

Chapter 2

Human Embryonic Stem Cells in Regenerative Medicine

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Abstract Human embryonic stem cells have the capacity for self-renewal and pluripotency, making them a primary candidate for tissue engineering and regenerative therapies. To date, numerous human embryonic stem cell (hESC) lines have been developed and characterized. In this chapter, we discuss how hESC lines are derived, the means by which pluripotency is monitored, and how their ability to differentiate into all three embryonic germ layers is determined. We also outline the methods currently employed to direct their differentiation into populations of tissue-specific, functional cells. Finally, we highlight the general challenges that must be overcome and the strategies being developed in order to generate highly purified hESC-derived cell populations that can safely be used for clinical applications.

Abbreviations

bFGF	Basic fibroblast growth factor
DKK1	Dickkopf homolog-1
hEB	Human embryoid body
hESC	Human embryonic stem cells
HLA	Human lymphocyte antigen
miR	MicroRNA
RPE	Retinal pigment epithelium

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TGF β Transforming growth factor- β
VEGF Vascular endothelial growth factor

2.1 Introduction

Stem cells have the ability to maintain long-term proliferation and self-renewal. Under specific conditions, stem cells can differentiate into a diverse population of mature and functionally specialized cell types. There are two main types of human stem cells classified according to their source and developmental potential: embryonic and adult, or tissue-specific, stem cells. Human embryonic stem cells are pluripotent cells that can differentiate into all types of somatic and in some cases, extraembryonic tissues. Human adult stem cells are derived from nonembryonic tissues and are capable of generating specific cells from its organ or tissue of origin. Because of the unrestricted potential of human embryonic stem cells (hESCs), these cells have become a highly desirable experimental tool for understanding human development, and are especially attractive for therapeutic applications.

2.2 Sources and Derivation of Human Embryonic Stem Cells

hESCs were first derived from the inner cell mass of the blastocyst-stage preimplantation embryo (Fig. 2.1). The inner cell mass is composed of pluripotent cells that are capable of differentiating into the extraembryonic endoderm and the three germ layers that will eventually generate all tissues of the embryo: ectoderm, mesoderm, and endoderm. To generate a hESC line, the cells encompassing the inner cell mass are microsurgically removed and cultured in vitro under specific conditions designed to select cell populations with the capacity to proliferate in the undifferentiated state. Thomson and colleagues reported the first derivation of pluripotent hESCs using this method [1] and were quickly followed by a number of other groups [2–4]. To date, there are 82 hESC lines that adhere to US federal guidelines, many of which are widely used in basic and clinical research. A current list may be found at http://www.grants.nih.gov/stem_cells/registry/current.htm.

hESC lines have also been derived from earlier stages of embryonic development, including single blastomeres of 4- or 8-cell stage embryos [5–8] and 16-cell morulae [9, 10] (Fig. 2.1). A single blastomere is considered totipotent and can produce an entire embryo. Thus, blastomere-derived hESCs could circumvent ethical issues surrounding the use of hESCs in biomedical research, since the removal of a single blastomere from an early-stage embryo will, theoretically, not impede the ability of the remaining blastomeres to develop into a normal embryo.

hESC lines can also be obtained from parthenogenetic embryos, which are generated when a single egg is fertilized in the absence of male sperm (Fig. 2.1). hESC lines derived using this method can circumvent ethical concerns about the use of embryonic cells since viable embryos are neither created nor destroyed. Parthenote-derived hESC lines have been generated through artificial fertilization of

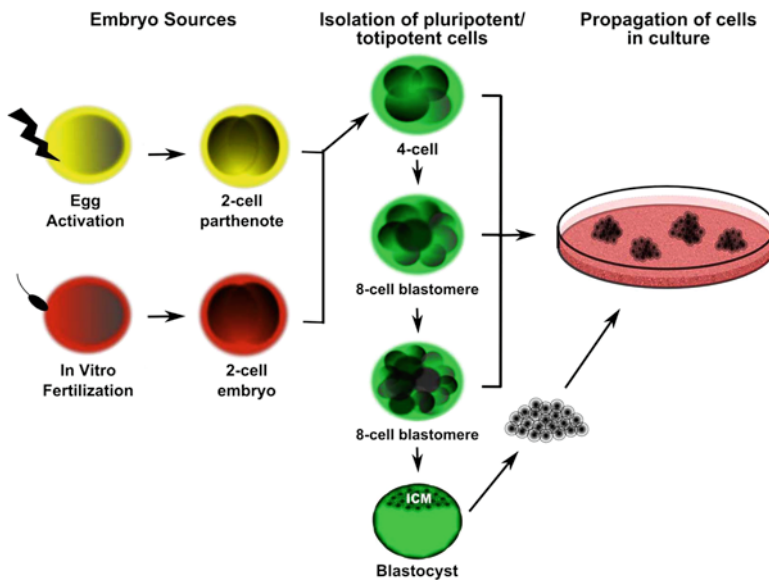


Fig. 2.1 Generation of hESC lines from various embryonic sources. Generation of hESC lines undergo three stages. First, donor embryos are obtained after in vitro fertilization or by egg activation (parthenogenetic embryos) and allowed to develop in vitro. Second, pluripotent cells are isolated either from the inner cell mass (ICM) of pre-implantation blastocysts or from 4, 8, or 16-cell stage morulae. Finally, isolated cells are plated in defined hESC medium with or without feeder cell layers to propagate and select for pluripotent cell populations

donor oocytes [11–14]. The ability to derive hESC lines from parthenote-blastocysts is especially attractive not only because of their normal karyotype and their pluripotent properties, but also because these lines contain homozygous major HLA alleles, which could circumvent immunological rejection involved in transplantation therapies (discussed below).

2.3 Characteristics of Pluripotent Human Embryonic Stem Cells

hESC lines have been derived from different sources using different methods which can introduce variability between lines. Thus, defining the specific properties and identifying the features of hESCs are critical to their use. In this section, we discuss the guidelines currently used when characterizing new hESC lines.

2.3.1 Cell Morphology and Density

Pluripotent hESCs maintain a specific cell morphology and density. hESCs have a high cell nucleus-to-cytoplasm ratio due to an enlarged nucleus and distinct nucleoli.

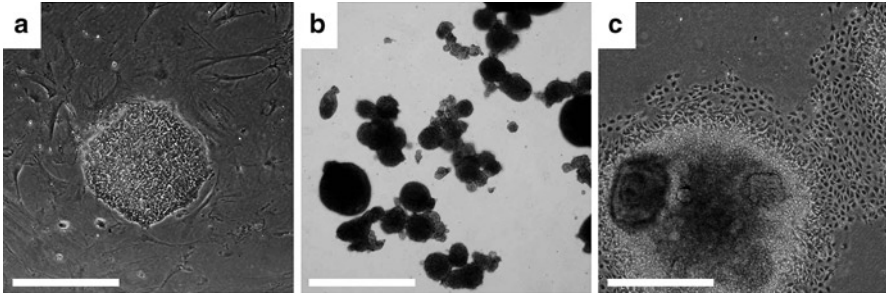


Fig. 2.2 Phase contrast images of undifferentiated and differentiating hESCs in culture. **(a)** A compact colony of proliferating pluripotent hESCs can be seen when cultured in defined medium on mouse embryonic fibroblasts. **(b)** Floating hEBs are observed at 2 days after induction of differentiation. **(c)** Differentiating cardiomyocytes appear within adherent cultures at 48 h after plating hEBs onto a gelatin-coated culture dish. Bar, 25 μ m

Proliferating pluripotent hESCs form compact and spherical cell colonies when grown on mouse embryonic fibroblast cell layers (Fig. 2.2a). Differentiating hESCs are easily distinguished by the loss of compact morphology and the appearance of flattened cells that form at the edges of the colony. This can be controlled with regular supplementation of fresh growth medium [15].

2.3.2 Expression Profiling

A systematic study has been conducted by the International Stem Cell Initiative, a consortium of stem cell researchers from more than 15 countries, on 59 independently derived and commonly used hESC lines in order to identify a panel of molecular markers that are consistently and strongly expressed in pluripotent hESCs [16]. These included developmentally regulated genes such as NANOG, POU domain class 5 homeobox 1 protein (POU5F/OCT4), teratocarcinoma-derived growth factor 1, DNA (cytosine-5-)-methyltransferase 3 β , γ -aminobutyric acid A receptor β 3, and growth differentiation factor 4. The study also established that the collective expression of Stage-Specific Embryonic Antigens 3 and 4, along with keratin sulfate (TRA-1-60, TRA-1-81, GDTM2, and GCT343) and protein (CD9 and Thy1) antigens, are reliable cell surface markers of pluripotent hESCs. Other characteristics of hESCs include the expression of the enzyme alkaline phosphatase, Stem cell factor (or c-Kit ligand), and class 1 HLA.

The expression profile of small, noncoding RNAs known as microRNAs, which regulate translational efficiency of target mRNAs [17], has been evaluated in hESCs by several groups [18–22]. These studies identified a number of miR family clusters specifically expressed in pluripotent hESCs. Among these are miR-92b, the miR-302 cluster, miR-200c, the miR-368 and miR-154* clusters, miR-371, miR-372, miR-373*, miR-373, and the miR-515 cluster [18, 22]. Functional studies of some

of these miRs, such as miR-302 and miR-92b, have established roles in pathways that control self-renewal and maintenance of the pluripotent stem cell state [23, 24]. To date, studies that compare the miR expression profiles of all available hESC lines are still lacking. A comprehensive analysis of miR expression profiles is warranted, as this will identify miRs that are expressed across hESC lines, and could be used to select for pluripotent populations, evaluate newly derived hESC lines, and understand mechanisms that regulate basic hESC biology.

2.3.3 Epigenetic Properties

Epigenetic mechanisms influence gene expression through heritable modifications in chromosomal or DNA structure, such as DNA methylation, histone modification, and X-chromosome inactivation. Similar to expression patterns of coding genes discussed above, the epigenetic properties of pluripotent hESCs can be used as molecular signatures to distinguish them from other cell types.

The chromatin structure of hESCs is generally in an open conformation, making it readily accessible to the transcriptional machinery necessary for the maintenance of pluripotency [25]. It has also been observed that hESC lines display DNA methylation profiles distinct from most other cell types [26]. One study of 14 different lines revealed markedly reduced methylation patterns of CpG dinucleotides when compared to somatic cells. Further analysis revealed that the observed differential methylation of hESCs was specific to promoter regions of pluripotency genes such as OCT4 and Nanog [27]. Thus, the unique epigenetic properties of hESCs likely promote maintenance of the pluripotent state and can be used as a hallmark of undifferentiated hESCs.

To date, almost all established female hESC lines analyzed exhibit partial or complete X-chromosome inactivation, a process that occurs as early as the blastocyst stage and leads to methylation of promoter regions. The states and levels of X-inactivation appear to differ between hESC lines, and also between subcultures of each hESC line that are propagated by different laboratories [28–31]. These observations point out that in addition to genetic heterogeneity, the environment and culture conditions can lead to variable and unstable epigenetic states. The variability in X-chromosome inactivation could result in inconsistencies as hESCs are developed for therapeutic applications. Thus, generating an epigenetically naïve hESC line, in which X-chromosome inactivation or other epigenetic modifications have not yet occurred, is an important goal.

2.3.4 Pluripotency of hESCs

hESCs are defined in part by their capacity to differentiate, which can be tested using *in vivo* and *in vitro* methods. A test of pluripotency *in vitro* involves determining

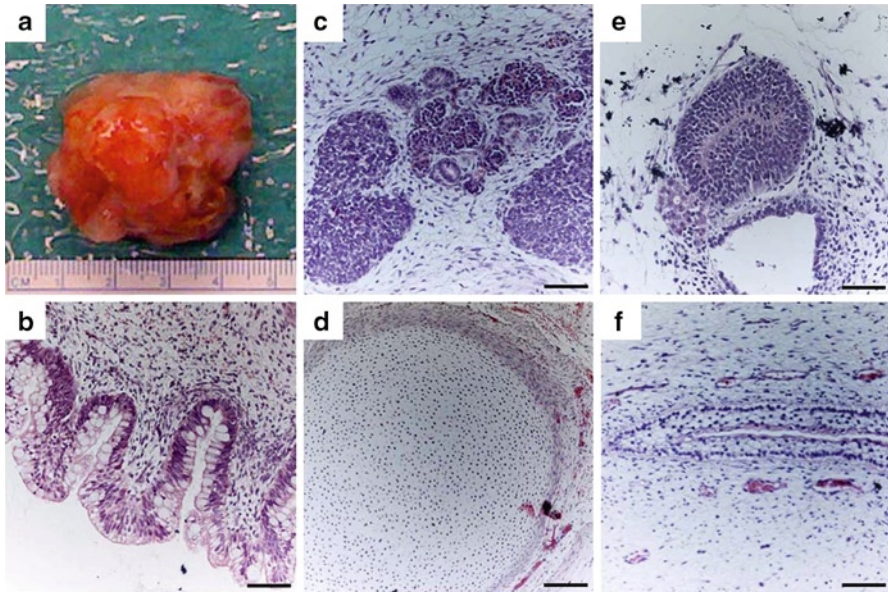


Fig. 2.3 In vivo differentiation of hESCs by teratoma formation. Proliferating cultures of hESCs were used to form teratomas by renal capsule grafting using established methods [35]. (a) An explanted teratoma is shown. (b–f) Teratomas were sectioned and stained with hematoxylin and eosin to identify embryonic tissues. Representative tissues from all three embryonic germ layers can be seen, including endoderm (b), mesoderm (c, d), and ectoderm (e, f). (b) Glandular intestinal structure. (c) Nascent renal tubules and glomeruli within a bed of primitive renal epithelium. (d) Cartilage surrounded by capsule of condensed mesenchyme. (e) Nascent neural tube. (f) Primitive squamous epithelium. Bar, 100 μ m

the ability of hESCs to form hEBs when cultured in a nonadherent cell suspension in the absence of feeder cell layers (Fig. 2.2b). hEBs are spherical colonies of differentiating hESCs that contain cell types representative of all three embryonic germ layers [32]. hEBs can be differentiated into specific tissues under suitable culture conditions (Fig. 2.2c).

The most commonly used in vivo method to test pluripotency involves the transplantation of undifferentiated hESCs into immunodeficient mice to induce the formation of teratomas [33–36]. Teratomas are benign tumors comprised of disorganized tissue structures characteristic of the three embryonic germ layers. Analysis of embryonic tissues found in teratomas from engrafted hESCs can be used to test their differentiation potential (Fig. 2.3).

The ability of hESCs and hEBs to mimic in vitro and in vivo the events occurring during human embryonic development makes them valuable tools for understanding the mechanisms involved in developmental processes, and steppingstones toward the generation of desired cell types suitable for cell therapies.

2.4 Stem Cell Derivatives and Their Uses for Cell-Based Therapies

Cellular insufficiency or deficiency, due to dysfunction or degeneration, respectively, is the root of diseases such as heart failure, neurodegenerative disorders, diabetes, bone marrow failure, and spinal cord injury. For centuries, therapeutic approaches have been limited to the surgical removal of damaged tissues or treatment with pharmacological therapies to ameliorate symptoms and fight infection. Thus, the prospect of replacing damaged or missing cells with new functional cells has shifted the therapeutic paradigm toward restoring tissue function.

Deriving specific cell populations from hESCs that could either replace damaged cells or coax neighboring cells to function normally provides a promising strategy for cell-based therapy. With hESCs, it is possible to generate lineage-restricted progenitors that are capable of differentiating into specialized postmitotic cell types such as cardiomyocytes, pancreatic islet cells, chondrocytes, hematopoietic cells, endothelial cells, or neurons. Furthermore, the ability of hESCs to divide indefinitely makes these cells an inexhaustible large-scale source of specific progenitors. Current research studies are focused on identifying and refining ways for directing the differentiation of hESCs that will enrich for pure, homogenous populations of specific cell types. In the following sections, we provide some examples of how differentiation of hESCs can be directed toward specific cell/tissue types, and the potential use of these cell types for clinical applications (Table 2.1).

2.4.1 *Endodermal Cell Derivatives of hESCs*

Endodermal derivatives include cells that populate the lung, liver, and pancreas. Directing the differentiation of hESCs toward definitive endoderm would help generate specific cell types, such as islet cells or hepatocytes, which could be used toward treatment of diseases such as diabetes or liver disease, respectively. D'Amour et al. [37] showed that selective induction of endoderm could be achieved through the addition of high concentrations of activin A, under low serum conditions, and in a stage-specific manner. Activin A mimics the action of Nodal, a ligand that activates TGF β signaling, which in turn leads to the induction of endoderm differentiation. The effect of activin A in inducing definitive endoderm is enhanced when additional factors such as Wnt3a [38] and Noggin [39] are present, or when coupled with the suppression of the phosphoinositide 3-kinase pathway [40].

Induction of definitive endoderm can lead to the generation of specific progenitor populations following the addition of other factors. Among the most successful examples to date is the generation of pancreatic islet progenitors devised by Kroon et al. [41], accomplished through the sequential exposure of hESCs to activin A and Wnt3A, followed by the addition of keratinocyte growth factor or fibroblast growth factor-7 to induce the formation of the primitive gut tube. Subsequently, retinoic

Table 2.1 Examples of methods used to differentiate hESCs into specific cell types

	Methods to induce differentiation	Differentiation factors and/or culture conditions	Example of differentiated cells
Derivation of endodermal cells from hESCs	Differentiation of hESC into definitive endoderm, followed by sequential exposure to differentiation factors	FGF, BMP4, hepatocyte growth factor, oncostatin M, dexamethasone	Hepatocytes [42, 43]
		Activin A, Wnt3A, keratinocyte growth factor/FGF7, retinoic acid, cyclopamine, noggin	Pancreatic islet progenitors [41]
	Genetic modification of hESCs followed by spontaneous differentiation	Recombinant keratinocyte growth factor	Lung alveolar cells [68, 69]
Derivation of mesodermal cells from hESCs	Human embryoid body formation	Serum-free conditions; BMP4	Dendritic cells [46]
		Micromass of dissociated embryoid bodies; BMP2	Chondrocytes [70]
		High-density culture of dissociated embryoid bodies; ascorbic acid, dexamethasone	Chondrocytes [73]
		Serum-free conditions: bFGF	Cardiomyocytes [50]
	Spin embryoid body formation	Serum-free conditions	Blood cells [45]
	Co-culture with stromal cells	Co-culture with stromal cell line M210-B4 for enhanced proliferation of CD34 ⁺ /CD45 ⁺ hematopoietic progenitor cells	T and NK cells [47]
	Directed differentiation from hESCs by sequential exposure to differentiation factors	Dense monolayer of hESCs; activin A, BMP4	Cardiomyocytes [55]
		BMP4, BMP4/bFGF/activin A, VEGF/DKK1, VEGF/DKK1/bFGF	Cardiomyocytes [56]
	Directed differentiation from hESCs by the addition of differentiation factors on 3D polymeric scaffolds	Co-culture with primary chondrocytes; poly-D, L-lactide scaffold	Chondrocytes [72]
	Genetic modification of hESCs followed by spontaneous differentiation	Cardiac-specific reporter	Cardiomyocytes [53]

Derivation of ectodermal cells from hESCs	Co-culture with stromal cells and addition of differentiation factors	FGF8, SHh	Dopaminergic neurons [59]
	Formation of neural rosettes and addition of differentiation factors	FGF8, SHh Ciliary neurotrophic factor, neuregulin 1β, dbcAMP Retinoic acid, SHh Withdrawal of FGF2, BDNF; addition of GDNF, NGF, dibutyl cyclic AMP Serum-free conditions; activin A, nicotinamide	Dopaminergic neurons [62] Schwann cells [64] Motor neurons [63] Peripheral sympathetic and sensory neurons [64] Retinal pigment epithelium [66] Oligodendrocytes [65]
	Directed differentiation from hESCs with sequential exposure to differentiation factors	B27, thyroid hormone, retinoic acid, FGF2, EGF, insulin BMP4, ascorbic acid	Basal keratinocytes [67]
	Direct differentiation with sequential exposure to differentiation factors on 3D culture with extracellular matrix components		

acid, cyclopamine, and Noggin are added to inhibit hedgehog and TGF β signaling, and thus induce the differentiation of posterior foregut cells, the source of pancreatic cell progenitors. These are cultured further to generate pancreatic endoderm cells. When these cells are engrafted in immunodeficient mice, they display the histological and structural characteristics of pancreatic islet cells, and are able to sustain insulin production for at least 100 days [41].

In a similar manner, hepatocytes can be obtained after differentiation of hESCs into definitive endoderm [42, 43]. A robust population of functional hepatocytes was generated with the sequential addition of low serum medium, collagen I matrix, and hepatic differentiation factors that include FGF, BMP4, hepatocyte growth factor, oncostatin M, and dexamethasone [43]. These cells expressed known markers of mature hepatic cells, exhibited appropriate function, and were able to integrate and differentiate into mature liver cells when injected into mice with liver injury [43].

2.4.2 *Mesodermal Derivatives of hESCs*

Directing the differentiation of hESCs into mesoderm requires the activation of the TGF β signaling pathway and can be accomplished through the stepwise and dosage-dependent addition of activin A, BMP4, and growth factors, VEGF and bFGF [44]. Mesodermal derivatives have also been successfully obtained by spontaneous differentiation of hESCs through hEB formation without first directing them toward mesoderm. Robust differentiation of hESCs into hematopoietic lineage cells, which give rise to all blood cell types and components of the immune system, has been achieved under serum-free conditions through spin hEB formation [45]. Specific hematopoietic cells, such as functional dendritic cells, have been successfully differentiated from hESCs through spontaneous hEB formation under serum-free conditions with the addition of BMP4 at specific time points [46]. Hematopoietic progenitor cells that give rise to functional T and natural killer cells capable of targeting human tumor cells both in vitro and in vivo have also been derived from hESCs co-cultured with stromal cells [47]. Thus, the ability to differentiate hESCs into hematopoietic lineage cells promises to be useful in improving existing therapies that require blood cell transplantation, and in immune therapies that require induction of the immune response in an antigen-specific manner [48].

Cardiomyocytes, which represent another therapeutically important derivative of mesoderm, have been successfully generated from hESCs using several methods [49]. Through hEB formation, hESCs can spontaneously differentiate into cardiomyocytes under appropriate culture conditions. These cardiomyocytes exhibit morphological, molecular, and electrophysiological properties similar to adult cardiomyocytes [50], and display quantifiable responses to physiological stimuli reminiscent of atrial, ventricular, and pacemaker/conduction tissue [51–54]. Cardiomyocytes have also been generated by directed differentiation with activin A and BMP4 on a dense monolayer of hESCs; these cells successfully form specific

cardiac lineages when transplanted in vivo [55]. Another study used additional medium supplements that included VEGF, and the Wnt inhibitor, DKK1, followed by the addition of bFGF to promote cardiomyocyte differentiation from hEBs [56]. Success of these studies was measured by the expression of proteins specific for mature cardiac cells such as cardiac troponin T, atrial myosin light chain 2, and the cardiac transcription factors, Tbx5 and Tbx20.

2.4.3 *Ectodermal Derivatives of hESCs*

The dominant differentiation pathway in hESC cultures leads to the formation of ectoderm, which makes up cells of the nervous system and the epidermis. hESC-derived neural progenitor cells are characterized by rosette-like neural structures that form in the presence of growth factors, FGF2 or EGF, through either spontaneous differentiation from an overgrowth of hESCs or after hEBs are plated onto adherent substrates [57, 58]. These neural rosettes have become the signature of hESC-derived neural progenitors, capable of differentiation into a broad range of neural cells in response to appropriate developmental signals. Thus, many studies are exploring ways to enhance the formation of neural rosettes in order to generate an enriched population of specific neural cell types. One example is the use of specific stromal cell lines [59]. With this method, stromal cells provide ectodermal signaling factors required for neural induction, as determined in animal model studies, and therefore promote the formation of neural rosettes [60, 61].

The withdrawal of FGF2 and EGF, and the addition of specific compounds can lead to the differentiation of neural rosettes into specific neural subtypes. For example, hESC-derived neural progenitors treated with FGF8 and sonic hedgehog give rise to dopaminergic neurons [62], while treatment with sonic hedgehog and retinoic acid induce motor neuron differentiation [63]. Neural crest stem cells derived from neural rosettes can differentiate into peripheral sympathetic and sensory neurons by withdrawing FGF2/EGF and adding BDNF, GDNF, NGF, and dbcAMP, or into Schwann cells in the presence of CNTF, neuregulin 1 β , and dbcAMP [64]. Neuroglial cells, such as oligodendrocytes, are generated with B27, thyroid hormone, retinoic acid, FGF2, epidermal growth factor, and insulin [65].

In 2010, the biotechnology firm, Geron, initiated the first clinical trial with hESCs in the USA using hESC-derived oligodendrocytes to treat acute spinal cord injuries (<http://www.clinicaltrials.gov/ct2/archive/NCT01217008>). Oligodendrocytes are rapidly lost following acute spinal cord injury leading to demyelination and neuronal loss. In these trials, purified oligodendrocyte progenitor cells derived from hESCs will be injected into the spinal cord of paralyzed patients within 2 weeks of the acute injury. While this first trial is a safety study, the expectation is that these progenitor cells will terminally differentiate into oligodendrocytes and produce myelin, which insulates neuronal cell membranes and is critical for efficient conduction of nerve impulses. Thus, the transplantation of newly differentiated oligodendrocytes is expected to restore myelination of damaged neurons preventing further neuronal death and restoring function.

Retinal pigment epithelium (RPE) cells are another specific cell type derived from neuroectoderm. These support the neural retina by phagocytosing and renewing the photoreceptor outer segments of rhodopsin. Recent reports have shown that RPE can be induced from hESCs in the presence of nicotinamide and activin A under serum-free conditions [66]. hESC-derived pigmented cells exhibit the morphological and functional properties of RPE cells after transplantation in an animal model of macular degeneration, a disease caused by dysfunction and loss of RPE. These data have led to the second and third clinical trials using hESCs by the biotechnology company, Advanced Cell Technology. For these trials, hESC-derived RPEs will be transplanted directly into the degenerating retinæ of patients with Stargardt's Macular Dystrophy, a juvenile form of macular degeneration, or Dry Age-Related Macular Degeneration, to rescue visual acuity. The launch of these clinical trials heralds the translation of hESC research into therapy for neurodegenerative disease.

2.5 The Promise of hESCs in Tissue Engineering

Tissue engineering and regeneration utilize biological substitutes to restore or maintain tissue function. As with cell transplantation, a successfully engineered tissue depends on the generation of the appropriate cell type that is able to provide normal cellular function. Thus, cells suitable for tissue engineering should have the ability to enter a desired differentiation program to produce a specific cell type, and be expandable *in vitro* to meet the needs of cell transplantation. hESCs provide much promise in tissue engineering and regeneration since hESCs can act as an inexhaustible *in vitro* source of differentiated cell types. The potential use of hESCs in tissue engineering include, but are not limited to, organ substitutes, vascularization, and *ex vivo* cartilage/bone construction. While these applications are discussed in detail in subsequent chapters, brief examples are provided below.

Basal keratinocytes, the cells that make up the pluristratified epidermal layer of the skin, have been successfully differentiated from hESCs. Guenou et al. [67] have shown that long-term culture of hESCs in defined medium supplemented with BMP4 and ascorbic acid leads to the directed differentiation of hESCs into basal keratinocytes. These cells express keratins 14 and 5, α 6- and β 4-integrins, collagen VII, and laminin 5 at levels comparable to postnatal keratinocytes. More importantly, these hESC-derived keratinocytes form a cohesive pluristratified epidermis when placed in 3D culture or when engrafted into immunodeficient mice. These findings prove the feasibility of using hESC-derived keratinocytes as a source of allograft for patients requiring skin restoration.

The use of hESCs to treat lung injury has also been an area of active investigation. A significant step toward directed differentiation of lung-specific cells was reported by Wang et al. [68, 69], in which genetically modified hESCs carrying lung-specific reporters under the control of promoters from tissue-specific genes such as surfactant

protein C, aquaporin 5, and T1 α , resulted in the purification of type I and type II alveolar epithelial cells. When engrafted into mice suffering from acute lung injury, these cells terminally differentiated *in vivo* into type I and type II alveolar epithelial cells and exhibited functional properties that include the capacity for gas exchange and histological amelioration of lung injury.

hESCs readily form connective tissue, such as bone or cartilage, as can be appreciated from teratoma formation assays (Fig. 2.3). Thus, hESCs are a valuable source of cells suitable for connective tissue replacement therapy for a number of bone and joint diseases, such as osteoarthritis, which is characterized by the breakdown of cartilage within joints. Most successful and efficient protocols for directing chondrocyte differentiation from hESCs utilize 3D culture systems created by seeding hESCs at high density leading to the formation of a pellet, or by introducing the cells into a synthetic 3D scaffold. Such systems enable cell–cell signaling between the undifferentiated hESCs and mature chondrocytes to stimulate homogeneous and sustained chondrogenic differentiation. For example, single-cell suspension of dissociated hEBs cultured as high-density micromass with BMP2 leads to efficient chondrocyte formation [70]. hESCs co-cultured with primary chondrocytes, or in the presence of osteogenic supplements and polymeric scaffolds, yield cartilaginous- or osteogenic-like cells [71, 72]. More recently, feeder-free 3D culture systems have successfully derived multipotent connective tissue progenitors from hESCs yielding tendon-like structures [73]. The engraftment of these *in vitro* differentiated tendon structures in injured immunosuppressed mice restored ankle joint movements that rely on an intact Achilles tendon [73]. Furthermore, there is evidence that transplanted chondrogenic cells may exert a stimulatory effect through paracrine mechanisms that promote growth and repair of endogenous cells [74].

2.6 Current Challenges

As discussed above, cell therapy with hESCs has begun to enter clinical trials. The International Stem Cell Banking Initiative has been created by the International Stem Cell Forum, a group of national and international stem cell research funding bodies, to develop a set of best practices and principles when banking, testing, and distributing hESCs for clinical application [75]. In the USA, the Food and Drug Administration also monitors these guidelines and has issued recommendations for reviewers of proposals for clinical trials of stem cell therapy (<http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Xenotransplantation/ucm074131.htm>). It is important to note that these recommendations do not ensure the quality or efficacy of hESC-derived cells used for clinical application. Rather, these guidelines warrant that the cells used for therapy are reproducible and meet specific criteria to ensure patient safety (Table 2.2). The major safety concerns for the use of hESCs are discussed in the following sections.

Table 2.2 Requirements for standardization and optimization of hESCs for clinical use

Important factors	Examples of test methods
Cell line identity: must match all alleles of parent cell line	Short tandem repeat (STR) testing or human leukocyte antigen (HLA) testing
Sterility and pathogen screening	Bacteria/fungi/mycoplasma testing by microbiological culture; qPCR analysis for murine viral short interspersed elements (SINE)
Genetic/chromosomal stability	Analysis of multiple single nucleotide polymorphisms (SNP); karyotype by G-band analysis of 20 metaphase spreads or fluorescent in situ hybridization
Epigenetic stability	MicroRNA profiling, methylation analysis, X-inactivation
Pluripotency	Formation of teratomas in immunodeficient mice; flow cytometry to determine hESC-specific antigens such as SSEA-3/4, TRA-1-60, TRA-1-81
Quality and differentiation ability	Gene expression profiling by DNA microarray or qPCR analysis to analyze expression of markers of pluripotency or differentiated cell types; ability to form embryoid bodies
Functional assays	Report on potency, efficacy, and lot-to-lot variability

2.6.1 *Xenobiotic-Free Conditions*

Many of the hESC lines currently in use have been exposed to animal products during isolation of the inner cell mass and propagation of hESCs in vitro. Under these conditions, hESCs could possess animal viruses and other unknown substances capable of eliciting a detrimental immune response in transplanted hosts. Currently, hESC lines under development for clinical use undergo extensive microbiological testing as strictly recommended by the International Stem Cell Banking Initiative. In the USA, the Food and Drug Administration legally requires documentation of the source, potential genetically modified components, and pathogenic agents in any hESC-derived cell intended for therapeutic use. Thus, avoiding exposure to xenobiotics is emphasized by law. Recently, replacement media have been developed that would allow maintenance of hESCs in xenobiotic-free conditions. These include xenobiotic-free serum replacements such as knockout serum replacer (KSR; Invitrogen) or xenobiotic-free culture media such as HESGRO (Millipore) or TeSR (STEMCELL).

Feeder-free culture systems are now being developed to reduce the risk of contamination with foreign agents when hESCs are cultured on animal feeder cell layers. Feeder-free and xenobiotic-free, defined culture media that consist of a combination of recombinant growth factors known to inhibit differentiation and maintain hESCs in the pluripotent state are now commercially available. However, some reports have associated feeder-free culture conditions with greater chromosomal instability and an increased risk of propagating genetically altered hESCs [76]. For this reason, most hESC laboratories practice a surveillance program for genomic instability in cultured lines [36, 53].

hESC lines derived using human feeder cells have been reported. For example, hESC lines have been successfully derived on human fibroblasts generated from neonatal foreskin [77, 78] and adult skin fibroblasts [79]. Some laboratories deriving new lines have moved exclusively to xenobiotic-free conditions [80]. The ability to derive and maintain new hESC lines using human fibroblast feeder cells represents a significant step toward generating clinical-grade hESCs.

2.6.2 Genetic Abnormalities in hESC Lines

The best characterized hESC lines to date are among the earliest lines derived. However, they may not be the best lines for therapeutic applications as many of these lines were derived using animal products. Chromosomal and genomic instability has been detected among several hESC lines, including loss of heterozygosity or copy-number variation in cancer-related genes [81, 82]. Many of these mutations appeared to be induced by prolonged culture, since these changes were not observed in low passage cells. It has been proposed that such karyotypic aberrations occurred with adaptation to the original culture conditions used when the first few lines were being derived and expanded [83]. These observations emphasize the need for complete characterization of hESC lines, particularly the effects of long-term culture, and the design of guidelines for designating therapeutic-grade hESCs.

2.6.3 Enrichment, Directed Differentiation, and Purification Protocols for hESCs

A primary safety concern when using pluripotent hESCs is their potential to form germ layer tumors. As discussed above, in vivo transplantation of undifferentiated hESCs in immunodeficient mice results in teratoma formation. Evidence of tumor-like growths has also been observed in differentiated hESC derivatives transplanted in vivo [84, 85]. Thus, it is essential that candidate hESC derivatives intended for use in cell transplantation are free of tumorigenic cells. Another concern is the differentiation of hESC-derived cells into unwanted cell types. For example, the engraftment of inappropriate muscle cells into the myocardium could alter the electrical activity of recipient tissue, provoking arrhythmias [86]. Thus, developing and further optimizing differentiation and purification protocols are necessary to minimize the generation of unwanted cell types for preclinical transplantation experiments and clinical therapy.

As discussed earlier in this chapter, enrichment of specific cell types can be achieved using molecules introduced at specific time points during culture. However, many of these methods yield only moderate enrichment that is not yet scalable for clinical application. It may be desirable to enrich first for partially differentiated, proliferative hESC intermediates with specific cell fates. These could then be

expanded before further differentiation into cells for therapy. For example, the expression of the cell surface antigen, CD133, on proliferating hESCs identifies cells predestined toward a neuroectodermal fate [34]. CD133-positive cells have been selected from cultures of undifferentiated hESCs, and have been observed to differentiate primarily into neuroectodermal cells in vitro and in vivo [34].

In the absence of specific cell surface antigens such as CD133 to identify tissue-specific precursors, molecular beacons have been used to select for specific subpopulations of hESCs. King et al. [33] first demonstrated the utility of this system in isolating live Oct4-expressing pluripotent hESCs in a specific and high-throughput manner. Molecular beacons are single-stranded oligonucleotides that generate fluorescent signals when bound to their target mRNAs, making these cells detectable and selectable by fluorescence-activated cell sorting. More importantly, molecular beacons have a short lifespan within cells and do not alter the function or genomic structure of hESCs. Thus, this method can be used to enrich for desired hESC-derived cell populations or used to select against unwanted cell types, such as undifferentiated hESCs that could form tumors.

2.6.4 Circumventing Immune Rejection Using Transplanted hESC-Derived Cells

Transplanted hESCs encounter immune rejection [87] because proliferating and differentiated hESCs express class I and II HLA as well as minor histocompatibility antigens at levels sufficient to activate the immune system [87, 88]. Another potential barrier to hESC engraftment can occur through mismatch between donor hESC and recipient ABO blood group antigens. While studies to determine the effects of ABO incompatibility on hESC transplantation are still lacking, this has long been a criterion for successful organ transplantation and thus, it is likely that ABO incompatibility between hESC-donor cells and the recipient would also trigger immune rejection.

Ideally, having genetically identical donor and patient cells is the best way to circumvent immune rejection. Thus, there is high interest in developing and using somatic cell nuclear transfer to generate patient-specific hESC lines. Using this technique, the DNA obtained from either a patient's skin or muscle cell would be transferred into an unfertilized egg that has had its DNA removed. Subsequently, the egg is artificially fertilized and allowed to develop until it reaches the blastocyst stage to derive hESCs. The resultant hESC line would have an immunologic profile matching the patient and could be used for cell therapy. This technique has been conducted successfully in animals using species-specific ESCs, but the bona fide derivation of hESCs through somatic cell nuclear transfer has not yet been reported.

Another strategy is to generate hESC lines with the closest match to potential transplant patients. Suggestions have included engineering "universal donor hESCs," a blood antigen O cell in which the expression of HLA is suppressed, or chimeric hematopoietic cells derived from hESCs capable of inhibiting the immune response when co-transplanted with the desired hESC-derived cells [89]. Alternatively, creating

hESC banks that store lines representing HLA/ABO combinations that match the majority of potential patients has been proposed. Studies have provided estimates on how many hESC lines would be needed in order to support the needs of a specific population. Taylor et al. [90] estimated that approximately 150 hESC lines could provide an HLA match for most of the population in the United Kingdom. Alternatively, approximately ten parthenote-derived hESC lines that are homozygous for HLA types could be sufficient for a majority of the population. Studies by Nakajima et al. [91] estimated that approximately 170 hESC lines, or 55 hESC lines with homozygous HLA types, would be sufficient for 80% of patients in the Japanese population. These findings demonstrate the feasibility of creating and maintaining a hESC bank with sufficient representation to support a large number of patients. However, in countries such as the USA, many more hESC lines would need to be established to serve its ethnically and genetically diverse population. Given the ethical issues and restrictions on hESC research, and the small number of approved hESC lines currently available, the creation of a hESC bank with a highly diverse collection of cell lines will undoubtedly face enormous challenges.

2.7 Conclusions

Research on hESCs has progressed significantly since their first derivation in 1998. The international scientific community has discovered the enormous potential of hESCs as newly derived lines continue to be developed, and differentiation methods into various types of cells are optimized for scientific investigation and clinical use. It is clear that there are still major scientific challenges as well as ethical and legislative issues that must be addressed, especially in the USA. Certainly more questions will emerge as more is understood in the coming years. However, it is encouraging to see that clinical trials involving the use of hESCs in spinal cord injury and macular degeneration have begun. These studies will pave the way toward determining the therapeutic benefit of hESCs in regenerative medicine.

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