis solution; pipet up and down and vortex vigorously to generate a high yield.
3. Add 500 μL of 64% ethanol and invert the microfuge tube several times.
4. Label a new microfuge tube and place a filter column inside.
5. Add 500 μL of the mixture to the filter column and centrifuge at 13,000*g* for 1 min and discard the flow through.
6. Repeat **step 5** using the remaining mixture.
7. Apply 700 μL of wash solution no. 1 to the filter, centrifuge at 13,000*g* for 1 min, and discard the flow-through.
8. Apply 500 μL of wash solution no. 2/3 to the filter, centrifuge at 13,000*g* for 1 min, and discard the flow-through.
9. Repeat **step 8**.
10. Centrifuge at 13,000*g* for 1 min and discard the flow-through.

3.5.1. RNA Elution

1. Place the filter into a clean collection tube, provided in the kit.
2. Apply 30 μL of elution solution, prewarmed at 80°C; wait 2 min and centrifuge at 13,000*g* for 1 min.
3. Repeat **step 2**.
4. The RNA will be in the tube and the filter should be discarded.

3.5.2. Elimination of Contaminating DNA

1. Add 18 µL of 10X DNase I Buffer to the sample obtained in **Subheading 3.5.1., step 4**.
2. Add 3 µL of DNase I.
3. Incubate at 37°C in a heating block for 90 min.
4. Remove from the heating block and add 18 µL of inactivation reagent; tap the tube to mix and leave at room temperature for 2 min.
5. Centrifuge at 10,000*g* for 1 min. The pellet contains the inactivation reagent and the contaminating DNA.
6. Transfer the supernatant to a new microfuge tube. The solution very close to the pellet should not be transferred to avoid DNA contamination in the final product.
7. Freeze the RNA sample at –80°C unless it is going to be used immediately.

3.5.3. Spectrophotometry of RNA

1. Set the wavelength to 260 nm on the spectrophotometer.
2. Fill a cuvet with 995 µL of sterile water; place in the spectrophotometer and set the reference.
3. Add 5 µL of the RNA sample to the cuvet, and cover with Parafilm and invert several times to mix.
4. Put the cuvet into the spectrophotometer and read the absorbance. Determine the RNA concentration (*see* **Note 8**).

3.6. RT-PCR

3.6.1. Generation of cDNA

cDNA is produced using the RT system kit. This kit produces first strand cDNA by RT of mRNA using avian myeloblastosis virus reverse transcriptase (AMV-RT) and oligo-dT primers. Usually, 800 ng/µL of DNase-treated total RNA is used in each 20 µL volume RT reaction.

1. Prepare RT reaction master mix: of 25 m*M* $MgCl_2$ (4 µL); 10X reverse transcription buffer (2 µL); 10 m*M* dNTP mixture (2 µL); of recombinant RNasin inhibitor (40 U/µL) (0.5 µL); and of oligo-dT primer (500 µg/mL) (0.5 µL).
2. Add 9 µL of master mix to the RNA.
3. Add 0.6 µL of the AMV-RT (25 U/µL) to each RT reaction.
4. Add ddH_2O to 20 µL.
5. For each RT reaction, a parallel reaction omitting the AMV-RT is set up (*see* **Note 9**).
6. A parallel reaction is set up without any DNase-treated total RNA, as a negative control for the RT process.
7. Add nuclease free water to each reaction for a final volume of 20 µL.
8. Reverse transcription is carried out at 42°C for 2 h to produce cDNA in a MJ Research engine PTC 200 machine.

9. Following reverse transcription, dilute the cDNA template to 100 µL by adding 80 µL of nuclease free water to the RT reaction.
10. Store the cDNA at 4°C before the final PCR.

3.6.2. PCR

1. 10 µL of diluted cDNA, produced by reverse transcription of DNase-treated total RNA, is amplified in each PCR.
2. Set each PCR up as a 50-µL reaction containing the cDNA template and a master mix.
3. In addition, a parallel PCR is set up with no cDNA template to act a negative control for the PCRs.
4. Further, a parallel PCR is set up with 200 ng/µL total DNA to act as a positive control for the PCRs.
5. Add the appropriate volume of ultra pure water to each PCR to reach a final volume of 50 µL.
6. PCR master mix: 10X NH_4 (no Mg^{2+}) buffer (5 µL); 50 m*M* $MgCl_2$ solution (1.5 µL); 100 m*M* total dNTPs (0.8 µL); forward primer (50 pmol/µL*) (0.5 µL); reverse primer (50 pmol/µL*) (0.5 µL); Bio*Taq* Polymerase (5 U/µL) (0.5 µL). (*For primer sequences, *see* **Table 1** [*see* **Note 10**]).
7. All PCRs are performed on an MJ Research DNA engine PTC 200 machine using the following conditions as standard: 5 min at 94°C; then 35 cycles of 1 min at 94°C; 1 min at specific annealing temperature for the primers (*see* **Table 1**); and 3 min at 72°C.
8. PCR products are kept at 4°C before loading onto agarose gels.

Figure 1 shows the expression of some key intron A nuclear-encoded transcription factors.

3.7. Sequencing

DNA sequencing is performed on PCR products isolated from a DNA agarose electrophoresis gel to ensure that all impurities are eliminated.

3.7.1. Purification of the PCR Product

The PCR product is purified from the agarose using the QIAquick Gel Extraction Kit.

1. Excise DNA bands from gel.
2. Transfer the bands into individual 1.5-mL sterile microfuge tubes and add 300 µL buffer QG.
3. Incubate at 50°C for 10 min and vortex each for 2–3 min to completely dissolve the gel.
4. Add 100 µL 100% isopropanol and vortex to mix.
5. Place the QIAquick spin column in a 2-mL collection tube and apply the sample.
6. Centrifuge for 1 min.
7. Discard flow through and replace the column in the same tube.

Table 1

Primers Used for Conventional and Real Time Reverse Transcriptase Polymerase Chain Reaction to Analyze Transcript Levels

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)	Annealing temperature (°C)	Product size (bp)
D-Loop	cga aag gac aag aga aat aag g	ctg taa agt ttt aag ttt tat gcg	55	181
COX I	tca taa tcg gag gct ttg gc	caa cct caa cac cac ctt ct	55	421
COX II	atc atc cta gtc ctc atc gc	aga cgt ctt gca ctc atg ag	55	280
COX III	cgc gat gta aca cga gaa ag	tca gag tac ttc gag tct cc	55	379
NRF1	gga gtg atg tcc gca cag aa	cgc tgt taa gcg cca tag tg	58	584
Tfam	tat caa gat gct tat agg gc	act cct cag cac cat att tt	55	441
NRF2α	tag acc tca cca cac tca ac	gtg acc aaa cgg ttc aac tc	58	627
NRF2β	gag ctc cct tta cta cag ac	aac tgt ggt gtt gca gca tg	58	462
PolG	cat tgg aca tcc aga tgc tc	cct gat atg agc tcg gtc aa	58	679
TFB1M	tct gca atg ttc gac aca tc	acc tat ata aga agc tcc ac	49	501
TFB2M	aga agc agt tcc ttg gac ag	agt ggt cta tta cag tgg cg	57	463
B-ACTIN	tgg cac cac acc ttc tac aat gag c	gca cag ctt ctc ctt aat gtc acg c	55	400
Oct-4	cga cca tct gcc gct ttg ag	ccc cct gtc ccc cat tcc ta	60	241
α-MHC	gga gga gca agc caa cac caa	gca gtg agg ttc ccg tgg ca	59	179
ANF	tag gga cag act gca aga gg	cga gga agt cac cat caa acc ac	59	577
Nkx2.5	tgg cta cag ctg cac tgc cg	gga tcc atg cag cgt gga c	60	165
Rex-1	gcg tac gca aat taa agt cca ga	cag cat cct aaa cag ctc gca gaa t	56	306
SOX-2	ccc ccg gcg gca ata gca	tcg gcg ccg ggg aga tac at	55	448
FGF-4	cta caa cgc cta cga gtc cta ca	gtt gca cca gaa aag tca gag ttg	55	370

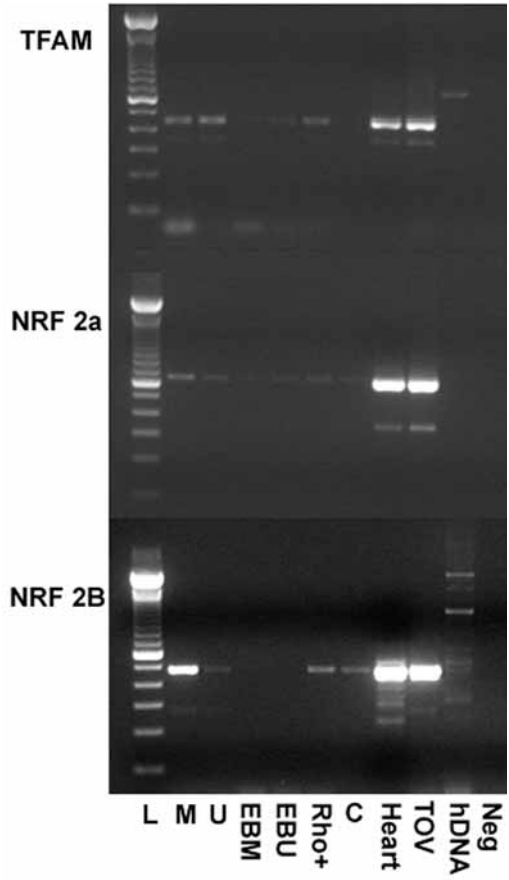

Fig. 1. Regulation of mtDNA transcription and replication factors. Differentiating and undifferentiating hESCs exhibit low levels of mtDNA transcription and replication factors, TFAM and NRF2α, as do hESC-derived cardiomyocytes. However, migratory hESCs have higher levels of NRF2α. Adult cells, human heart and TOV cells, consistently express high levels of all three transcription factors. U, undifferentiated and M, migratory hESCs i.e., those cells migrating from the central colony; EBM, ethidium bromide-treated M; EBU, ethidium bromide-treated U; Rho+, hESCs cultured in the same media as ethidium bromide-treated cells; C, hESC-derived cardiomyocytes; heart, adult human heart; TOV, an ovarian tumor cell line; L, 100 bp DNA ladder (Invitrogen); neg, negative control reaction. For further details *see* **ref. *46***.

8. Add 0.5 mL of buffer QG and centrifuge for 1 min.
9. Wash the DNA with 0.75 mL of buffer PE containing ethanol and centrifuge 1 min.
10. Discard flow-through, then centrifuge for 1 min and place column into a new 1.5-mL microcentrifuge tube.

11. Elute the DNA in 50 µL autoclaved sterile ddH_2O and centrifuge for 1 min.
12. Store at –20°C.

3.7.2. Cycle Sequencing: Preparing the Reaction

1. For each PCR reaction, add 3.2 pmol of forward primer to 20 ng of purified DNA product, obtained from the purified PCR, in a final reaction volume of 10 µL.
2. Apply the total sample volume in one of the wells in the 96-well reaction plate.
3. Add 8.0 µL Terminator Ready Reaction Mix consisting of 2 µL of Reaction Premix and 6 µL of BigDye Sequencing Buffer.
4. Mix well and spin briefly.

3.7.3. Cycle Sequencing: PCR Conditions Used

1. Perform 25 cycles of the following parameters: 96°C for 10 s; 50°C for 5 s; 60°C for 4 min; and 4°C (infinity).
2. Following the cycling reaction, spin down the contents of the tubes in a microcentrifuge and proceed to purify the extension products.

3.7.4. Purification of Extension Products

1. Pipet entire contents of the reaction into a 1.5-mL microcentrifuge tube.
2. Add 2 µL EDTA 250 m*M*.
3. Add 64 µL of nondenatured 95% ethanol.
4. Close the tube and vortex briefly.
5. Incubate at room temperature for 15 min (*see* **Note 11**).
6. Centrifuge the tubes for 20 min at maximum speed and proceed immediately to **step 7**.
7. Carefully aspirate the supernatant with a separate tip for each sample and discard (*see* **Notes 12** and **13**).
8. Add 250 µL of 70% ethanol and vortex briefly. Repeat.
9. Spin for 10 min at maximum speed.
10. Aspirate the supernatant carefully as in **step 7**.
11. Dry the samples in a vacuum centrifuge for 10–15 min or to dryness (*see* **Notes 14** and **15**).

3.8. Allele-Specific PCR for the Analysis of hESCs for Homoplasmy and Heteroplasmy

1. D-loop analysis by PCR (Conventional) amplification of total DNA extracted from hESCs using primers specific to the human mtDNA D-loop. The D-loop has polymorphic variants that can determine specific mtDNA lineage or lineages, relevant to homo- or heteroplasmy.
2. Perform PCR as follows: 95°C for 5 min; then 25 cycles of 94°C for 30 s; 60°C for 30 s; 72°C for 2 min; followed by 72°C for 10 min.
3. Purify D-loop fragments once resolved on a 2% agarose gel (either by excising bands from the gel or from the PCR product).

4. Calculate the DNA concentration in the purified D-loop samples using absorbance spectrophotometry.
5. Prepare PCR products for DNA sequencing (*see* **Subheading 3.7.**).
6. Align DNA sequences, using ClustalW Multiple Sequence Alignment Tool (EMBL-EBI), to identify SNPs (*see* **Note 16**).
7. Prepare 20X primer mix (*see* **Subheading 2.8., item 4**).
8. Dilute purified D-loop fragments sequenced in **step 5** to 2 ng/μL before use.
9. Generate a series of 10-fold dilutions using purified D-loop fragments in **step 5** of known concentration for the absolute quantitation of mtDNA heteroplasmy.
10. Prepare reaction by combining 19 μL of 1X master mix with 1 μL purified D-loop product (2 ng/μL).
11. Reactions are performed on a 72-well Rotorgene-3000 4 Channel Multiplexing System Real Time PCR machine with a Rotorgene (version 6) software interface. The reaction conditions are: 95°C for 3 min; then 45 cycles of 95°C for 5 s, 55°C for 20 s (annealing and data acquisition on FAM/Sybr channel), 72°C for 20 s.

3.9. Real-Time PCR to Determine mtDNA Copy Number mtDNA- and Cardiac-Specific Transcript Levels

1. Adjust the concentrations of total DNA and cDNA to 20 ng/μL and 625 ng/μL, respectively and determine the amount of mtDNA and mtDNA-transcripts by quantitative real time PCR analysis. MtDNA primers are designed based on the human mtDNA sequence ***(45)***, *see* **Table 1** (*see* **Note 17**).
2. Generate a series of 10-fold dilutions of either a total DNA or a total cDNA sample of known concentration for the absolute quantitation of mtDNA and mtDNA-transcript levels, respectively.
3. Determine mtDNA copy number (D-Loop primers) by preparing a 20-μL volume reaction as follows: 19 μL 1X master mix (*see* **Subheading 2.9.**) and 1 μL total DNA (20 ng/μL).
4. Determine MtDNA- and cardiac-specific transcript levels as follows: add 16 μL 1X master mix (*see* **Subheading 2.9.**) to 4 μL total cDNA (625 ng/μL).
5. Reactions are performed in a 72-well Rotorgene-3000 4 Channel Multiplexing System Real Time PCR machine with a Rotorgene (version 6). Reaction conditions are: 95°C for 15 min; followed by 45 cycles of 95°C for 5 s; 60°C for 20 s; and 72°C for 20 s (extension and data acquisition on FAM/Sybr channel).
6. Melt curve analysis: ramp from 72°C to 99°C, 1°C per step. Wait 30 s for the first step and then 5 s for each subsequent step. Acquire melt data from the FAM/Sybr channel.

3.10. Determination of Mitochondrial Membrane Potential and Functionality With JC-1 Staining and Fluorescent Microscopy

1. Culture cells in hESC medium without Phenol Red and allow to proliferate overnight under normal incubation conditions.
2. Wash cells two to three times with 1X PBS to remove any residual Phenol Red.

3. Incubate the cells in 0.3 μg/mL JC-1 and 2 ng/mL Hoechst 33342 for 15 min in phenol-free hESC medium.
4. Rinse the cells twice in phenol-free hESC medium.
5. Observe with a Nikon E1000 fluorescence microscope and/or Leica TCSSP2 confocal microscope. JC-1 stained cells are excited at 485 nm and green (low membrane potential) and red (high membrane potential) fluorescence are viewed separately using 535 nm and 610 nm emission fitters, respectively. Domains of active and less active mitochondria within the cells are viewed simultaneously by merging the red and green fluorescent images. **Figure 2** shows examples of hESCs stained with JC-1 and Hoechst 33342. For extended imaging, a heated stage or CO_2 source should be available to ensure viability.

3.11. Immunocytochemistry of Proteins Encoded by the Mitochondrial Genome in Human Embryonic Stem Cells

1. Grow the cells on cover slips as previously described (*see* **Subheading 3.1.2.**).
2. Fix the cover slips by placing them in fixation solution, and incubating for 40 min at room temperature.
3. Permeabilize the cell membranes by incubating the cover slips with the permeabilization solution for 20 min at room temperature (*see* **Note 18**).
4. Place the cover slips in blocking solution for at least 30 min at room temperature (*see* **Note 19**).
5. Incubate cover slips with the primary antibody diluted in blocking solution at 37°C for 1 h, in a staining chamber (*see* **Note 20**). As an alternative, incubate overnight at room temperature or 4°C (*see* **Note 21**).
6. Wash the cover slips twice at room temperature with washing solution for 10–30 min. This removes loosely bound antibody (unspecific binding).
7. Incubate with the appropriate secondary antibody (diluted 1:200 in blocking solution) for 40 min to 1 h at 37°C in a staining chamber.
8. Rinse the cover slips twice with washing solution for 10–30 min at room temperature.
9. Mount the cover slips carefully on a microscope slide in a drop of Vectashield mounting medium, and seal with nail polish.
10. Observe under an epifluorescence microscope, or with a confocal microscope. **Figure 3** demonstrates the low number of mitochondria present through expression of COXI and COXViC. It also demonstrates the large nucleus to cytoplasmic ratio, as evidence by DAPI staining.

4. Notes

1. The formaldehyde fixation solution should be prepared immediately before use. Furthermore, other primary antibody manufacturers may recommend different fixation solutions, such as different concentrations of formaldehyde or paraformaldehyde. Cold methanol is also a widely used fixative. An antibody may require a very specific fixation, or be more permissive. Also note that some antibodies used for immunolocalization may not be suitable for other techniques (such as Western blotting), and vice versa.

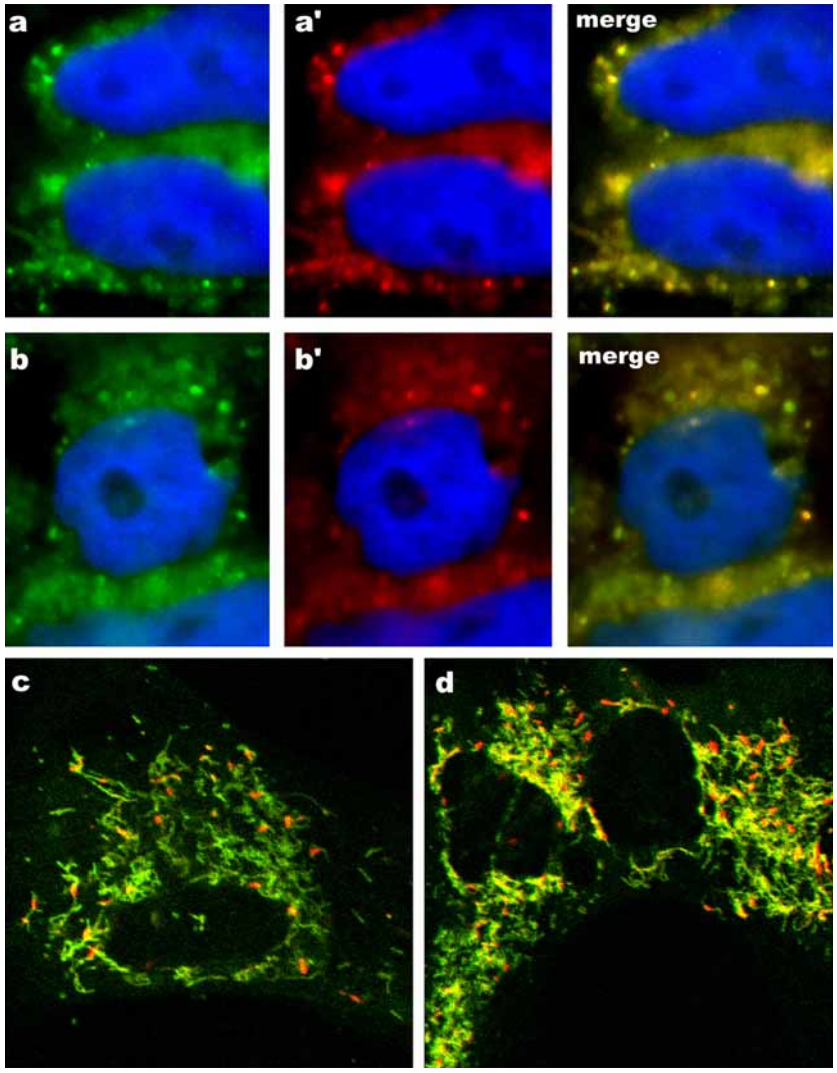

Fig. 2. Assessment of mitochondrial membrane potential with JC-1 in human embryonic stem cells. Undifferentiated and migratory stem cells cultured on feeder layers. Low membrane potential is observed through green fluorescence (**A**,**B**), though the cells tend to have high membrane potentials because of the presence of red fluorescence (**A**,**B**). This is further clarified in the merged images (merge). The early migratory and differentiating cells, as demonstrated in the merged confocal images (**C**,**D**), tend to exhibit mitochondrial polarity forming an "engagement ring"-like structure (**C**) as they start to proliferate in the cytoplasm and further expand during differentiation (**D**). The nucleus is marked by staining with Hoechst 33342. For further detail *see* **ref.** *46*. (Please *see* the companion CD for the color version of this figure.)

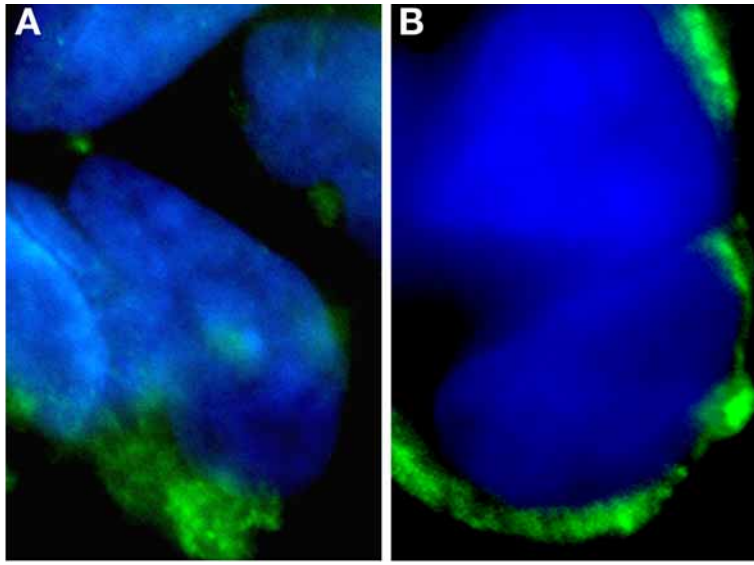

Fig. 3. The use of immunocytochemistry to identify subunits associated with OXPHOS. Immunocytochemistry using antibodies specific to genes encoding for the subunits of the electron transfer chain. **(A)** COXI specific to the mitochondrial genome; and **(B)** COXViC, a nuclear-encoded gene, highlights the low numbers of mitochondria present in undifferentiated human embryonic stem cells. The large nuclear-cytoplasmic volume is demonstrated by the nuclear stain (blue) DAPI. For further detail *see* **ref. *46***. (Please *see* the companion CD for the color version of this figure.)

2. We typically use 100 × 15-mm compartmentalized Petri dishes with vertical stacking rings. These are often known as X-Dishes, where dividers have created four small compartments from a traditional Petri dish (for example, Sigma; cat. no. P6481). The Staining chamber is prepared by filling the four compartments of the X-Dish with washing solution.
3. pDSRed2-Mito Vector has been successfully transfected and expressed in cells using the agent lipofectamine. Fluorescence can usually be detected within 24 h, although this will depend on the rate at which cells are replicating/cycling. Once incorporated, cells in culture continue to express the vector for a number of weeks allowing the nucleocytoplasmic interactions to be monitored over time. We have recently excised the CMV promoter and inserted the EF1 promoter into the rector, and achieved higher levels of transfection.
4. A list of guidelines for this design is set out on the Ambion web site: www.ambion.com. We typically design four or five siRNAs from different regions of the same gene as some are likely to be more effective than others.
5. This is done by multiplying the absorbance value at 260 nm by the dilution factor and by 20 μg/mL (used to compensate for the non-full length oligonucleotide that

is typically present in chemically synthesized oligonucleotide preps). Calculate from this value the molar concentration by dividing the (µg/mL concentration by 9.7 (there are 9.7 µg of DNA in 1 nmol of an average 29mer).

6. siRNA concentration used is critical to success as too much causes nonspecific reductions in gene expression and toxicity to the transfected cells. Transfecting too little will not produce the required decrease in gene expression.
7. The concentration of vector can be varied and the optimum is determined by performing several repeats with a range of dilutions (suggested range 0.1–10 µ*M*). Increasing the vector concentration further may result in the opposite to the desired effect. In the case of siRNAs, this may decrease their inhibitory action on the targeted gene and with the pDSRed2-Mito Vector higher concentrations cause too much fluorescence in those cells that are successfully transfected resulting in a lack of definition in these cells when viewed microscopically. Other variables should be investigated including the media in which transfection is performed, length of incubation in the transfection media and the transfection reagent used. We found lipofectamine to give a significantly better result than oligofectamine, but this will vary with cell type and the vector to be transfected. Furthermore, serum severely inhibits vector incorporation.
8. The RNA concentration of a sample can be measured using a spectrophotometer at a wavelength of 260 nm. The absorbance, which is calculated by the spectrophotometer, can be converted into an RNA concentration using the following formula:

$$\frac{\text{Absorbance at 260 nm} \times 40}{\text{Dilution factor}}$$

where 40 is the quantity of RNA in nanograms at an absorbance of 1.
For example: an absorbance of 0.040 at 260 nm, where 5 µL of RNA was added to a cuvet containing 995 µL of water (a 0.005 dilution factor), would yield:

$$\frac{0.040 \times 40}{0.005} = 320\,\text{ng}/\mu\text{L RNA}.$$

9. For each RT reaction, a parallel reaction is set up in the absence of the AMV reverse transcriptase enzyme. This allows screening for the presence of genomic DNA contamination in the DNAse-treated RNA samples. Any products seen in these "no-enzyme controls" indicate the presence of contaminating genomic DNA in the RNA samples, which must be taken into account when analyzing sample results. This is especially vital when analyzing mtDNA transcripts, as there are no introns present in or between mtDNA genes.
10. Different mitochondrial and nuclear genome specific primer pairs are designed and used to amplify the cDNA of interest. Examples of mtDNA and nuclear encoded genes associated with mtDNA transcription and replication and also genes associated with pluripotency are listed in **Table 1**.
11. Precipitation times longer than 15 min will result in loss of very short extension products. Precipitation times greater than 24 h will increase the precipitation of

unincorporated dye terminators. Consequently, 15 min is considered optimal though slightly longer precipitation times may be required.

12. Careful pipetting is vital as the pellet may not be visible and will therefore be lost. It is useful to load the tube into the microcentrifuge with the hinge of the cap facing the outside of the centrifuge so that the products will be isolated to this side of the tube wall and then the supernatant can be removed from the other side.
13. The supernatant must be removed completely as unincorporated dye terminators are dissolved in them. The presence of unincorporated dye terminators could result in either poor quality or inaccurate sequence data.
14. Do not overdry. Overdrying will reduce the chances of successful rehydration in Hi Di Formamide.
15. After drying, it is recommended that 10 μL of Hi Di Formamide is added to each sample and left for 30 min to allow the DNA to be resuspended before proceeding with the sequencing reaction.
16. Allele specific PCR primers are designed by ensuring that the 3′ end of the primer contains the SNP. The primer should be approximately 20 bp long and have annealing temperatures of approx 55°C. These primers should then be tagged with the sequence specific to the FAM (green: gaa ggt gac caa gtt cat gct) or JOE (red: gaa ggt cgg agt caa cgg att). Using Diamond DNA polymerase ensures that only the allele with the appropriate SNP will be amplified. However, we recommend first using the allele-specific primers individually with the common reverse primer to test for specificity. These reactions are normally preformed in separate conventional PCR reactions (*see* **Subheadings 2.6.2.** and **3.6.2.**) using the same reagents as for allele-specific PCR, except for the red and green fluorescent labeling tags. The products are resolved on 3% agarose gels.
17. The primers described in **Table 1** can also be used for conventional RT-PCR. Examples for TFAM, NRF2α, and NRF 2β can be seen in **Fig. 1**.
18. Permeabilization is necessary to allow the antibody to penetrate into cells to label intracellular antigens after formaldehyde or paraformaldehyde fixation (methanol fixation dissolves the plasma membrane, and thus this step is not required). Mitochondrial ligands are internal and, although the plasma membrane may have been damaged by the fixation procedure itself, it is advisable to further permeabilize the cells, for optimal antibody penetration. Permeabilization should not be used if the antibody ligand is on the plasma membrane (e.g., the SSEA antigens), because it would decrease the intensity of the signal. If it is necessary to simultaneously detect a plasma membrane and intracellular ligand (e.g., SSEA-3 or -4, and mitochondrial proteins) a compromised may be reached using milder permeabilization conditions. This may include less time in the permeabilization solution (1–2 min), or the use of a permeabilization solution with lower detergent content, such as PBS with 0.1% Triton X-100. PBS without detergent could also be used as a washing solution in this case.
19. This will prevent nonspecific reactions. Formaldehyde fixation often creates radicals that may allow unspecific antibody binding. These sites need to be blocked using an appropriate solution, in this case composed of bovine serum

Table 2

Primers for the Analysis of the Human mtDNA D-Loop

Primer	Sequence (5′-3′)	T_m (°C)	Annealing temperature	Product size (bp)
D1 (forward)	tca aag ctt aca cca gtc ttg taa acc	57	55	1060
D2 (reverse)	ttg agg agg taa gct aca ta	53		

albumin and glycine that will bind the unspecific binding sites. In addition, it is often suggested to include goat or bovine serum (10% v/v) in the blocking medium. It is possible to stop the assay at this step for a few days. In this case, store the cover slips sealed with Parafilm in blocking solution at 4°C. It is always advisable to prepare more cover slips of the same sample than immediately needed, in case optimizations, repetitions, or further stainings are required.

20. The cover slip to be stained can be suspended in the center of the Staining chamber (X-Dish) using the compartment dividers, and the antibody solution carefully placed on it. Water surface tension will keep the solution on the cover slip. Washing solution in the four chambers is used for washes, by merely dropping the cover slip into a compartment (twice after primary antibody incubation; twice after secondary antibody incubation). In addition, the presence of washing solution prevents the cover slips from drying during staining. Drying may result in unspecific binding of antibody to the cover slip, generating high background levels or unspecific staining.
21. The antibodies are diluted to the proper final concentration, according to manufacturers' instructions. It should be noted that primary antibody concentration and incubation conditions may need to be optimized for each system. Note that the antibody may decrease in reactivity through time. There may also be variations between batches or suppliers. To enhance weak staining the experimenter should try to increase antibody concentration or time of staining. If the signal is too strong, the opposite may be required.

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Human Embryonic Stem Cell Protocols

Edited by

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Ottawa Health Research Institute, Ottawa, Ontario, Canada

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