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. These cells also provide an opportunity to study the development of human tissues ex vivo. To date, numerous human embryonic stem cell lines have been derived and characterized. In this review, we will detail the strategies used to direct tissue-specific differentiation of embryonic stem cells. We also will discuss how these strategies have produced new sources of tissue-specific progenitor cells. Finally, we will describe the next generation of methods being developed to identify and select stem cell-derived tissue precursors for experimental study and clinical use.

Abstract

Human embryonic stem cells have the capacity for self-renewal and pluripotency and thus are a primary candidate for tissue engineering and regenerative therapies. These cells also provide an opportunity to study the development of human tissues ex vivo. To date, numerous human embryonic stem cell lines have been derived and characterized. In this review, we will detail the strategies used to direct tissue-specific differentiation of embryonic stem cells. We also will discuss how these strategies have produced new sources of tissue-specific progenitor cells. Finally, we will describe the next generation of methods being developed to identify and select stem cell-derived tissue precursors for experimental study and clinical use.

Enrichment of tissue-specific precursors

Pluripotency is defined as the potential of a stem cell to differentiate into tissues originating from all of the three embryonic germ layers: endoderm (for example, gastrointestinal tract and lung), mesoderm (for example, heart, muscle, bone, blood, and urogenital), and ectoderm (for example, nervous system and epidermis). At the molecular level, highly regulated transcriptional circuitry as well as extrinsic factors control the pluripotent state [1,2]. In addition, chromatin remodeling and small non-coding RNAs have been implicated in the regulation of pluripotency [2,3]. Pluripotency can be tested by using in vitro and in vivo methods. A test of pluripotency in vitro involves determining the ability of hESCs and iPSCs to form human embryoid bodies (hEBs) when cultured in a non-adherent cell suspension in the absence of feeder cell layers. hEBs are spherical colonies of differentiating stem cells that contain cell types representative of all three embryonic germ layers [4]. The most commonly used in vivo method to test pluripotency involves the transplantation of undifferentiated stem cells into immunodeficient mice to induce the formation of teratomas [4-6]. Teratomas are benign tumors composed of disorganized tissue structures characteristic of the three embryonic germ layers. Analysis of embryonic tissues found in teratomas from engrafted stem cells can be used to test their differentiation potential (Figure 1).

The ability of hESCs and iPSCs to mimic in vitro and in vivo the events occurring during human development makes them not only valuable tools for understanding the mechanisms involved in developmental processes but also stepping stones toward the generation of desired cell types suitable for cell therapies. Recent studies have shown that it is possible to generate lineage-restricted...
progenitors that are capable of differentiating into specialized post-mitotic cell types such as cardiomyocytes, pancreatic islet cells, chondrocytes, hematopoietic cells, endothelial cells, and neurons. Furthermore, the ability of pluripotent stem cells to divide indefinitely makes these a potential large-scale source of specific progenitors. In the following sections, we will provide examples of how stem cell differentiation can be directed toward specific cell/tissue types.

Developmental programming

Some of the most effective strategies for directing the differentiation of pluripotent stem cells into specific cell types have taken advantage of our understanding of human development (Table 1). Endodermal derivatives include cells that populate the lung, liver, and pancreas. Directing the differentiation of hESCs and iPSCs toward definitive endoderm would help generate specific cell types, such as islet cells or hepatocytes, which could be used in the treatment of diseases such as diabetes or liver disease, respectively. D’Amour and colleagues [7] 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 transforming growth factor-beta (TGFβ) 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 [8] and Noggin [9] are present or when coupled with the suppression of the phosphoinositide 3-kinase pathway [10].

Induction of definitive endoderm can lead to the generation of specific progenitor populations after the addition of other factors. Among the most successful examples to date is the generation of pancreatic islet progenitors, devised by Kroon and colleagues [11] and 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 acid, cyclopamine, and Noggin are
added to inhibit hedgehog and TGFβ 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 engrafted in immunodeficient mice, these cells display the histological and structural characteristics of pancreatic islet cells and are able to sustain insulin production for at least 100 days [11].

In a similar manner, hepatocytes can be obtained after differentiation of hESCs into definitive endoderm [12,13]. 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 fibroblast growth factor (FGF), bone morphogenetic protein 4 (BMP4), hepatocyte growth factor, oncostatin M, and dexamethasone [13]. 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 [13].

Directing the differentiation of stem cells into mesoderm requires activation of the TGFβ signaling pathway and can be accomplished through the stepwise and dosage-dependent addition of Activin A, BMP4, vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (bFGF) [14]. 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 [15]. 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 [16]. 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 [17]. 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 [18].

Cardiomyocytes, which represent another therapeutically important derivative of mesoderm, have been successfully generated from pluripotent stem cells by using several methods [19]. Spontaneous differentiation of stem cells under appropriate culture conditions can produce cardiomyocytes that exhibit morphological, molecular, and electrophysiological properties similar to those of adult cardiomyocytes [20] and display quantifiable responses to physiological stimuli reminiscent of atrial, ventricular, and pacemaker/conduction tissue.

### Table 1. Examples of directed differentiation of human embryonic stem cells into specific cell types

| Embryonic germ layers | Differentiation factors or culture conditions or both | Example of differentiated cells |
|-----------------------|------------------------------------------------------|--------------------------------|
| Endoderm              | FGF, BMP4, hepatocyte growth factor, oncostatin M, dexamethasone | Hepatocytes [12,13] |
|                       | Activin A, Wnt3A, keratinocyte growth factor/FGF7, retinoic acid, cyclopamine, noggin | Pancreatic islet progenitors [11] |
| Mesoderm              | Serum-free conditions; BMP4 | Dendritic cells [16] |
|                       | Micromass of dissociated embryoid bodies; BMP2 | Chondrocytes [100] |
|                       | High-density culture of dissociated embryoid bodies; ascorbic acid, dexamethasone | Chondrocytes [101] |
|                       | Co-culture with primary chondrocytes; poly-D, L-lactide scaffold | Chondrocytes [102] |
|                       | Serum-free conditions | Blood cells [15] |
|                       | Dense monolayer of hESCs; Activin A, BMP4 | Cardiomyocytes [25] |
|                       | BMP4, BMP4/FGF/Activin A/VEGF/DKK1, VEGF/DKK1/bFGF | Cardiomyocytes [26] |
| Ectoderm              | FGF8, SHH | Dopaminergic neurons [29,103] |
|                       | Ciliary neutrotrrophic factor, neuregulin 1β, dbcAMP | Schwann cells [34] |
|                       | Retinoic acid, SHH | Motor neurons [33] |
|                       | Withdrawal of FGF2, BDNF; addition of GDNF, NGF, dbcAMP | Peripheral sympathetic and sensory neurons [34] |
|                       | Serum-free conditions; Activin A, nicotinamide | Retinal pigment epithelium [104] |
|                       | B27, thyroid hormone, retinoic acid, FGF2, EGF, insulin | Oligodendrocytes [35] |
|                       | BMP4, ascorbic acid | Basal keratinocytes [105] |

BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; BMP, bone morphogenetic protein; dbcAMP, dibutyryl cyclic adenosine monophosphate; DKK1, Dickkopf-related protein; EGF, epidermal growth factor; FGF, fibroblast growth factor; GDNF, glial cell-derived neurotrophic factor; hESC, human embryonic stem cell; NGF, nerve growth factor; SHH, sonic hedgehog; VEGF, vascular endothelial growth factor.
Cardiomyocytes have also been generated by directed differentiation with Activin A and BMP4 of a dense monolayer of stem cells; these cells successfully form specific cardiac lineages when transplanted in vivo [25]. Another study used additional medium supplements that included VEGF and the Wnt inhibitor, DKK1, followed by the addition of bFGF to promote cardiomyocyte differentiation in culture [26]. The 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.

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 either through spontaneous differentiation from an overgrowth of hESCs or after hEBs are plated onto adherent substrates [27,28]. 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 [29]. 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 [30,31].

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 [32], whereas treatment with sonic hedgehog and retinoic acid induces motor neuron differentiation [33]. Neural crest stem cells derived from neural rosettes can differentiate into peripheral sympathetic and sensory neurons by withdrawing FGF2/EGF and adding brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), nerve growth factor (NGF), and dibutyryl cyclic adenosine monophosphate (dbcAMP) or into Schwann cells in the presence of ciliary neurotrophic factor (CNTF), neuregulin 1β, and dbcAMP [34]. Neuroglial cells, such as oligodendrocytes, are generated with B27, thyroid hormone, retinoic acid, FGF2, epidermal growth factor, and insulin [35].

### Chemical enrichment

In some cases, the manipulation of key pathways during germ layer development has allowed significant enrichment of specific cell types without the complete recapitulation of embryonic development. Treatment with 5-azacytidine at days 6 to 8 of hESC differentiation significantly increases cardiac alpha-myosin heavy chain (αMHC) expression and enhances cardiomyocyte differentiation, suggesting that DNA demethylation is a key factor in directing tissue-specific differentiation [36]. Similarly, exposure to SB203580, a small-molecule inhibitor of p38MAPK, has been shown to significantly improve cardiomyocyte differentiation of hESCs grown in medium conditioned by mouse END2 cells, supporting a role for p38MAPK signaling in regulating human cardiomyocyte differentiation [37]. SB203580-treated hEBs display an increase in expression of both early mesoderm markers (Brachyury T, Tbx6, and Mesp1) and cardiac αMHC as well as increased cardiomyocyte numbers. Gaur and colleagues [38] subsequently showed that p38MAPK inhibition occurs in a dose- and stage-dependent manner, that it also causes the accelerated differentiation of hESC-derived cardiomyocytes by using the standard hEB formation method, and that it appears to act at the ectoderm/mesoderm endoderm branch point during hESC differentiation.

In the original study with SB203580, cells were subjected to an adapted differentiation system in which hESCs were differentiated in a suspension culture by using serum-free medium conditioned by the mouse END2 cell line [39]. END2-conditioned medium alone exhibits cardiomyocyte-inducing activity during hESC differentiation [37], and biochemical as well as microarray analysis of END2-conditioned medium and END2 cells, respectively, identified PG12, a product of prostaglandin synthase enzymes, as an inducing factor in hESC cardiac differentiation [40]. Two key enzymes involved in PG12 synthesis are upregulated in END2 cells compared with control MES1 cells [41], which lack cardiogenic activity. PG12 levels are between 6- and 10-fold higher in END2-conditioned medium compared with control conditioned medium from MES1 cells. Moreover, insulin, a common supplement in media formulations, was discovered to be an inhibitor of hESC cardiac differentiation. END2-conditioned medium supplemented with increasing concentrations of insulin results in a dramatic decrease in hESC cardiomyocyte differentiation. Thus, addition of PG12 in combination with insulin-free, unconditioned medium yields effective cardiac induction similar to that produced by END2-conditioned medium. Cardiac differentiation is further augmented in the presence of SB203580. Taken together, these three components provide a basic synthetic recipe for directing cardiomyocyte differentiation of hESCs.

### Mechanical factors that influence differentiation

Since cardiac muscle is one of the few tissues that develop under the effects of dynamic force, it is not surprising...
that conditions generated by the force of fluids in motion can enhance cardiomyocyte differentiation as well. Supplying a constant rotary orbital motion for 7 days to suspension cultures of differentiating mouse EBs (mEBs) results in a significantly increased number of beating mEBs compared with mEBs cultured in static suspension [42]. Analysis of gene expression shows higher levels of mesodermal and cardiac proteins (Brachyury, GATA4, Nkx2-5, MEF2c, αMHC, and MLC2v) in rotary mEBs than in static mEBs. In addition, a greater proportion of rotary mEBs stain positive for α-sarcomeric actin compared with static EBs. The enhanced cardiomyocyte differentiation is independent of rotary speed ranging from 25 to 55 revolutions per minute as determined by the expression of cardiomyogenic genes [43].

Domian and colleagues [44] have examined the effects of surface tension on cardiomyogenic differentiation of murine cardiac progenitors. Progenitors derived from embryos and mouse ESCs (mESCs) are cultured on either fibronectin-coated slides or micropatterns of fibronectin alternating with a surfactant that blocks cell adhesion. When grown on these micropatterned surfaces, a population of cells form longitudinally aligned myocardi al fibers. In addition, culturing this population on micropatterned surfaces results in a statistically significant increase in the proportion of cardiomyocytes, supporting a role for microenvironmental forces in cardiac muscle differentiation.

Studies of substrate stiffness and elasticity during stem cell differentiation have also demonstrated effects on skeletal muscle and bone development. Myosin/actin striations occurred only when myoblasts were cultured on gels with stiffness typical of normal muscle [45]. In addition, muscle stem cells cultured on hydrogel substrates that mimic the elasticity of muscle self-renew and fuse to existing myofibers with greater efficiency than cells grown on rigid plastic [46]. Substrate stiffness has also been shown to favor mesodermal formation from ESCs, specifically osteogenic differentiation [47].

These observations, as well as an evolving understanding of the tissue environment in which stem cells differentiate in vivo, underline the recent application of biomaterials to promote the differentiation and retention of stem cells in a number of tissues [48,49]. For example, optimal sizing of pancreatic β-cell clusters derived from hESCs is essential to preventing nutrient and oxygen deprivation in β-cell transplant therapy for diabetes. As shown by Van Hoof and colleagues [50], the size of β-cell aggregates can be controlled by using micropatterned laminin. Polymer scaffolds have also been studied to facilitate transplantation of retinal progenitor cells for the treatment of macular degeneration. Steedman and colleagues [51] demonstrated that biodegradable thin films of polycaprolactone could be topographically fabricated to enhance the attachment and organization of progenitor cells and induce their differentiation to photoreceptor cells.

**Epigenetic reprogramming**

MicroRNAs (miRNAs) are small non-coding RNAs thought to regulate the expression of 30% of protein-coding genes [52]. The biological importance of these RNAs in stem cell biology is underscored by recent studies demonstrating that mESCs lacking the miRNA-processing enzyme Dicer display differentiation and proliferation defects [53-56]. miR-1 and miR-133 specifically are expressed in the mouse heart [57,58]. Targeted deletion or knockdown of these miRNAs results in dysregulation of cardiac morphogenesis, electrical conduction, cell-cycle, and cardiac hypertrophy [57-60]. Recently, Ivey and colleagues [61] showed that miR-1 and miR-133 regulate the differentiation of mESCs and hESCs into the cardiac lineage. Both miRNAs are enriched in mESC-derived cardiomyocytes. Lentiviral introduction of either miR-1 or miR-133 into mESCs enhances early mesoderm differentiation as evidenced by increased expression of Brachyury. miR-1 and miR-133 also reinforce mesoderm lineage decisions by repressing endoderm and neuroectoderm differentiation. When stimulated to differentiate into either endoderm or neuroectoderm lineages, mEBs expressing either miR-1 or miR-133 express lower levels of endodermal and neural markers compared with control mEBs. However, further differentiation revealed opposing roles of miR-1 and miR-133. miR-1 promotes differentiation of mesoderm into the cardiac and skeletal muscle lineages as determined by enhanced Nkx2-5 and myogenin expression, respectively, whereas miR-133 blocks induction of both markers. Importantly, the differentiation of hESCs in the presence of miR-1 behaves comparably to mESC differentiation. Overexpression of miR-1 in hESCs increases Nkx2-5 expression and yields more than a threefold higher number of beating hEBs compared with wild-type controls.

Whereas miRNAs direct cell lineage determination by controlling protein dosage, epigenetic regulation through chromatin remodeling has been shown to control cell fate as well. Takeuchi and Bruneau [62] identified a minimal set of factors necessary to execute the cardiac transcriptional program. Baf60c, a cardiac-enriched subunit of the Swi/Snf-like BAF chromatin remodeling complex, in combination with cardiac transcription factors GATA4 and Tbx5, is able to induce cardiac differentiation in mouse embryos when ectopically expressed. With this combination, 90% of the transfected embryos display expression of the early cardiac marker Actc1 and 50% of the transfected embryos exhibit beating tissue. GATA4, together with Baf60c, is essential in initiating the cardiac gene program as assessed by expression of Actc1. Neither
of the other transcription factors tested alone (Tbx5 and Nkx2-5) or in concert with Baf60c is able to induce Actc1 expression. GATA4/Baf60c, however, is not sufficient for generating spontaneously contracting embryonic tissue: Tbx5 is required to achieve beating cardiomyocytes.

Selection of tissue-specific precursors
As discussed earlier in this review, enrichment of specific cell types can be achieved by 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. In addition, it may be desirable to enrich first for partially differentiated, proliferative stem cell intermediates with specific fates. These could then be expanded before further differentiation into cells for therapy. In the following sections, we will discuss the variety of methods used to select tissue-specific precursors and their derivatives.

Use of cell surface markers
Even before the tools of genetic engineering were employed to manipulate stem cells, proteins expressed on the cell surface were used to identify distinct populations of stem cells and their ontogeny. Perhaps the best-known application of this approach is to the intermediates of hematopoiesis [63]. Over the past 10 years, similar attempts to create lineage maps of other tissues have focused on the identification of lineage-specific cell surface markers (Table 2). For example, pluripotent stem cells can be identified by the expression of stage-specific embryonic antigen (SSEA) 3 and 4 and the embryonal carcinoma marker Tra-1-60 on their surface [4]. The expression of the cell surface antigen CD133 on proliferating hESCs identifies cells predestined toward a neuroectodermal fate [4]. Hemangioblasts that ultimately give rise to hematopoietic stem cells, smooth muscle progenitors, and endothelial progenitors can be selected on the basis of the surface expression of CD143, E-cadherin, VE-cadherin, platelet endothelial cell adhesion molecule 1 (PECAM-1), and vascular endothelial growth factor receptor 2 (VEGFR2) [64]. Mesenchymal stem/stromal cells that differentiate into muscle, fat, cartilage, and bone cells can be identified by the expression of the cell surface proteins CD105/endoglin, CD73, and CD90/Thy-1 in the absence of surface expression of CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA-DR [65]. Recently, cardiomyocytes that have, until now, eluded detection by specific surface protein expression were shown to express signal-regulatory protein alpha (SIRPA) [66].

Two main approaches have been used to identify lineage-specific surface markers. One employs labeled cell fate mapping to assess the tissue-specific fate of subpopulations of pluripotent stem cells expressing specific surface proteins [4], whereas the other uses a high-throughput flow cytometry screen of available antibodies against stem cell derivatives [66]. Although there are still large gaps in our ability to identify and select derivatives of all three germ layers on the basis of surface marker expression, progress continues to be made.

Biochemical purification
Although detection of lineage-specific genes underlies most methods for selecting specific cell types from differentiating stem cell cultures, a few cell types, such as cardiac muscle cells, bear unique biochemical properties that aid in their isolation. For example, Percoll gradient centrifugation has been reported to purify hESC-derived cardiomyocytes [36,67], based on their buoyancy characteristics. Differentiating hESCs are applied to a discontinuous Percoll gradient consisting of 40.5% Percoll layered over 58.5% Percoll. After centrifugation, the majority of cardiomyocytes reside within the 58.5% Percoll layer and express cardiac troponin I, sarcomeric MHC, αMHC, βMHC, and N-cadherin. hESC-derived cardiomyocytes of almost 70% purity have been obtained by using this approach.

A second purification strategy is based on the observation that cardiomyocytes have high mitochondrial content compared with that of non-myoocytes [68]. Using the fluorescent dye tetracythylrhodamine methyl ester (TMRM), which freely diffuses into the mitochondrial matrix, Hattori and colleagues [68] found that TMRM fluorescence in embryonic rat hearts increases with developmental stage, suggesting that mitochondrial biogenesis is linked to myocardogenesis. In whole rat embryos, TMRM fluorescence in the heart is more robust than in other tissues, and when analyzed by flow cytometry, flow-sorted populations with the highest TMRM fluorescence are observed to express cardiac α-actinin. TMRM-labeled cardiomyocytes derived from mESCs are positive for both Nkx2-5 and α-actinin. The cardiomyocyte content in cultured cells sorted from day-12 to day-25 mEBs is greater than 99% as determined by Nkx2-5 and α-actinin expression. Most notably, greater than 99% cardiomyocyte purity is also obtained in cultured cells sorted from differentiating hEBs.

Transgenic reporter cell lines
The use of fluorescent reporters of gene expression offers an approach to selecting tissue-specific cells by high-throughput fluorescence-activated cell sorting [69]. Alternatively, the use of reporters that activate expression of a selectable marker can drive a population of cells to near-homogeneity by restricting survival to a specific cell type. The paucity of specific cell surface markers with which to select cardiac cells has led to the development
of a variety of reporter lines with which to select stem cell-derived cardiomyocytes and cardiac progenitors. Huber and colleagues [70] used lentiviral vectors to produce stable hESC lines in which enhanced green fluorescent protein (eGFP) is expressed under control of the cardiac-specific human ventricular myosin light chain 2 promoter. Xu and colleagues [71] generated stable hESC lines by using a reporter plasmid consisting of the cardiac-specific mouse αMHC promoter driving expression of the neomycin resistance gene. Kita-Matsuo and colleagues [72] designed a set of lentiviral vectors to generate multiple stable hESC lines with eGFP and mCherry reporters or with puromycin resistance downstream of the mouse αMHC promoter. Ritner and colleagues [23] generated a cardiac-specific hESC reporter line by using a lentiviral construct consisting of

| Stem cell | Progenitor cell | Differentiated cell |
|-----------|----------------|---------------------|
| Pluripotent stem cell | SSEA-3/4, TRA-1-60 | Neuron-restricted progenitor | Neuronal cell |
| Ectoderm | Neural stem cell | NCAM [108] | PSD-95, Synaptophysin [109] |
| | CD133, ABCG2 [4,106,107] | Glial-restricted progenitor | Oligodendrocyte |
| | | FGFR [110-112] | MOG, CD140a [113,114] |
| | | | Type 2 astrocyte |
| | | | Type 1 astrocyt |
| | | | FGFR3 [110,111] |
| Surface ectoderm | Motor neuron progenitor | Motor neuron |
| | | | |
| | Epidermal stem cell | a6-integrin [56], CD71 [115] |
| | | Myeloid/lymphoid lineages [118] |
| Mesoderm | Hemangioblast | CD34, CD44, CD150, VEGFR2, Sca-1 [63] |
| | CD143, E-cadherin, VE-cadherin, PECAM-1, VEGFR2 [64] | Smooth muscle progenitor | Smooth muscle cell |
| | | VE-cadherin [119] | VE-cadherin [119] |
| | | Endothelial progenitor | Endothelial cell |
| | CD31, CD105, CD144, CD146, VEGFR2, VWF [120] | | CD31 [120] |
| | Mesenchymal stem cell | Myogenic precursor | Cardiac muscle |
| CD105/endoglin, CD73, CD90/Thy-1 [65] | a7-integrin, M-cadherin [121] | SIRPA [66] |
| | | | Skeletal muscle |
| | | | a7β1-integrin [122] |
| | | | Pre-adipocyte |
| | | | Adipocyte |
| | | | Glut4 [123] |
| | | | Chondrocyte precursor |
| | | | Chondrocyte |
| | | | CD44 [124] |
| | | | Osteoblast |
| | | | Osteocyte |
| | | | |
| Endoderm | Primitive endoderm | Hepatic progenitor | Hepatocyte |
| | | c-Met, CD49f [125] | IL-6 [126] |
| | Definitive endoderm | Pancreatic progenitor | α cell |
| | | | β cell |
| | | | Glut2 [127] |
| | | | PPγ cell |
| | | | δ cell |
| | | | ε cell |

ABCG2, ATP-binding cassette sub-family G member 2; CEACAM-1, carcinoembryonic antigen-related cell adhesion molecule 1; EpCAM, epithelial cell adhesion molecule; FGFR, fibroblast growth factor receptor; Glut, glucose transporter; MOG, myelin oligodendrocyte glycoprotein; NCAM, neural cell adhesion molecule; PECAM-1, platelet endothelial cell adhesion molecule 1; P-SDF-95, post-synaptic density protein 95; SIRPA, signal-regulatory protein alpha; SSEA, stage-specific embryonic antigen; VEGFR2, vascular endothelial growth factor receptor 2; VWF, von Willebrand factor.
a fragment of the mouse αMHC promoter upstream of eGFP. The specific promoter fragment allowed the identification and analysis of early cardiac progenitors expressing Nkx2-5 but before the onset of cardiac troponin T or chamber-specific MLC expression [23].

Whereas isolation of hESC-derived cardiomyocytes from these reporter lines is based on positive selection, Anderson and colleagues [73] implemented a negative selection strategy to deplete undifferentiated proliferating hESCs from cultures of hESC-derived cardiomyocytes. Their hESC line used a herpes thymidine kinase/gancyclovir (HTK/GCV) suicide gene system under the control of a constitutive phosphoglycerate kinase promoter. After administration of the antiviral drug GCV, cells expressing HTK-phosphorylated GCV incorporated the guanosine analog into nascent DNA chains of proliferating cells and caused chain termination and cell death. The increased number of α-actinin-positive cells after GCV treatment led to an almost sevenfold enrichment of cardiomyocytes. An important caveat of this approach is that other non-proliferating cell types would remain in the culture while proliferating cardiomyocytes would be depleted. The culture would still need to undergo a cardiac purification step, and the excluded proliferating cardiomyocytes and cardiac progenitors may be of greater benefit for transplantation than fully differentiated, non-proliferating cells [19].

Reporter lines have similarly been derived for the isolation of other tissue-specific cells of biological and clinical interest, including motor neurons [74], germ cells [75], hepatocytes [76], and pancreatic progenitors [77]. However, the effects of random genomic integration on disrupting endogenous gene expression, as well as unregulated transgene expression, are potential limitations to this approach. Reporter lines must be rigorously evaluated to confirm authentic representation of gene expression without altering cell behavior [23].

Transgenesis through homologous recombination
To address the limitations of virally mediated introduction of reporter transgenes (for example, constitutive transgene expression and effects of random integration), many investigators have approached reporter development by using homologous recombination to ‘knockin’ a fluorescent protein into a specific genetic locus [78]. Initially, gene targeting of ESCs by homologous recombination was slowed by the difficulties of single-cell cloning [79-82], transfection efficiency using conventional methods [83,84], and karyotypic instability with dissociation of ESCs [85,86]. However, these obstacles have been overcome to some degree, and several successfully targeted ESC lines have resulted [78].

The hESC reporter lines currently in use represent a variety of targeting strategies. An H1.1-derived OCT4-eGFP line targeting exons 3 to 5 of the octamer-binding transcription factor 4 gene by positive selection alone has been used to sort pluripotent hESCs [83]. Similarly, an MIXL1-GFP line targeting exon 1 of the Mix1 homeobox-like 1 gene in HES3 cells by positive selection has been used to identify blood precursors [79]. Ruby and Zheng [84] used the FEZ family zinc finger 2 promoter to direct eYFP expression in HUES9 cells as a means of isolating neuronal precursors by using cre-mediated recombination with both positive and negative selection steps, and a similar approach using the oligodendrocyte lineage transcription factor 2 promoter driving eGFP expression in BG01 hESCs identified a subset of glial-restricted progenitors with oligodendrocyte fate [87]. Cre-mediated homologous recombination has also been used to generate two cardiac reporter lines, one using the second heart field-specific Islet-1 promoter directing dsRed expression in H9 hESCs [81] and another recently described pair of hESC lines in which the promoter of the early cardiac transcription factor NKX2-5 was used to drive eGFP expression in MEL1 (male) and HES3 (female) cells [88]. While these accomplishments demonstrate that the various unrelated ESC lines currently available are all amenable to homologous recombination, optimal targeting vector designs and selection strategies are still being explored.

Use of dual-FRET molecular beacons
Although homologous recombination technology avoids some of the concerns with transgenic reporter cell lines generated by random integration, the resulting cells have still undergone genomic modification. Seeking an alternative to genetically modified stem cell lines for tracking and isolating tissue-specific progenitors, King and colleagues [6] adapted dual-fluorescence resonance energy transfer (FRET) ‘molecular beacon’ technology for transient, real-time detection of gene expression during ESC differentiation. Molecular beacons are single-stranded oligonucleotide probes that have been employed to assay gene expression in vitro, as in real-time polymerase chain reaction assays, and in vivo by using microscopy [89]. These consist of short sequences capable of forming stem-loop structures bearing a fluorescent reporter group at one end and a fluorescent quencher at the opposite end [89]. In the absence of a target sequence, the oligonucleotide self-anneals, forming a stem that brings the reporter and quencher in close proximity, thereby quenching fluorescence (Figure 2). In the presence of a target sequence, the oligonucleotide anneals to the target, separating the reporter and quencher, thereby allowing fluorescence. These investigators showed that appropriately designed, dual-FRET molecular beacon pairs can identify the expression of specific mRNAs by microscopy and flow
cytometry and facilitate the collection of specific stem cell populations by cell sorting while leaving the stem cell genome intact [6].

Spectral flow cytometry: next-generation analysis

Conventional flow cytometers and cell sorters use a series of dichroic mirrors and filters matched to the fluorescence emission profile of reporter fluorochromes to identify and quantify specific biomarkers by using photomultiplier tubes (PMTs) to detect each parameter. Historically, this technology has relied on dichroic mirrors that reflect light of specific wavelengths and bandpass filters that allow incident light within a specific wavelength range to get to the detector. The detector, or PMT, detects photons and turns them into electrons or current. The fluorescent photon hits a photocathode and turns into an electron on a one-to-one basis; the electron then passes through a series of dynodes that amplify the electrons on the basis of the voltage generated across the dynode. One electron is multiplied and becomes two or more electrons, creating a cascade of electrons based on the single initial photon. At the end of the dynode chain, an anode collects the electron wave into an electronic signal; the more photons delivered to the PMT, the bigger the electronic signal.

A key criterion for distinguishing specific biomarkers over background is the optical signal-to-noise ratio of the detector [90]. For cytometric analysis of ESCs and iPSCs in altered differentiation states and treatment conditions, variable cellular autofluorescence and fluorescence crossover between multiple fluorochromes contribute to the background or ‘noise’ [91-93]. Increased noise decreases the resolving power for dim signals. A new cytometer developed by Sony Corporation (Tokyo, Japan) replaces filter-based optics with a spectral detection system based on a multi-anode spectral PMT. In the spectral cytometer, the light is directed on the basis of wavelength into discrete channels corresponding to different regions of the color spectrum by using a series of prisms. Over 30 channels are distributed across the 500- to 800-nm color spectrum, which allows almost all photons to be processed, whereas at least 20% of photons may be lost in the dichroic mirror/bandpass filter system.

The spectral cytometer is able to measure and subtract varying autofluorescence, permitting increased signal-to-noise ratios and improving the resolution of dim signals (Figure 3). Multiple fluorochromes are mathematically unmixed by using component analysis. Fluorochromes with overlapping emissions like fluorescein isothiocyanate and Alexa 532 can now be used simultaneously where...
their spectral overlap would prevent their use in a conventional filter-based cytometer. Unique spectral ‘fingerprints’ can be created to distinguish tissue precursors transfected with combinations of molecular beacons described above but without the need for genome integration of reporter constructs or surface antibody staining that might cause unintended pleiotropic cellular responses. This technology holds great promise for its ability to exploit functional biosensors [94-97], such as dual-FRET molecular beacons [6], to identify specific stem cell differentiation states of therapeutic value.

**Conclusions**

Research on pluripotent stem cells has progressed significantly since the first derivation of hESCs from discarded blastocysts in 1998 [98] and the discovery that somatic cells can be reprogrammed to a pluripotent stem-like state in 2006 [99]. The international scientific community has discovered the enormous potential of pluripotent stem cells as newly derived lines continue to be developed and differentiation methods into various types of cells are optimized. The ability to select and isolate stem cell-derived precursors and differentiated cells with tissue-specific properties holds the key to fully exploiting these cell lines and differentiation methods for scientific investigation and clinical use.

**Abbreviations**
bFGF, basic fibroblast growth factor; BMP4, bone morphogenetic protein 4; dbcAMP, dibutyl cyclic adenosine monophosphate; EGF, epidermal growth factor; eGFP, enhanced green fluorescent protein; ESC, embryonic stem cell; FGF, fibroblast growth factor; FRET, fluorescence resonance energy transfer.
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