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Differentiation of Embryonic Stem Cells to Clinically Relevant Populations: Lessons from Embryonic Development

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The potential to generate virtually any differentiated cell type from embryonic stem cells (ESCs) offers the possibility to establish new models of mammalian development and to create new sources of cells for regenerative medicine. To realize this potential, it is essential to be able to control ESC differentiation and to direct the development of these cells along specific pathways. Embryology has offered important insights into key pathways regulating ESC differentiation, resulting in advances in modeling gastrulation in culture and in the efficient induction of endoderm, mesoderm, and ectoderm and many of their downstream derivatives. This has led to the identification of new multipotential progenitors for the hematopoietic, neural, and cardiovascular lineages and to the development of protocols for the efficient generation of a broad spectrum of cell types including hematopoietic cells, cardiomyocytes, oligodendrocytes, dopamine neurons, and immature pancreatic β cells. The next challenge will be to demonstrate the functional utility of these cells, both in vitro and in preclinical models of human disease.

Introduction

Embryonic stem cells (ESCs) have the potential to give rise to any of the hundreds of cell types in the human body, raising exciting new prospects for biomedical research and for regenerative medicine. Many of the diseases that place the greatest burden on society are, at their root, diseases of cellular deficiency. Heart failure, diabetes, stroke, hematological disorders, neurodegenerative disorders, most cases of blindness and deafness, spinal cord injury, osteoarthritis, and kidney failure all result from the absence of one or more critical populations of cells that the body is unable to replace. Having the ability to generate clinically relevant numbers of defined cell populations places in our hands the basic elements for tissue repair and regeneration (see Essay by D. Scadden and G. Daley, page 544 of this issue). Additionally, the ability to derive patient-specific ESC equivalents (Park et al., 2008; Takahashi et al., 2007; Yu et al., 2007) provides powerful new tools to evade the immune system, study basic disease mechanisms, and establish screens for drug discovery (see Review by R. Jaenisch and R. Young, page 567 and Essay by L. Rubin, page 549 of this issue).

Although most scientists agree on the potential of ESCs, it has also become clear that pluripotency is a double-edged sword: the same plasticity that permits ESCs to generate hundreds of different cell types also makes them difficult to control. Three basic methods have been developed to promote differentiation of ESCs: (1) the formation of three-dimensional aggregates known as embryoid bodies (EBs), (2) the culture of ESCs as monolayers on extracellular matrix proteins, and (3) the culture of ESCs directly on supportive stromal layers. Although there is debate as to which approach is best, each method demonstrates that ESCs can differentiate into a broad spectrum of cell types in culture. Many of the early studies that documented the remarkable differentiation capacity of ESCs included fetal calf serum in the protocol. Given the poorly defined combination of factors in serum and the variability between different serum lots, these protocols were often difficult to reproduce, and most were not well optimized for the generation of any particular lineage. Several advances including the use of serum-free media with specific inducers to direct differentiation (Kubo et al., 2004; Ng et al., 2005a; Wiles and Johansson, 1999; Yasunaga et al., 2005) and the development of reporter ESCs to monitor and access early differentiation steps (Fehling et al., 2003; Gadue et al., 2006; Ng et al., 2005a; Tada et al., 2005; Ying et al., 2003) have overcome these obstacles. With these new tools, it became possible to approach ESC differentiation from a developmental biology perspective, taking advantage of the insights gained from studies in other model systems. This developmental biology approach has made it possible to recapitulate in ESC cultures the key events that regulate early lineage commitment in the embryo, resulting in the efficient and reproducible generation of highly enriched differentiated cell populations.

With access to a range of differentiated ESC-derived cell types, experiments are underway to begin testing the capacity of these cells to function following transplantation into different animal models. Here, we have chosen to focus on development of the hematopoietic, cardiac, neural, and pancreatic lineages,

as access to such cell populations may provide new therapies for some of society's most devastating diseases.

Gastrulation in the Mouse

One of the most important events during embryogenesis is the generation of the three primary germ layers: ectoderm, mesoderm, and endoderm during the process of gastrulation (Figure 1A). In the mouse, the beginning of gastrulation is marked by the formation of a transient structure known as the primitive streak (PS) in the region of the epiblast that will ultimately form the posterior end of the embryo (Tam and Behringer, 1997). During this process, uncommitted epiblast cells mobilize, egress through the PS, and exit either as mesoderm or definitive endoderm (Figure 1B). Molecular analyses and lineage mapping studies have defined posterior, mid, and anterior regions of the PS that differ in gene expression patterns and developmental potential. Several genes, including *Brachyury* (*T*) (Kispert and Herrmann, 1994) and *Mixl1* (Hart et al., 2002), are expressed throughout the PS, whereas others are found preferentially in anterior regions (*Foxa2* and *Goosecoid*) (Kinder et al., 2001; Sasaki and Hogan, 1993) or posterior regions (*HoxB1*, *Evx1*) (Dush and Martin, 1992; Forlani et al., 2003). Mapping studies have shown that the specification of distinct subpopulations of mesoderm and endoderm is not random but rather appears to be controlled both temporally and spatially. The first mobilized epiblast cells traverse the posterior PS and give rise to the extraembryonic mesoderm that forms the allantois and amnion as well as the hematopoietic, endothelial, and vascular smooth muscle cells of the yolk sac (Kinder et al., 1999). As gastrulation proceeds, cells migrate through more anterior parts of the PS and generate cranial and cardiac mesoderm, and subsequently paraxial and axial mesoderm. Definitive endoderm develops from epiblast cells that transit the most anterior region of the PS. In contrast to mesoderm and definitive endoderm, ectoderm derives from the anterior region of the epiblast that does not enter the PS.

The temporal and spatial segregation of cell fates observed during gastrulation strongly suggests that the different regions of the PS, or cell populations in the immediate vicinity of the PS, constitute different signaling environments that are responsible for the induction of specific lineages. Although the precise regulation of PS formation and germ layer induction is not fully understood, expression analyses and gene-targeting studies have shown that members of the TGFβ family including BMP4 (Hogan, 1996) and Nodal (Conlon et al., 1994; Schier, 2003) as well as members of the Wnt family (Yamaguchi, 2001) are essential for these developmental steps. Moreover, different levels of expression of agonists of these pathways, together with regionalized expression of inhibitors, combine to form signaling domains that regulate germ layer induction and specification (reviewed in Gadue et al., 2005). Thus, germ layer development is a dynamic process that is controlled, in part, by the coordinated activation and regional inhibition of the Wnt, Nodal, and BMP-signaling pathways.

Translating Embryology to ESCs

Manipulation of the BMP, Wnt, and Nodal pathways in ESC cultures reveals that they are involved in the regulation of germ layer development in vitro. In many ESC differentiation studies, expression of *Brachyury* is used to monitor the formation of a PS-like population and the onset of mesoderm induction. Although the population generated in ESC differentiation cultures will not share all properties of the PS that develop in the embryo, it will be referred to as the PS throughout this review. When added to ESC differentiation cultures in the absence of serum, BMP4 efficiently induces the formation of a *Brachyury*-positive PS population as well as the subsequent development of Flk-1$^+$ mesoderm (Wiles and Johansson, 1999; Park et al., 2004; Ng et al., 2005a; Nostro et al., 2008). Blocking Wnt signaling at early stages of differentiation in serum-stimulated cultures inhibited the formation of cells expressing *Brachyury* and mesoderm development, indicating that this pathway is required for the generation of the PS (Lindsley et al., 2006; Naito et al., 2006). Conversely, addition of Wnt to serum-containing cultures at the onset of differentiation accelerated the formation of a PS population and enhanced cardiac mesoderm development (Ueno et al., 2007). Activation of the Nodal pathway by the

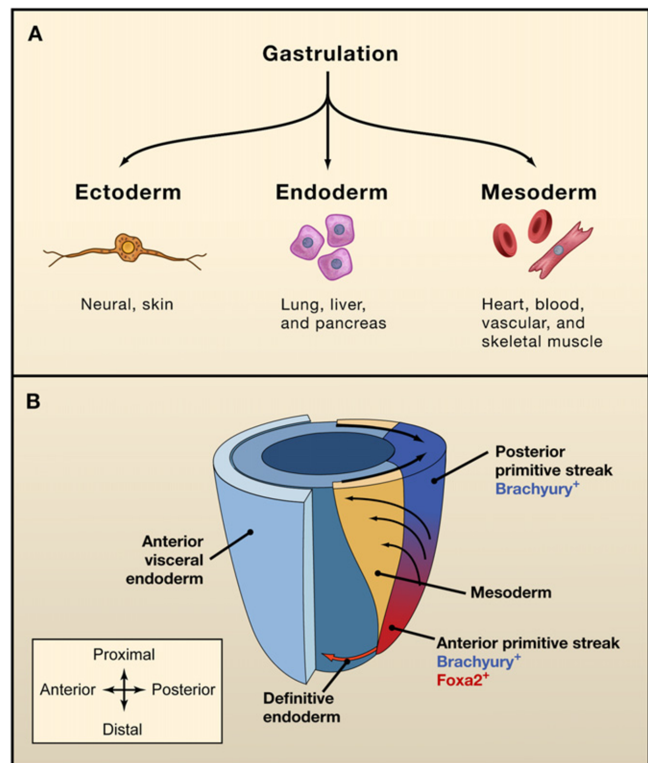

Figure 1. Mouse Gastrulation and Germ Layer Formation

(A) The derivatives of the three primary germ layers—ectoderm, mesoderm and endoderm—generated during gastrulation.

(B) Gastrulation in the mouse embryo. Shown are the posterior region of the primitive streak that expresses the marker Brachyury (blue), and the anterior region of the primitive streak that coexpresses both Brachyury and Foxa2 (red). At the top of the embryo, epiblast cells are shown entering the primitive streak (thick black arrows). The yellow/orange region depicts newly formed mesoderm, and migration of these cells from the primitive streak is indicated by thin black arrows. Also depicted is the movement of the earliest definitive endoderm cells (red arrow at bottom).

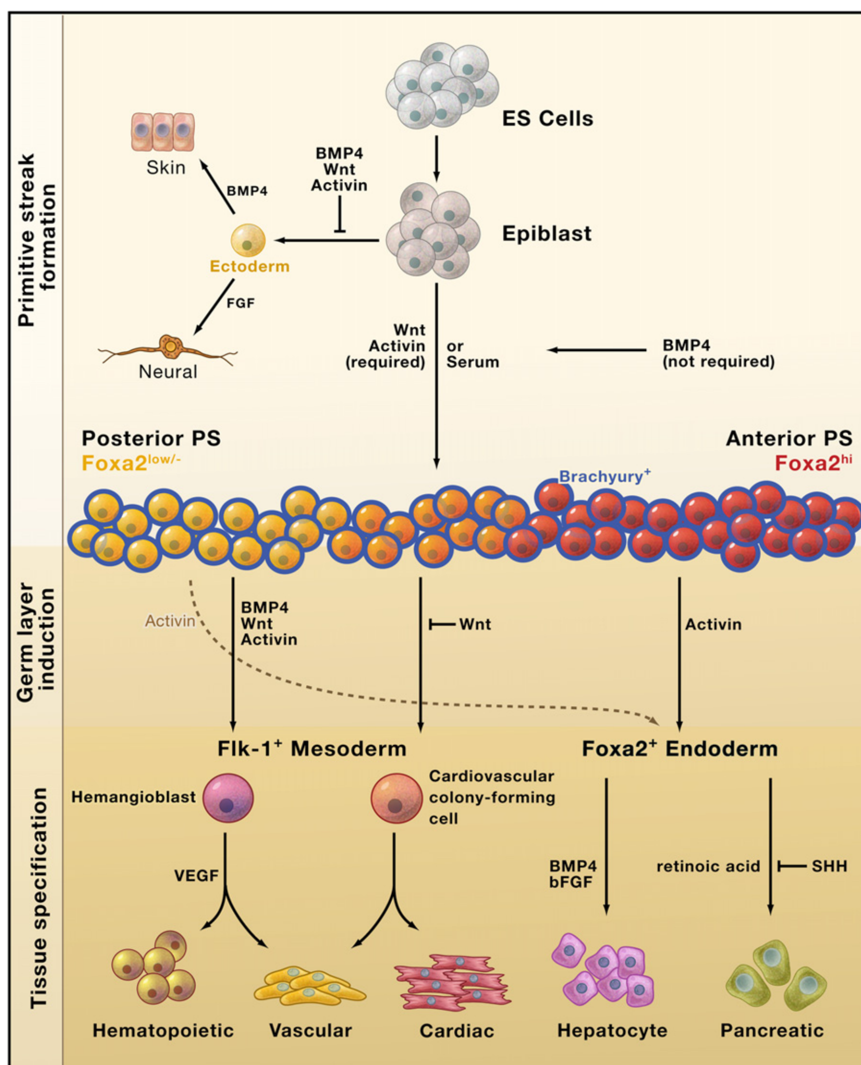

Figure 2. ESC Differentiation in Culture

This model depicts the regulation of primitive streak formation, primary germ layer induction, and tissue specification from differentiated mouse ESCs. The first step in the differentiation pathway is the development of a population resembling the epiblast of the mouse embryo. When induced with Wnt, activin, BMP4, or serum, these cells will generate a primitive streak (PS)-like population (indicated by the row of cells outlined in blue). If these pathways are not activated, the epiblast population will differentiate into the ectoderm lineage. Ectoderm differentiation is blocked by BMP, Wnt, and activin signaling. Following PS induction, the posterior PS cells (yellow) are specified to Flk-1^{+} mesoderm, whereas the anterior streak cells (dark orange) are fated to generate Foxa2^{+} definitive endoderm. These fates are not firmly established at this stage, as activin can induce endoderm from the posterior PS population (indicated by the brown stippled arrow below the PS). The pathways that specify Flk-1 mesoderm to the hematopoietic lineage and Foxa2^{+} definitive endoderm to either the hepatocyte or pancreatic lineages are shown.

addition of activin A (activin) induced a PS population and the subsequent formation of endoderm or mesoderm, depending on the strength of the signal (Kubo et al., 2004). Collectively, these observations indicate that these signaling pathways play some role in the early stages of ESC differentiation. However, the precise stage at which they function and their interactions with each other were not established in these studies.

While PS formation and germ layer induction are often considered part of gastrulation, these stages likely represent distinct developmental steps that are regulated by different signaling pathways. To investigate regulation of these early stages, Gadue et al. (2006) engineered a reporter mouse ESC cell line to model PS formation in vitro. This cell line contains the green fluorescent protein (GFP) cDNA targeted to the PS gene *Brachyury (T)* and the human CD4 cDNA targeted to the anterior PS gene *Foxa2*. Stage-specific analyses with this reporter mouse ESC line revealed that both Wnt and Nodal signaling are simultaneously required for the development of the GFP-T^{+}CD4-Foxa2^{+} PS cell population (Figure 2). When added individually, each factor displayed distinct inducing properties: Wnt generated a GFP-T^{+}CD4-Foxa2$^{lo\text{-}med}$ posterior PS population, whereas activin led to the development of a GFP-T^{+}CD4-Foxa2$^{med\text{-}hi}$ anterior PS population. In contrast to Wnt and activin/Nodal, BMP4 signaling was dispensable for generation of the PS (Nostro et al., 2008). Although not required at this stage, BMP4 alone did induce the development of a GFP-T^{+}CD4-Foxa2^{lo} posterior PS population, consistent with other reports that this factor does promote the development of the PS and mesoderm (Ng et al., 2005a; Park et al., 2004). This effect was not direct but rather was mediated through the induction of Wnt and Nodal. Interestingly, when added together with Wnt and activin, BMP displayed a dominant posteriorizing effect, promoting the development of a GFP-T^{+}CD4-Foxa2^{lo} population while inhibiting the formation of the CD4-Foxa2^{hi} cells.

These studies provide important insights into formation of the PS, the earliest stage of ESC differentiation. First, they demonstrate that signaling pathways that regulate PS development in the early embryo, namely Wnt and activin/Nodal, are also required for this differentiation step in vitro. Second, they highlight the importance of monitoring more than one signaling pathway when studying the regulation of ESC differentiation, allowing one to distinguish direct from indirect effects. Third, they raise a cautionary note that the sequence of addition of factors can dramatically alter the differentiation pathway.

Endoderm Induction

A number of endoderm-derived organs such as the liver and pancreas are potential targets for cell-based therapy, and so

there is great interest in understanding the pathways that regulate the induction and specification of this germ layer. High levels of activin/Nodal signaling will efficiently induce definitive endoderm in mouse ESC cultures (Kubo et al., 2004; Yasunaga et al., 2005). When analyzed at the PS stage, one step prior to induction of definitive endoderm, activin-induced populations identified either by the coexpression of Brachyury and Foxa2 (GFP-T^{+}CD4-$Foxa2^{hi}$) or expression of the anterior marker Goosecoid (tagged with GFP, Gsc-GFP) were found to contain both mesoderm and endoderm (Gouon-Evans et al., 2006; Tada et al., 2005). Clonal analysis revealed that individual cells within the Gsc-GFP population had the potential to generate both endoderm and mesoderm derivatives, suggesting that they may represent mesendoderm progenitors. Thus, the first step in the generation of definitive endoderm may be the formation of mesendoderm. Future studies should determine if all endoderm and anterior mesoderm is derived from such progenitors.

Progression of the anterior PS population (GFP-T^{+}CD4-$Foxa2^{hi}$) to definitive endoderm (GFP-T^{neg}CD4-$Foxa2^{hi}$) depends on sustained activin signaling (Gadue et al., 2006), consistent with increased Nodal signaling required for definitive endoderm formation in the early embryo (Schier, 2003). Interestingly, when exposed to high levels of activin, the GFP-T^{+}CD4-$Foxa2^{lo}$ posterior PS population was also able to generate endoderm, indicating that germ-layer fates are not yet fixed at the PS stage in mouse ESC differentiation cultures. Once induced, endoderm forms an epithelial sheet that undergoes specification to distinct regions known as foregut, midgut, and hindgut (Wells and Melton, 1999). This specification is controlled in part by factors secreted by surrounding mesoderm-derived tissues. By translating findings from the embryo to ESC cultures, FGF and BMP4 together were shown to induce a hepatic fate in activin-induced endoderm (Gouon-Evans et al., 2006), whereas retinoic acid combined with inhibition of sonic hedgehog (SHH) resulted in specification to a pancreatic fate (D'Amour et al., 2006).

Mesoderm Induction

The hematopoietic, vascular, cardiac, and skeletal muscle lineages develop from subpopulations of mesoderm induced in a defined temporal pattern. Understanding the mechanisms that regulate mesoderm induction is a prerequisite for generating each of these cell types. The early stages of mesoderm induction from the PS can be monitored by the upregulation of Flk-1 and PDGFR, receptors that are broadly expressed on subpopulations of this lineage (Ema et al., 2006; Kataoka et al., 1997). Although most studies have not distinguished PS formation from mesoderm induction, several have provided insights into the signaling pathways that regulate this step. Park et al. (2004) showed that BMP signaling is required to induce Flk-1^{+} hematopoietic mesoderm from populations that express *Brachyury*, indicating that this pathway functions at the level of mesoderm induction. In a more recent study, Nostro et al. (2008) demonstrated that the generation of hematopoietic mesoderm from a Brachyury-GFP tagged PS population is dependent on a combination of Wnt, activin/Nodal, and BMP signaling (Figure 2), and that upregulation of Flk-1 correlated with commitment to a mesoderm fate (this population could no longer undergo respecification to endoderm following activin treatment).

Although the emergence of cardiac mesoderm from the PS has not been investigated in as much detail as hematopoietic mesoderm, findings from several studies suggest that transient inhibition of Wnt/β-catenin signaling at this stage is essential for the generation of this population (Naito et al., 2006; Ueno et al., 2007). Wnt/β-catenin is thus required for mesoderm induction, whereas its inhibition is subsequently required for specification of precardiac mesoderm. Recent studies using various concentrations of BMP4 or a combination of BMP4 and activin indicate that subpopulations of mesoderm can be generated in mouse ESC cultures by manipulating different signaling pathways at appropriate developmental stages (Era et al., 2007).

Ectoderm Induction

The neural lineages and skin are derived from ectoderm, which is induced from epiblast cells in the anterior region of the embryo that do not traverse the PS. The induction of ectoderm in ESC cultures is often referred to as the "default" pathway, as neuroectoderm readily develops in cultures that contain no serum or other PS inducers. Neuroectoderm induction is inhibited by BMP, Wnt, and activin/Nodal signaling, consistent with the observation that these pathways are not active in the region of ectoderm induction in the early embryo (Aubert et al., 2002; Kubo et al., 2004; Ying et al., 2003). Ying et al. (2003) studied development of the neuroectoderm lineage from mouse ESCs using a reporter cell line with GFP targeted to the neuroectoderm-specific gene *Sox1*. They clearly showed that induction of neuroectoderm is dependent on FGF signals endogenously produced by the differentiating ESCs. Thus, "default" differentiation to neuroectoderm still proceeds through signaling pathways involved in embryogenesis, similar to the formation of mesoderm and endoderm. Neuroectoderm generated from ESCs can be specified to neuronal subtypes, using factor combinations known to regulate these steps in the early embryo. In addition to the neural lineages, ESC-derived ectoderm can also generate epidermal lineage cells. As observed in vivo, the BMP-signaling pathway does play a role in ectoderm specification in ESC cultures as BMP signaling blocked neural differentiation and promoted epidermal development (Kawasaki et al., 2000).

PS formation and Germ-Layer Induction in Human ESCs

Mouse and human ESCs may represent different stages of development and clearly display different requirements for growth and maintenance in the undifferentiated state (Reubinoff et al., 2000; Thomson et al., 1998). However, the signaling pathways that regulate human ESC differentiation are similar to those that regulate these processes in other organisms and in mouse ESC cultures. As with mouse ESCs, the most successful human ESC differentiation strategies are those that recapitulate normal development. For example, activin signaling leads to the efficient induction of definitive endoderm in human ESC cultures (D'Amour et al., 2005). Molecular analysis showed that the induced populations progressed through a PS stage to definitive endoderm in a time frame similar to that observed in the mouse cultures. Mesoderm induction from human ESCs also shows similarities to mouse. Studies with wild-type human ESCs and a human ESC reporter line expressing GFP from the *MIXL1* gene (GFP-MIXL1) demonstrated that induction with BMP4 leads to a rapid increase in *BRACHYURY* and *MIXL1* expression and the

subsequent formation of KDR$^+$ (Flk-1$^+$) and PDGFR$^+$ mesoderm (Davis et al., 2008; Kennedy et al., 2007; Ng et al., 2005b; Pick et al., 2007). PS induction by BMP appears to require active FGF and TGF-β/Nodal/activin signaling, as inhibition of these pathways blocked the BMP4 response (Zhang et al., 2008). Blocking Wnt signaling early in serum-induced cultures reduced hematopoietic development, suggesting that this pathway acts early in either PS formation or mesoderm induction (Woll et al., 2007). Stimulating human ESCs with activin in addition to BMP4 led to the induction of cardiac mesoderm, suggesting that different subpopulations of mesoderm can be induced by manipulating different signaling pathways (Laflamme et al., 2007). These findings document the success of translating differentiation strategies from mouse ESC to the human ESC system.

ESCs and the Hematopoietic Lineage

As many of the early ESC differentiation studies focused on blood cell development, hematopoiesis is one of the best-studied programs in ESC cultures (reviewed in Keller, 2005; Olsen et al., 2006). The goal of many investigators working in this area has been to induce the formation of transplantable hematopoietic stem cells (HSCs). With the relative ease of generating blood cell lineages from ESCs, it was assumed that differentiation to HSCs would be straightforward. However, despite extensive efforts, the development of HSCs from ESCs that have not been genetically modified remains a challenge, which may reflect the complexities of embryonic hematopoietic development where different hematopoietic programs are generated at different times from different embryonic sites.

Hematopoiesis in the early mouse embryo is initiated independently at two distinct sites: the yolk sac and the para-aortic splanchnopleura (P-Sp), an intraembryonic region in the caudal portion of the embryo that later contains the developing aorta, gonads, and mesonephros (AGM) (reviewed in Cumano and Godin, 2007; Dzierzak, 2005; see Review by S.H. Orkin and L. I. Zon, page 631 of this issue). Of significance for ESC differentiation studies is the finding that these sites display different hematopoietic potential. The yolk sac generates primitive erythrocytes as well as a subset of other hematopoietic populations including macrophages and progenitors of the definitive erythroid, megakaryocyte, and mast cell lineages. When analyzed prior to the onset of circulation, the yolk sac displays little, if any, lymphoid or HSC potential. In contrast, the P-Sp-derived hematopoietic population generates HSCs as well as multipotential progenitors that give rise to myeloid, lymphoid, and definitive erythroid lineages in vitro. Hematopoietic development in the human embryo follows a similar pattern (reviewed in Tavian and Peault, 2005). Given these differences, it will be necessary to recapitulate these developmental stages in vitro and to identify the equivalent of P-Sp-derived hematopoiesis in order to isolate HSCs from ESCs.

Recapitulating Hematopoiesis in Mouse ESC Cultures

Mouse ESCs undergo rapid and synchronous differentiation to the hematopoietic lineages when cultured with serum or under serum-free conditions supplemented with inducers (reviewed in Keller, 2005). Gene expression and progenitor cell analysis revealed that the differentiation program in these cultures closely parallels that in the early embryo, progressing through a PS stage, to Flk-1$^+$ mesoderm, and subsequently to a yolk sac-like hematopoietic program. Detailed analysis of these early stages led to the identification of the hemangioblast, a progenitor that displays hematopoietic and vascular potential and one that defines the onset of hematopoiesis (Choi et al., 1998). A comparable progenitor was identified in the posterior PS region of the early mouse embryo and may represent the yolk-sac hemangioblast (Huber et al., 2004). After the hemangioblast appears, primitive erythroid progenitors develop in ESC cultures, establishing the primitive erythropoiesis phase of hematopoiesis. In addition to primitive erythrocytes, other progenitors including those of the macrophage, definitive erythroid, megakaryocyte, and mast cell lineages develop in the differentiation cultures with a kinetic pattern similar to that observed in the yolk sac. HSCs and progenitors of the lymphoid lineage are not generated during this early stage of hematopoiesis. These patterns of lineage development suggest that the first hematopoietic population to develop from ESCs represents the equivalent of yolk-sac hematopoiesis. The striking similarities in yolk-sac hematopoietic development observed between the ESC cultures and the early embryo suggests that lineage commitment in this in vitro system recapitulates that found in vivo.

The yolk-sac stage of hematopoiesis is well characterized in mouse ESC cultures, but little is known about specification and development of P-Sp hematopoiesis. Lymphoid potential is one characteristic defining the P-Sp and mouse ESCs do generate these lineages under appropriate conditions. When cocultured with OP9 stromal cells, mouse ESCs will give rise to cells of the B lymphoid lineage (Cho et al., 1999; Nakano et al., 1994). If cocultured with OP9 stromal cells engineered to express the Notch ligand Delta-like 1, mouse ESCs will differentiate along the T cell rather than the B cell lineage (Schmitt et al., 2004). These observations suggest that a population equivalent to the P-Sp region is generated in these cultures. However, given that the ESCs were differentiated in complex cultures with stromal cells in serum-based media, isolation of the lymphoid progenitors and identification of the signaling pathways involved remains a challenge.

The differentiation of mouse ESCs to HSCs that are capable of long-term engraftment in recipient animals remains one of the greatest challenges in the field of ESC biology. Although the development of transplantable cells from ESCs in serum-stimulated cultures was reported (Burt et al., 2004), the routine isolation of HSCs using this approach is not widespread, suggesting that success may depend on a specific batch of serum. As factors regulating the specification of HSCs have yet to be defined, protocols for the efficient generation of these cells from ESCs do not exist. To promote the development, survival, and expansion of mouse ESC-derived HSCs, Daley and colleagues (Kyba et al., 2002; Wang et al., 2005b) forced the expression of *HoxB4* and the caudal-related homeobox-containing factor *Cdx4* in mouse ESC-derived hematopoietic populations prior to transplantation. *Cdx4* promotes hematopoiesis when expressed in mouse ESC cultures (Davidson et al., 2003), and *HoxB4* induces extensive self-renewal and expansion of bone marrow-derived HSCs when expressed in this population (Sauvageau et al., 1995). The ESC-derived cells expressing these two genes yielded multilineage repopulation of recipient animals, demonstrating that

these cells can acquire hematopoietic repopulating potential if manipulated to express appropriate levels of specific transcription factors. The next step on the road to generating clinically useful HSCs will be to induce development of this population without genetic modification.

Hematopoietic Development of Human ESCs

Hematopoietic development of human ESCs has been demonstrated by multiple groups using different induction schemes (Kaufman et al., 2001; Vodyanik et al., 2005; Chadwick et al., 2003; Ng et al., 2005b; Zambidis et al., 2005; Kennedy et al., 2007; Pick et al., 2007). Kinetic analysis revealed that the differentiating populations progressed through a PS stage defined by either *BRACHYURY* or *MIXL1* expression, then to KDR$^+$ (Flk-1$^+$) or PDGFR$^+$ mesoderm and subsequently to a yolk-sac hematopoietic program (Davis et al., 2008; Kennedy et al., 2007; Ng et al., 2005b; Zambidis et al., 2005). Progression through these stages is fast, as hematopoietic progenitors were detected within the first week of differentiation (Davis et al., 2008; Kennedy et al., 2007; Vodyanik et al., 2006). As observed in the mouse system, the predominant population generated during the first 7–10 days of human ESC differentiation is primitive erythroid progenitors, indicating that the equivalent of yolk-sac hematopoiesis develops first in these cultures (Kennedy et al., 2007; Zambidis et al., 2005). As observed with mouse ESCs and the mouse embryo, the onset of hematopoiesis in human ESC cultures is marked by development of the hemangioblast between days 2 and 4 of differentiation, prior to establishment of the primitive erythroid lineage (Davis et al., 2008; Kennedy et al., 2007; Lu et al., 2007).

Although the early stages of development in human ESC cultures appear to represent the yolk-sac phase of hematopoiesis, more mature hematopoietic populations develop after extended periods of time. Analysis of cell surface phenotypes revealed progression from populations that expressed KDR, CD31, and CD34 to those that also expressed CD45, a marker found on fetal and adult hematopoietic cells (Kennedy et al., 2007; Vodyanik et al., 2005; Woll et al., 2007). Hemoglobin analysis at different stages demonstrated maturation from an embryonic to a fetal-like pattern of erythropoiesis (Qiu et al., 2008). These changes demonstrate that the hematopoietic population does mature with time, but it is unclear if they reflect maturation of a yolk sac-like population or the onset of P-Sp-derived hematopoiesis. Given this uncertainty, the generation of HSCs and progenitors with lymphoid potential remains the defining characteristic of this hematopoietic program. T lymphoid progenitors have been generated from human ESCs following differentiation directly on OP9 stromal cells in serum-containing media (Galic et al., 2006). To promote maturation of the lineage, CD34$^+$ cells isolated from the differentiation cultures were transplanted into human thymic grafts in SCID-hu mice. Low numbers of human ESC-derived T cells were detected within a month of transplantation, suggesting that the differentiation scheme led to the development of a P-Sp hematopoietic program. Although this approach documents the lymphoid potential of human ESCs in culture, the complexities of the culture system preclude detailed analysis of the population that gave rise to these progenitors or of the signaling pathways that regulate its development.

Several groups have described the development of human ESC-derived populations with limited in vivo hematopoietic repopulating potential. Wang et al. (2005a) demonstrated that human ESC-derived cells mature and produce hematopoietic progeny when transplanted directly into the femur of a NOD/SCID recipient animal. Although the cells did show some differentiation potential, they did not display normal HSC properties as they failed to migrate from the femur to other sites in the recipient animal. Tian et al. (2006) transplanted human ESC-derived hematopoietic cells into NOD/SCID recipients by intravenous or intrafemoral injection and found donor cells in both sets of recipients more than 3 months later. However, engraftment in all recipients was exceptionally low and appeared to be restricted to the myeloid lineage, raising the possibility that the transplanted population may be reflective of the yolk sac rather than P-Sp hematopoiesis. A fetal sheep model has also been used to evaluate the repopulating potential of human ESC-derived hematopoietic progenitors (Narayan et al., 2006). Low levels (<1.0%) of human hematopoietic cells were detected in the bone marrow and peripheral blood of recipient animals 5 and 17 months following transplantation of CD34$^+$ human ESC-derived cells. As with the NOD/SCID mouse model, there was limited engraftment in the sheep, and this was predominantly myeloid, again suggesting that the population used for transplantation may be reflective of the yolk-sac program.

These transplantation studies show that the identification of human ESC-derived HSCs that provide robust sustained multilineage engraftment is likely to be as challenging as the identification of this population in mouse ESC cultures. Elucidating the pathways that regulate the development of P-Sp hematopoiesis in ESC differentiation cultures will be a prerequisite for establishing differentiation protocols for generating HSCs from both mouse and human ESCs.

Differentiation of ESCs into Cardiomyocytes

The heart originates from lateral plate mesoderm and develops in at least two distinct waves of myogenesis from regions called the primary and secondary heart fields (see Essay by S.M. Wu et al., page 537 of this issue). Lineage-tracing studies indicate that both heart fields are marked by expression of *Flk-1* and the transcription factor *Nkx2.5*, whereas the transcription factor *Isl1* selectively marks the secondary heart field, giving rise to much of the right ventricle and outflow tracts (Ema et al., 2006; Moretti et al., 2006; Wu et al., 2006). These markers have proven useful in the identification of cardiac progenitors from ESCs. Embryoid body-based differentiation of ESCs stimulated with serum generates cardiomyocytes, which are readily detected by their spontaneous beating activity (Doetschman et al., 1985). The efficiency of this process is low, typically 1%–3% from mouse ESCs and <1% from human ESCs. An early approach for directing human ESCs along a cardiac differentiation pathway involved using medium conditioned with the endodermal cell line, End-2 (which produces activin A and BMPs, among other factors). This technique was recently improved using a small molecule inhibitor of p38 MAP kinase, which almost doubled the yield of cardiomyocytes from human ESCs (from 12% to 25%) by enhancing induction of mesoderm (Graichen et al., 2007).

A clearer picture is emerging of the signals that control cardiomyocyte differentiation (Zeineddine et al., 2005), and progenitors for cardiovascular cells are being defined. Signals mediated through Wnt/β-catenin and TGF-β family members including activin and BMPs promote differentiation of mouse ESCs into mesoderm (Gadue et al., 2006; Lindsley et al., 2006; Naito et al., 2006; Ueno et al., 2007). Once mesoderm is induced, however, Wnt/β-catenin signaling inhibits cardiac differentiation and may redirect the cells to alternate mesodermal fates (Naito et al., 2006; Ueno et al., 2007). Wnt signaling thus has a biphasic role in cardiac differentiation in mouse ESCs, being procardiac prior to PS formation and antagonizing cardiac differentiation thereafter. Part of the procardiac pathway initiated by Wnt/β-catenin signaling depends on upregulation of the endodermal transcription factor Sox17, which appears to regulate downstream signals that specify precardiac mesoderm (Liu et al., 2007b) possibly through combinatorial interactions with residual Oct4 (Zeineddine et al., 2006). Two groups have recently shown that human ESCs can be induced to form cardiomyocytes efficiently (Laflamme et al., 2007; Yao et al., 2006). Both used defined media and induced differentiation with activin and BMP4 in serum-free cultures. Laflamme et al. (2007) reported that their populations contained >30% cardiomyocytes and could be enriched to 80%–90% cardiomyocytes using density-gradient centrifugation (Figures 3 and 4).

Three recent studies used a developmental approach to identify multipotent cardiovascular progenitor cells in mouse ESC differentiation cultures. Wu et al. (2006) identified progenitors based on activity of the promoter for *nkx2.5*, a homeobox gene expressed in the earliest cardiomyocytes. These progenitors could be isolated both from developing transgenic mouse embryos and differentiating mouse ESC cultures, and they exhibited the capacity for both cardiac and smooth muscle differentiation (bipotential). Using a similar strategy, Moretti et al. (2006) used the promoter for the secondary heart field marker, *Isl-1*, to identify progenitors from mouse embryos and differentiating mouse ESCs. They showed that these progenitors could be expanded on feeder layers and that 12% of the resulting colonies gave rise to cardiomyocytes, endothelial cells, and smooth muscle cells (that is, they were tripotential). Finally, Kattman et al. (2006) used the VEGF receptor Flk-1, known to mark progenitors for multiple mesodermal lineages, to isolate hematopoietic and cardiovascular progenitors from mouse ESCs. By analyzing embryoid bodies derived from mouse ESCs over time, they found that the earliest Flk-1^+ population to emerge contained "hemangioblasts," progenitors for blood cells and endothelium. A later Flk-1^+ population contained cardiovascular progenitors (cardiovascular colony-forming cells, Figure 2) that were able to generate cardiac, endothelial, and vascular smooth muscle cells (tripotential). Thus, commitment to the blood lineage occurs in mesoderm cells prior to cardiovascular commitment. Moreover, three of the major cell types in the heart can be derived from a common progenitor. These progenitors provide a new population for transplantation with the capability of contributing both to remuscularization and revascularization of the heart. It remains to be determined whether or not a comparable progenitor exists in human ESC cultures.

In vitro study of human ESC-derived cardiomyocytes has provided some of the first insights into human cardiac development.

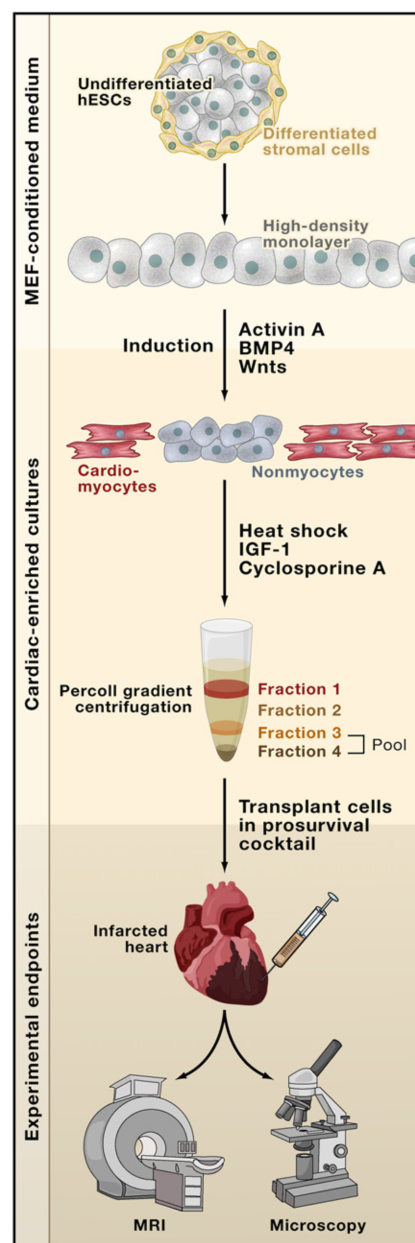

Figure 3. Making Human Cardiomyocytes

The scheme shows the directed differentiation of human ESCs to cardiomyocytes and their application for cardiac repair in a rat model of cardiac infarct (Laflamme et al., 2007). Undifferentiated human ESC colonies are replated as high-density monolayers, expanded, and then induced to differentiate by sequential treatment with activin A (day 0) and BMP4 (day 1). Differentiation along the cardiac lineage can be further enhanced by activating the Wnt/β-catenin pathway, followed by its inhibition. Cultures typically exhibit vigorous beating activity 10–14 days postinduction. These populations are then subjected to heat shock and treated with IGF-1 24 hr prior to transplantation to enhance viability, and then enriched for cardiomyocytes using Percoll density-gradient centrifugation. They are then suspended in a "prosurvival cocktail" to block cell-death pathways, and are delivered to the infarcted heart by direct injection. Experimental endpoints are assessed by microscopy and magnetic resonance imaging.

Electrophysiological studies indicate that, even during early differentiation, cells with atrial, ventricular, and pacemaker/conduction system phenotypes can be readily distinguished (He et al., 2003; Mummery et al., 2003; Satin et al., 2004). Interestingly, repeated impalement of a single colony with an electrode gave reproducible recordings, consistent with this colony containing predominantly one subtype of cardiomyocyte. This suggests that cardiac lineage diversity is established early in differentiation, although the timeline has not been precisely defined. Ionic currents in human ESC-derived cardiomyocytes show some maturation in vitro (Sartiani et al., 2007; Satin et al., 2004), but these are slower to mature than in murine cells, a phenomenon that may reflect the 13-fold difference in gestation time between the species. Catecholamines enhance cardiomyocyte beating rate and contractility (He et al., 2003; Xu et al., 2002), indicating the presence of intact adrenergic signal transduction pathways and a functional sarcoplasmic reticulum that regulates contraction through calcium-induced calcium ion release (Liu et al., 2007a). The early human cardiomyocytes show readily quantifiable responses to many other pharmacological agents, raising hopes that these cells may be useful in drug screens to predict efficacy and toxicity (see Essay by L. Rubin).

One of the most interesting differences between cardiomyocytes derived from human and mouse ESCs is their proliferative status. Mouse ESC-derived cardiomyocytes, like those derived from mouse or rat fetuses, exhibit little proliferation in vitro (Klug et al., 1995; McDevitt et al., 2005). This has impeded studying cardiomyocyte cell cycle control as such studies require the routine isolation of primary cells. In contrast, human ESC-derived cardiomyocytes proliferate robustly in culture (McDevitt et al., 2005; Snir et al., 2003; Xu et al., 2002). The early human cardiomyocytes proliferate in the absence of serum or other exogenous growth factors, suggesting autocrine/paracrine pathways are involved. Their proliferation can be reduced markedly by inhibition of either PI-3 kinase or Akt kinase and may be driven in part by signaling through the IGF-1 receptor (McDevitt et al., 2005); inhibition of ERK signaling has no impact on proliferation. Studies are underway to identify the growth factors and intracellular signaling pathways that control human ESC-derived cardiomyocyte proliferation and to understand the mechanisms that mediate cell-cycle withdrawal and further differentiation.

Transplantation of ESC-Derived Cardiomyocytes

Most of the initial cardiac transplantation experiments have been performed in the uninjured heart. Klug et al. (1996) were the first to show that cardiomyocytes derived from mouse ESCs could be purified using genetic selection and could form stable grafts in the mouse. When cardiomyocytes from human ESCs were similarly transplanted into the uninjured hearts of immunocompromised mice, rats, and pigs, they formed grafts of human myocardium (Dai et al., 2007; Kehat et al., 2004; Laflamme et al., 2005). Interestingly, when relatively impure cell preparations from embryoid bodies derived from human ESCs were used, contaminating epithelial cells were progressively lost from the heart, but human endothelial cells expanded and formed microvessels that interconnected with the host circulation, perhaps helping to support the human cardiomyocytes (Laflamme et al., 2005). The human ESC-derived cardiomyocytes continued to proliferate after transplantation, such that myocardial graft size increased 7-fold in a 4 week period. This correlated with their relatively high proliferative capacity in vitro and suggests that it might be possible to implant a subtherapeutic dose of cardiomyocytes that could expand over time to more fully repopulate a region of injury.

A major goal has been to generate ESC-derived cardiomyocytes to repair heart damage, particularly that from myocardial infarction. Transplantation of mouse ESC-derived cardiomyocytes improves contractile function of the infarcted mouse heart (reviewed in Cai et al., 2007; Ebert et al., 2007; Kolossov et al., 2006; Singla et al., 2007). Remuscularization also appears to normalize electrical conduction through the infarct, reducing susceptibility to arrhythmias (Roell et al., 2007). There also has been progress in using human ESCs in myocardial infarct repair thanks to two advances. First, protocols for the directed differentiation of human ESCs to cardiomyocytes (such as the activin/BMP and p38-inhibitor systems) enabled production of large numbers of cells for transplantation. Second, a "prosurvival cocktail" was developed that counteracted the potentially fatal stimuli that cells encounter upon transplantation into the harsh infarct environment. By combining these two advances, researchers have generated significant amounts of human myocardium in the infarcted rat heart, reaching up to 11% of the infarct's volume (Figure 4) (Laflamme et al., 2007). The human myocardium prevented the progression to heart failure seen in untreated rats and in control animals receiving noncardiac derivatives of human ESCs. Magnetic resonance imaging demonstrated that systolic thickening of the infarct wall was increased 2.5-fold by human cardiomyocyte engraftment. This effect is consistent with the human cardiomyocytes beating synchronously with the rat tissue, although it could also result from an indirect effect of the graft on host myocardium. Similar beneficial effects on cardiac repair have been reported by others (Caspi et al., 2007; van Laake et al., 2007), although in one study the benefit from the transplanted cells was only transient and could not be detected 3 months after transplant (van Laake et al., 2007).

In addition to the repair of infarcted myocardium, ESC-derived cardiac cells may be able to become "biological pacemakers" after transplantation. For example, in an immunosuppressed pig model where the atrioventricular node was destroyed thereby preventing normal pacing signals from reaching the ventricles, implantation of ESC-derived human cardiomyocytes into the left ventricular wall resulted in new pacemaker activity originating from the implantation site (Kehat et al., 2004). This provides physiological evidence that the grafted cardiomyocytes had electrically integrated with the host myocardium, and was corroborated by structural evidence showing gap junctions between graft and host cardiomyocytes.

Despite this recent progress, important issues in cardiac repair with ESC derivatives remain to be addressed. We do not know whether the ESC-derived cardiomyocytes in the infarct scar are electrically coupled by gap junctions to the host myocardium. If they are coupled, it is not known whether human cardiomyocytes can keep pace with the rapid rodent heart rate (~450 beats/min for the rat). Thus, it may be important to use hosts with slower heart rates. It remains to be seen whether human ESC-derived cardiomyocytes stop dividing and undergo

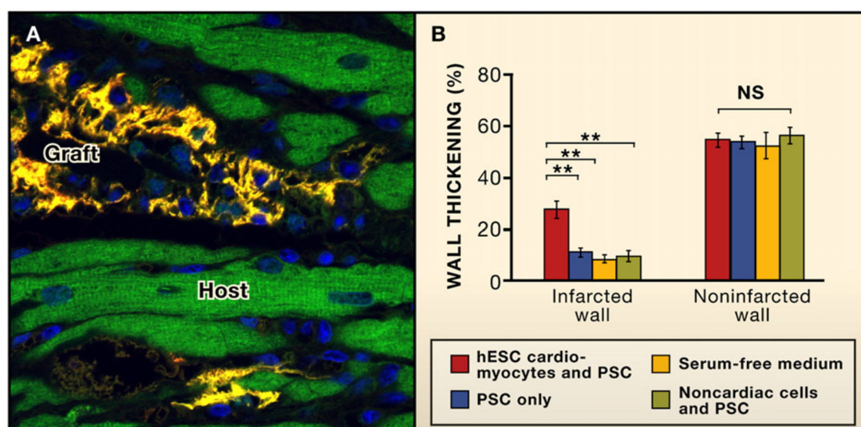

Figure 4. Human ESC-Derived Cardiomyocytes in a Rat Model of Cardiac Infarct

(A) Shown is a confocal fluorescent micrograph of a human myocardial graft in an infarcted rat heart. The peri-infarct zone is stained with human-specific β-myosin heavy chain (red) and pan-cardiac marker cardiac troponin I (green) revealing immature human cardiomyocytes (yellow) in close apposition to host cardiomyocytes (green). (Reprinted with permission from MacMillan Publishers Ltd., *Nature Biotechnology* 25, 1015, 2007).

(B) Human cardiomyocyte engraftment and cardiac contractile function. Magnetic resonance imaging demonstrates a 2.5-fold enhancement of systolic wall thickening in the infarct region of the rat heart receiving a human cardiomyocyte graft. Control groups received noncardiac human ESC derivatives in prosurvival cocktail (PSC), PSC only, or serum-free media (SFM only). NS, no significant difference. (Adapted from Laflamme et al., 2007.)

hypertrophic growth to match their host counterparts. Finally, which stage of ischemic heart disease would benefit most from human ESC-derived cardiomyocytes: hearts with acute infarcts or those with old scars and established heart failure? It will be essential to answer these questions to move the field forward.

Differentiation of ESCs into Neural Phenotypes

Early methods to direct the differentiation of ESCs to neural fates used treatment with retinoic acid (Bain et al., 1995), sequential culture in serum and serum-free media (Okabe et al., 1996), or coculture with specific stromal cell lines such as PA6 (Kawasaki et al., 2000). It is well established that trilineage neural progenitors—capable of giving rise to neurons, astrocytes, and oligodendrocytes—can be generated from ESCs (reviewed in Joannides et al., 2007). Neural progenitors are commonly derived from differentiating ESC cultures by growing them under conditions optimized for adult neural progenitors, including growth as three-dimensional spheroids (neurospheres) in the presence of EGF and FGF2.

Although ESC-derived neural progenitors resemble adult and fetal neural progenitors in their trilineage capacity, microarray and DNA methylation assays indicate that there are many differences between these two progenitor populations (Shin et al., 2007). These differences are important to keep in mind as the two cell populations can be expected to behave differently in many settings. Although generating cells with a neural phenotype from ESCs is relatively straightforward, it should be noted that there are many neuronal and glial subtypes with different physiological functions. Obtaining a purified subtype for basic research or for tissue repair is considerably more challenging.

Many signaling pathways known to regulate neural cell fate in the embryo have been exploited to control neural differentiation from ESCs, including Notch (reviewed in Androutsellis-Theotokis et al., 2006; Hitoshi et al., 2002; Lowell et al., 2006), sonic hedgehog (Maye et al., 2004), Wnts (Davidson et al., 2007; Lamba et al., 2006), the FGF family (Rao and Zandstra, 2005), and members of the TGF-β superfamily (Smith et al., 2008). The Notch pathway has emerged as a particularly important axis for controlling neural differentiation. Hitoshi et al. (2002) showed that neural progenitors could form in the absence of Notch signaling, but that these cells did not self-renew and hence were quickly lost to differentiation. Other investigators demonstrated that activation of Notch in mouse ESC derivatives after withdrawal of leukemia inhibitory factor (LIF) promoted exclusively neural differentiation, whereas inhibition of Notch blocked formation of neural progenitors. The ability of Notch ligands to promote neural progenitor formation required FGF receptor-mediated signaling (Lowell et al., 2006). Taken together, these data implicate Notch signaling as a key player in establishment of neural progenitor cells, principally through effects on cell survival and promoting expansion of the progenitors by blocking their differentiation.

Joannides et al. (2007) have developed a protocol for neural induction of human ESCs that uses chemically defined media at each step. Supplements include common amino acids and taurine; trace metals; vitamins; and the growth factors insulin, EGF, and FGF2. After optimizing techniques for passaging to generate small clumps of human ESCs, cells were induced to form neural progenitors and were expanded in defined media. Some cultures approached 90% nestin-negative/Pax6-positive cells that were trilineage-competent, and these cells could undergo 5- log expansion with a stable karyotype. Thus, defined media facilitate mechanistic studies and should help to promote translation to the clinic.

Wichterle et al. (2002) were the first to derive a protocol for the directed differentiation of ESCs to a specific neural type, using induction with retinoic acid and a sonic hedgehog analog to induce transplantable murine spinal motor neurons (Wichterle et al., 2002). Following this pioneering work, multiple investigators developed techniques to induce differentiation of ESCs into specific neuronal populations, including progenitors for retinal photoreceptors, cerebellar granule neurons, and cerebral-type neurons that use glutamate, GABA, and dopamine as their major neurotransmitters. Different lines of human ESCs appear to preferentially make one neuron type over another.

Differentiation to Dopamine Neurons

Dopamine neurons are of particular interest because of their central role in Parkinson's disease. Many studies now show that mouse and human ESCs can form dopamine neurons, and they appear to arise through the neural progenitor stage described above. These neurons express tyrosine hydroxylase (required

for dopamine synthesis), release dopamine upon depolarization, and form at least rudimentary synapses in vitro with transmitter reuptake abilities (reviewed in Kim et al., 2007). The combined use of FGF8 and SHH effectively induces dopamine neurons from ESC-derived neural progenitors generated from either mouse ESCs (Lee et al., 2000) or human ESCs (Yan et al., 2005). Although recombinant factors are now routinely used, most protcols do include undefined reagents at one or more stages of dopamine neuron production, due to coculture with stromal cell lines or the use of conditioned media. One of the best-defined protocols for human ESC differentiation into dopamine neurons was validated in three human ESC lines and two monkey ESC lines (Perrier et al., 2004). Neural progenitors were induced in this study using stromal cell coculture, followed by SHH and FGF8 to specify a neuronal fate. Addition of ascorbate, BDNF, glial-derived neurotrophic factor, dibutyryl cyclic-AMP, and TGF-β3 yielded cultures that were 30%–50% neurons expressing β-III tubulin. Of these neurons, 65%–80% expressed tyrosine hydroxylase, and the majority fired action potentials that could be blocked by tetrodotoxin, a Na^+ channel blocker. The remainder of the culture comprised nestin-positive progenitors with low frequencies of other differentiated neural cell types. It would be interesting to determine whether combining the above-mentioned protocol for producing neural progenitors (Joannides et al., 2007) with this regimen for inducing midbrain neurogenesis would yield dopamine neurons from human ESCs using completely defined factors.

Differentiation to Oligodendrocytes

Astrocytes and oligodendrocytes are the two neuroglial types in the central nervous system. Diseases of the central nervous system typically involve proliferation of astrocytes and loss of oligodendrocytes and the protective myelin sheath they produce. Thus, derivation of oligodendrocytes from ESCs has been an important goal for cell replacement therapy. The most common protocols involve an initial differentiation step to neural progenitors, followed by expansion, further differentiation, and selection. Oligodendrocytes were first efficiently derived from mouse ESCs (Brustle et al., 1999), where medium containing FGF2 and EGF was used to expand progenitors, followed by a switch to FGF2 and PDGF to yield bipotential glial progenitors. These glial progenitors were transplanted into the spinal cords of rats with a genetic deficiency in myelin production, yielding myelinated fibers in the majority of animals. Transplantation of these glial progenitors into the brains of developing rats (at embryonic day 17) resulted in widespread myelin-producing cells of mouse origin. Oligodendrocytes were first generated from human ESCs by Zhang et al. (2001b), who used a similar strategy involving FGF treatment followed by growth as neurospheres. They reported occasional cells expressing the oligodendrocyte marker O4, with many more cells expressing neuronal or astrocytic markers. No human oligodendrocytes were detected after transplantation into the brains of newborn mice, although human neurons and some astrocytes were found to have engrafted.

The first detailed protocol for directed differentiation of oligodendrocytes from human ESCs involved generation of neurospheres, followed by several rounds of expansion and selection in various media containing, among other things, the multicomponent additive B27, thyroid hormone, retinoic acid, FGF2, EGF, and insulin (Nistor et al., 2005). After ~42 days of culture, the desired cells were found in yellow spheroids, which upon differentiation as low-density monolayers formed 85%–95% oligodendrocytes (based on expression of the markers GalC, RIP, and O4). The remaining cells were astrocytes or neurons. The authors observed significant amounts of cell death and noted that cells seemed to be selected based on preferential adherence. Importantly, the derived oligodendrocyte progenitors were able to myelinate host axons when transplanted into the *shiverer* mouse, which has a genetic defect preventing myelination. Kang et al. (2007) recently reported a simplified protocol for isolation of oligodendrocyte progenitors from human ESCs, using a multistep procedure that yielded ~80% oligodendrocytes that were capable of myelinating fetal neural explants in vitro. These experiments show that human oligodendrocytes can be generated in large numbers and used to restore myelination under some circumstances in mice.

Transplantation for Parkinson's Disease

Parkinson's disease is characterized by loss of a critical population of dopamine-producing midbrain neurons with cell bodies in the substantia nigra. These neurons project to the striatum and are essential for motor processing. Parkinson's patients suffer from multiple motor problems including a resting tremor, difficulty in walking, and loss of facial expression. The disease is typically progressive due to ongoing loss of neurons. Parkinson's disease was one of the first diseases of solid tissue to be treated with cellular therapy, in this case with cells derived from human fetal midbrains transplanted into the striatum of patients (Goya et al., 2007). Early clinical trials with fetal tissue appeared promising (Lindvall and Hagell, 2001), but the results of randomized, controlled studies were less robust with modest improvements seen only in younger patients (Freed et al., 2001). Of note, some patients receiving these transplants developed dyskinesias, movement disorders associated with excessive dopamine levels in the brain. Dyskinesias may have resulted from overdosing with graft cells, although this is controversial (Hagell et al., 2002). These mixed results may stem, in part, from the variability inherent in using human fetal tissue as a source of therapeutic cells. One advantage afforded by human ESCs is their ability to reproducibly generate well-characterized cell populations for transplantation.

In a common animal model for Parkinson's disease, 6-hydroxydopamine, a toxin for dopamine neurons, is injected into the midbrain to produce a unilateral lesion. Treating the animal with an agent that stimulates motion, such as amphetamines or opioids, results in a characteristic unilateral turning behavior that can be readily quantified. Using 6-hydroxydopamine in a rat model, Rodriguez-Gomez et al. (2007) demonstrated long-term benefit of dopamine neural grafts derived from mouse ESCs. They showed that mouse ESC neural derivatives survived for 32 weeks in the brains of immunosuppressed Parkinsonian rats, and that the grafts produced dopamine and induced persistent improvements in rotational behavior.

We are aware of one study that attempted a head-to-head comparison of fetal-derived midbrain cells to mouse ESC-derived cells (Yurek and Fletcher-Turner, 2004). These investigators induced mouse ESCs toward a neural phenotype, achieving ~10% dopamine neurons, which were transplanted into the

striatum of immunosuppressed rats previously injured with 6-hydroxydopamine. Another group of rats received transplants of intact rat fetal ventral mesencephalic tissue, apparently without immunosuppression. Both cell populations yielded viable grafts in the striatum with comparable numbers of cells expressing tyrosine hydroxylase. However, the rats receiving mouse ESC derivatives did not show an improvement in their rotational score, whereas rats receiving fetal rat midbrain tissue did. The lack of benefit with mouse ESC derivatives may reflect comparison of a xenograft under immunosuppressive conditions with an allograft without immunosuppression.

In one of the most detailed studies involving human ESC derivatives, Ben-Hur et al. (2004), transplanted human ESC-derived neurospheres into the chemically lesioned brains of immunosuppressed rats. The human cells persisted for 12 weeks and formed nestin$^+$ progenitor cells, astrocytes, and tyrosine hydroxylase-positive neurons (these neurons comprising ~0.2% of the total human cells in the graft). Despite the relatively small population of dopamine neurons, the grafts reduced turning behavior by 25%–50% after opiate or amphetamine treatment. Interestingly, there was a linear correlation between the number of dopamine human neurons and the reduction in turning behavior, and no benefit was seen in lesioned rats that did not receive a surviving graft. The percentage of dopamine neurons was similar to that observed with in vitro differentiation of human ESCs, suggesting that differentiation signals are absent from the brain and need to be provided before transplantation.

A drawback to extensive predifferentiation in vitro was highlighted by Park et al. (2005), who directed differentiation of human ESCs to a population comprising >95% neurons or nestin$^+$ progenitors. Although 40% of these cells expressed tyrosine hydroxylase and released dopamine upon depolarization in vitro, the cells did not survive after transplantation into the lesioned brains of immunosuppressed rats and had no influence on turning behavior. Thus, more differentiated dopamine neurons may be more likely to die after transplantation.

Stem Cells and Spinal-Cord Injury

Trauma to the spinal cord kills neurons, myelinating cells, blood vessels, and other resident cells (reviewed in Coutts and Keirstead, 2008; Kim et al., 2007). The main determinants of morbidity are the extent of tissue disruption (mild contusion versus full transection) and the point at which the spinal cord sustains injury (cranial versus caudal). Hemorrhage and inflammation are thought to inhibit axon regeneration by promoting glial scarring and through direct chemical signals that block axon growth. Stem cells could have beneficial effects by being directly incorporated into tissue or by promoting repair by endogenous cells. Possible benefits include new neurons to bridge the gap, enhanced remyelination, and controlling host responses like cell survival, axon sprouting, inflammation, angiogenesis, and gliosis.

Neural progenitors derived from mouse ESCs promote hindlimb motor recovery when implanted in a mouse (Kimura et al., 2005) or rat spinal cord injury model (McDonald et al., 1999). Tracking with bromodeoxyuridine suggested that the cells differentiated into oligodendrocytes, astrocytes, and neurons. Liu et al. (2000) reported that these cells promoted remyelination of a chemically demyelinated spinal cord in immunosuppressed rats. Human ESC-derived oligodendrocyte progenitor cells populate a ~1 cm region when engrafted in a rat model of spinal-cord contusion (Keirstead et al., 2005). The human cells promoted remyelination of host axons and enhanced motor function when administered 7 days postinjury. However, when the human ESC-derived oligodendrocyte progenitor cells were transplanted into a 10-week-old rat spinal injury model with glial scarring, remyelination was inhibited and there was no improvement in motor function. This suggests that, like fibrosis in the heart, glial scarring in the spinal cord is a barrier to regeneration. Despite these setbacks, spinal cord injury is likely to be the first clinical setting in which human ESC derivatives will be tested, with a multicenter clinical trial expected to commence in 2008–2009.

Differentiation of ESCs to Pancreatic Cells

The potential to generate functional pancreatic β cells from ESCs differentiated in culture has raised the exciting possibility of a new source of insulin-producing cells for transplantation to treat type I diabetes. Given the therapeutic potential of ESC-derived β cells, significant efforts have focused on isolating such cells in both mouse and human ESC cultures. Initial attempts to generate the pancreatic lineage used mouse ESCs (reviewed in Spence and Wells, 2007), but the most successful differentiation along this pathway has been recently achieved with human ESCs (D'Amour et al., 2006). The key to generating pancreatic lineage cells from human ESCs relies on recapitulating the critical signals that regulate endocrine pancreas development in the embryo.

The pancreas develops from foregut endoderm, and the earliest stages of induction are controlled in part by retinoic acid (RA) and the inhibition of SHH signaling (reviewed in Collombat et al., 2006; Murtaugh, 2007; Spence and Wells, 2007). The first indication of pancreas morphogenesis is the upregulation of *Pdx1*, a gene encoding a transcription factor that is essential for development of this tissue (Figure 5). Although indicative of pancreas specification, expression of *Pdx1* is not restricted to pancreatic tissues as it is also found in the region of the foregut that will give rise to the pyloric region of the stomach and the proximal duodenum. Coexpression of the transcription factor encoded by the *Ptf1a/P48* gene together with *Pdx1* marks the population that will give rise to the pancreas. Recent evidence suggests that expansion of the pancreatic progenitor population is supported by the surrounding mesenchyme through FGF10 secretion. FGF10 enhances Notch signaling, which represses expression of the transcription factor *Ngn3* and promotes expansion of pancreatic progenitors. Expression of *Ngn3* within the pancreatic epithelium defines the development of a progenitor population for all endocrine lineages, including the β cells. With further maturation, cohorts of factors function to establish the different endocrine lineages. β cell development is dependent, in part, on the combined activity of *Nkx2.2*, *Nkx6.1*, *Pax4*, *Pax6*, and *MafA*.

Through the sequential activation of different signaling pathways, D'Amour et al. (2006) demonstrated that it is possible to recapitulate many of these developmental stages in human ESC cultures. In this study, endoderm induced by activin signaling in monolayer cultures was specified to a pancreatic fate through a combination of FGF and retinoic acid signaling as

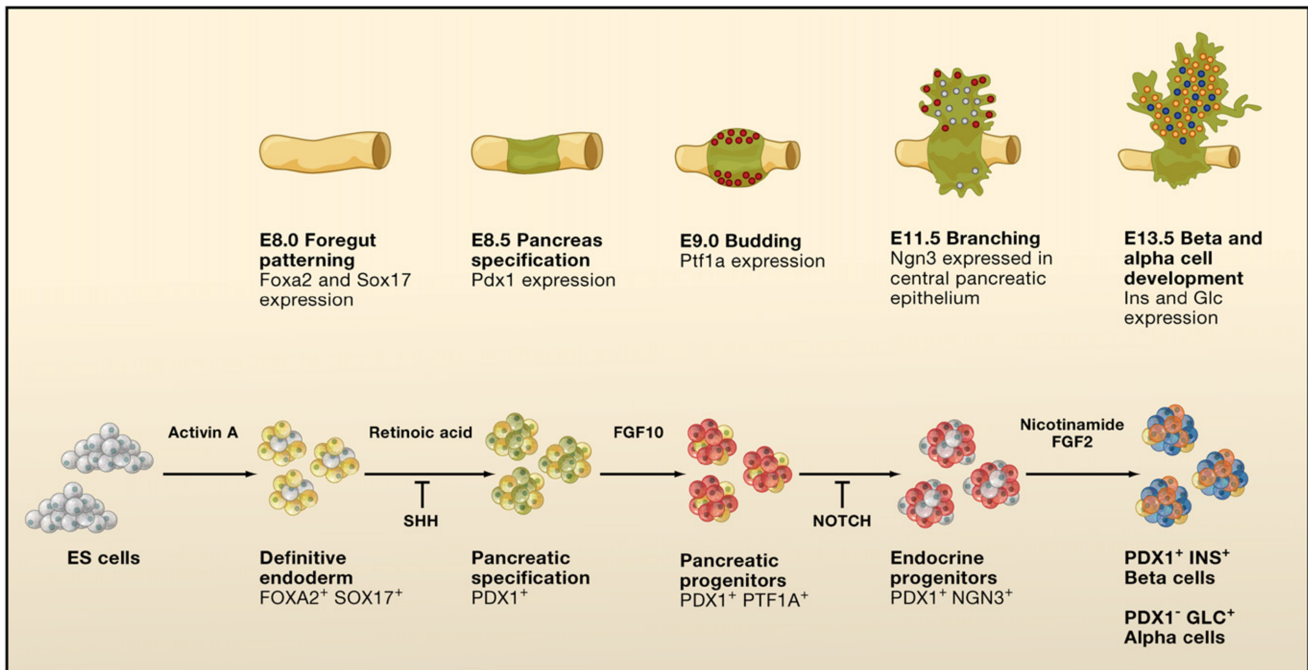

Figure 5. Pancreatic Development in the Mouse Embryo and in Human ESC Cultures

(Top) Pancreatic development in the mouse embryo, highlighting the key transcription factors involved in the specification of this organ from embryonic foregut endoderm. The first indication of pancreas morphogenesis is expression of PDX1. Coexpression of PDXI and PTF1A marks the population that will give rise to the pancreas. Expression of NGN3 within the pancreatic epithelium defines a progenitor population for all endocrine lineages, including β cells.

(Bottom) The differentiation scheme used to generate insulin-producing cells from human ESCs. Although the cells are represented as aggregates, the initial protocol was carried out in monolayer cultures (D'Amour et al., 2006). The different stages of development in the human ESC differentiation cultures are positioned to approximate those in the developing embryo.

well as inhibition of SHH signaling (Figure 5). Following specification, the cultures were treated with a γ-secretase inhibitor to inhibit Notch signaling and a combination of exendin-4, IGF1, and hepatocyte growth factor (HGF), which are known to promote β cell maturation. With this protocol, the population progressed through normal stages associated with pancreas development, including the induction of $FOXA2^+$ $SOX17^+$ $CXCR4^+$ endoderm, the formation of $HNF1\beta^+HNF4\alpha^+$ gut tube-like cells, specification of $PDX1^+$ progenitors, development of $NGN3^+$ $NKX2.2^+$ endocrine progenitors, and finally maturation to insulin-producing cells. Differentiation with this protocol was fast and reasonably efficient: ~7% of the population was insulin-positive within 16 days of differentiation. In addition to insulin-positive cells, mesoderm and ectoderm derivatives were also present in the cultures at this stage, indicating that not all cells underwent endoderm induction during the early stages of differentiation. The cells generated in these cultures expressed high levels of insulin and released C-peptide following depolarization with potassium chloride. The presence of C-peptide, released when proinsulin is converted to insulin, is a clear demonstration that the insulin is produced by the human ESC-derived cells and not absorbed from the culture media. Although the cells displayed many characteristics of β cells, they did not show a glucose response and so may represent an immature stage within the lineage.

Several other groups have analyzed the potential of activin-induced human ESC-derived populations to generate functional β cells using different differentiation schemes. Jiang et al. (2007a) induced endoderm with a combination of activin and sodium butyrate and promoted further maturation to $PDX1^+$ populations and subsequently $insulin^+$ cells by culturing the cells as aggregates, initially in the presence of bFGF, EGF, and the BMP inhibitor Noggin and finally in the presence of nicotinamide and IGF2. Development with this protocol was somewhat slower with cultures maintained for up to 36 days. At this stage, C-peptide-positive cells were detected in small clusters that also contained glucagon- and somatostatin-positive cells, reminiscent of pancreatic islets. In contrast to the population described by D'Amour et al. (2006), the cells in these clusters did release C-peptide in response to glucose, a key characteristic of mature β cells.

An important test for β cells is their physiological ability to produce insulin following transplantation into animal models of diabetes. Two groups have addressed this issue with human ESC-derived populations. Jiang et al. (2007b) used a modification of the protocol developed by D'Amour et al. (2006) to generate C peptide-positive cells capable of releasing insulin in response to glucose. When transplanted under the renal capsule of mice treated with streptozocin to induce diabetes, this cell population was able to reverse the hyperglycemic state of 30% of the recipients, suggesting that it contained functional insulin-producing cells. Analysis of the grafts in the kidneys of recipient mice revealed cells expressing human C-peptide and PDX1. The 70%

of recipients that still showed hyperglycemia had far fewer C peptide-positive cells in the graft than did the mice that achieved normal glucose levels. No teratomas were detected up to 3 months after transplantation, indicating that the graft did not contain significant numbers of undifferentiated human ESCs.

In the second study, Shim et al. (2007) used a combination of activin and retinoic acid to induce differentiation of human ESCs to the PDX1^{+} progenitor stage. These immature cells were transplanted to the kidney capsule of streptozocin-treated recipients to determine if this environment would promote survival and maturation of the transplanted population. Insulin-positive cells, not present in the population prior to transplantation, were detected 28 days following transplantation, indicating that further differentiation had occurred in vivo. Some of these insulin-positive cells also expressed glucagon, suggesting that they still represent an immature stage of development. These grafts appeared to rescue the streptozocin-induced hyperglycemia as the animals showed a significant reduction in blood glucose levels within 4 days of transplantation. This is somewhat surprising, given the immature status of the population used for transplantation and the presumptive requirement that the grafts be vascularized for proper endocrine function. Despite the immature stage of development, the transplanted cells appeared to be responsible for the reversal of hyperglycemia as the mice reverted back to a hyperglycemic state following removal of the kidney with the graft. Given the reversal of hyperglycemia by immature cells in streptozocin-treated animals, it will be important to use additional preclinical models of diabetes to verify the therapeutic potential of insulin-producing cells generated by this approach.

Taken together, these studies highlight remarkable progress over the last two years in the development of β cells from human ESCs. Although the populations generated may not represent mature functional cells, differentiation in most studies was efficient and relied on protocols that incorporated aspects of pancreatic development in the embryo. A major hurdle is the establishment of culture conditions that will promote efficient maturation of the population to end stage functional β cells. The transplantation studies of Brolen et al. (2005) suggest that the fetal pancreas may provide a source of regulators that promote these maturation steps. These investigators cotransplanted differentiated human ESC populations expressing PDX1 together with fetal mouse pancreas under the kidney capsule of recipient mice. The human ESC-derived cells matured to generate insulin-positive cells when transplanted with mouse pancreatic tissue, but not when transplanted with fetal liver or telencephalon cells, suggesting that factors within the pancreatic environment induced the human cells to mature. Future studies will be required to determine if the fetal pancreas can influence human ESC differentiation in vitro.

Challenges for Clinical Applications

Safety

Pluripotent cells such as ESCs or iPS cells present a safety concern because of their potential to form tumors. When these cells are transplanted in the undifferentiated state they form teratomas, tumors derived from all three germ layers. In our experience, normal or injured adult tissues lack the cues required to induce ESCs to form appropriate cell types (Nussbaum et al., 2007). Currently, the only way to ensure that teratomas do not form is to differentiate the ESCs in advance, enrich for the desired cell type, and screen for the presence of undifferentiated cells. When such procedures were rigorously followed, teratomas were not observed in over 200 animals transplanted with human ESC-derived cardiomyocytes (Laflamme et al., 2007). But more differentiated tumor-like growths can occur. For example, transplanting a population of human ESC neural derivatives enriched for dopamine neurons into Parkinsonian rats resulted in a primitive population of nestin^{+} neuroepithelial cells that continued to proliferate in the striatum 70 days after transplantation (Roy et al., 2006). This raises a cautionary flag and suggests that even committed progenitors can proliferate excessively after transplantation, a problem that may be solved by improving purification methods.

Other types of safety concerns exist not only with ESC-based approaches but also with adult cell grafts. For example, cell grafts into the heart may provoke arrhythmias (due, for example, to aberrant pacemaker activity or conduction properties) or, similarly, induce seizure activity in the central nervous system. Also, it is possible to give patients too many normal cells. This has been suggested as an explanation for Parkinson's patients receiving fetal neural tissue, who subsequently developed dyskinesias (Hagell et al., 2002). It will be critical to choose the initial patient population correctly to minimize risk, likely starting with the sickest patients, such as patients awaiting heart transplants or those with longstanding complete spinal-cord transaction injuries. Unfortunately, although beginning with the sickest patients minimizes risks, it also reduces the chance of success. It undoubtedly will be easier to prevent physiological deterioration in the early phases of illness rather than to try and reverse severe and chronic damage.

Purification

Despite considerable progress in directing the differentiation of ESCs, purification of desired differentiated cell types is required for most applications. Microdissection of cellular aggregates with a visible phenotype (e.g., beating cardiomyocytes or fluorescent reporter gene expression) is perhaps the simplest approach, but the purity of the resulting cultures is only moderate, and the system cannot be scaled up for the clinic. Density-gradient centrifugation can be used for purifying human ESC-derived cardiomyocytes (Laflamme et al., 2005, 2007; Xu et al., 2002), but in our experience, offers only ~5-10-fold enrichment. Thus, the final purity is highly dependent on the purity of the input population.

Surface molecules on cells are commonly used to select a desired population or to remove unwanted cell populations. Fluorescence-activated cell sorting (FACS) has been used to select many ESC derivatives, including hematopoietic cells, mesodermal progenitors, and endothelial cells (Gadue et al., 2006; Kattman et al., 2006; Kouskoff et al., 2005; Wang et al., 2004). FACS offers many advantages, including the ability to analyze multiple surface markers simultaneously to refine selection of the desired subpopulation. The biggest drawback to FACS is its relatively low throughput, necessitating expansion of some cell types after selection. A related technique, magnetically activated cell sorting (MACS), permits higher throughput selection of

ESC derivatives based on surface markers (Vodyanik et al., 2006) but is less amenable to the use of multiple markers than is FACS.

Finally, genetic selection of cells based on expression of a selectable marker driven by a lineage-restricted promoter offers excellent purity and scalability. This approach was first developed to select cardiomyocytes at > 99% purity by expressing an antibiotic resistance gene via a cardiac-restricted promoter (Klug et al., 1996). Genetic selection has been expanded to include fluorescent reporter proteins and has been used with excellent results to purify endothelial cells and insulin-producing islet cells from ESCs. Importantly, genetic selection of clinically relevant numbers of highly purified cells has been achieved in bioreactors (Zandstra et al., 2003). The principal drawback to genetic selection is the necessity (at present) of inserting a selection cassette into the host genome, which may increase the risk of tumorigenesis.

Controlling Graft Size

Death of transplanted cells remains a major limitation for repair of the heart (Murtuza et al., 2004; Zhang et al., 2001a), for treatment of Parkinson's disease (Cicchetti et al., 2002; Emgard et al., 2003; Marchionini et al., 2004; Schierle et al., 1999), in islet cell transplantation for diabetes (Contreras et al., 2002; Nakano et al., 2004), and for myogenic precursor transplantation for muscular dystrophy (Guerette et al., 1997; Skuk et al., 2003). Current evidence suggests that cell-death pathways are initiated by multiple stresses associated with transplantation, including ischemia, loss of matrix attachments, and inflammation. In studies of human ESC-derived cardiomyocytes in the infarcted heart, a prosurvival cocktail that targeted multiple points in the death signaling network succeeded in generating physiologically significant graft sizes, whereas individual interventions were without benefit (Laflamme et al., 2007). This suggests that there are multiple pathways through which transplanted cells can die, and that blocking individual paths simply leads to death through alternate routes. It will be of interest to determine whether the prosurvival cocktail developed for cardiomyocytes can benefit other cell types.

Proliferation of grafted cells offers the prospect of exponential growth. Delivery of growth factors to the cells may regulate their proliferation, but it has the potential to induce undesired effects on the host such as stimulation of fibrosis or glial scarring. One route to control proliferation involves genetic modification of the graft cells to render them responsive to a signaling pathway controlled by a small molecule, to which the host cells are unresponsive. One such approach has been to fuse the cytoplasmic domains of growth-factor receptors to drug-binding domains, typically variants of FK506-binding proteins (Belshaw et al., 1996). Administering bivalent ligands for the modified binding proteins induces dimerization of the receptor domains, reproducing signaling induced by a native ligand selectively in the graft cells. This approach has been used to promote hematopoiesis after bone-marrow transplantation (Weinreich et al., 2006) and was recently used selectively to control graft cell proliferation in the heart (Stevens et al., 2007), yielding a pharmacologically tunable system for infarct repair.

Scarring

In the heart, scar tissue associated with the infarct typically forms a barrier that interferes with integration of grafted cardiomyocytes into host myocardium. As a consequence, grafts may remain electrically insulated from the host tissue and beat asynchronously, or they may have delayed activation that leads to arrhythmias. In the central nervous system, proliferation of astrocytes following injury leads to "glial scarring" (Keirstead et al., 2005). In this context, cytoplasmic projections of astrocytes insulate axonal projections and prevent them from finding their distal tracks or forming new synapses with transplanted neural progenitors. The simplest approach to these problems would be to intervene relatively early, before the scar tissue forms. Unfortunately, this option is not always clinically feasible. The pathogenesis of scarring in the setting of stem cell transplantation has not been well studied. Additional research is needed to rationally identify targets to address this important problem.

Finding the Right Model for Preclinical Testing

Preclinical research with human ESC derivatives requires xenotransplantation, raising the question of which species are best suited as hosts. An ideal model would (1) support human xenografts, (2) recapitulate key aspects of the disease, (3) have physiological properties that make it predictive of the human response to therapy, and (4) be cost-effective enough to permit high-throughput screening necessary at this early stage of development. Clearly, no single model meets all of these criteria. A large animal with drug-induced immunosuppression, such as a dog, pig, sheep or nonhuman primate, probably provides the best physiological mimic of the human. These models are extraordinarily expensive, however, and studies requiring multiple iterations are difficult to do in large animals. On the other end of the spectrum, the mouse is inexpensive, and there are many murine models for human diseases, including genetically immunodeficient strains. Murine physiology differs significantly from human physiology; for example, the mouse heart rate is 600 beats/min and that for human is 70 beats/min. This makes electrophysiological results from mouse less predictive of human responses. Furthermore, scaling up a process 2500-fold for the human can raise new problems in cell production or delivery that were not anticipated from murine studies. One clear drawback to all animal studies is that the immune response to xenogeneic transplantation does not predict the response to allogeneic transplantation. From an immune standpoint, we probably will learn more from animal-to-animal allotransplantation studies than we will from xenotransplantation. Most investigators support the development of therapeutic approaches in small animals where higher throughput permits more rapid progress. However, key concepts should be tested in large animals, including nonhuman primates, where real-world issues of scale, delivery, and assessment of efficacy can be developed before attempting studies in patients.

Immune rejection

The immune system presents a formidable barrier to allogeneic cell transplantation. ESCs express few class I major histocompatibility antigens and have virtually no expression of class II molecules (Drukker et al., 2002; Nussbaum et al., 2007; Swijnenburg et al., 2005), which initially raised hopes that ESC derivatives might not induce an immune response when transplanted. However, once transplanted, ESC derivatives show increased expression of histocompatibility antigens, with further increases in response to cytokine stimulation. Although cell transplants

may not have the full antigenicity of solid organs (due, for example, to the absence of professional antigen-presenting cells), there is now clear evidence that differentiated progeny of ESCs will be rejected in an allogeneic setting (Nussbaum et al., 2007).

There are several strategies to address this problem (reviewed in Cabrera et al., 2006; Drukker, 2004). The first approach is immunosuppression, similar to that used for solid organ transplantation, and this is likely where clinical trials will begin. Banking of ESCs can provide cells with a repertoire of tissue types, but large numbers of cell lines (likely thousands) would be needed to cover a genetically diverse population such as that in the United States (Taylor et al., 2005). The number of required cells can be reduced exponentially by using human ESCs that are homozygous at the HLA histocompatibility loci. Others have suggested development of a "universal donor cell," for example by using blood type O cells and suppressing expression of HLA molecules (Cabrera et al., 2006; Drukker, 2004). HLA suppression, although conceptually appealing, makes ESC derivatives targets for killing by NK cells of the immune system. Another notion is engineering ESC progeny to secrete locally immunosuppressive molecules such as a soluble interleukin-1 receptor or agents that block coreceptors for antigen stimulation. A final approach is to take a lesson from hematopoietic cell transplantation, where animals with hematopoietic chimerism tolerate solid organ transplants from the marrow donors (Menendez et al., 2005; Priddle et al., 2006). If hematopoietic cells capable of inducing tolerance could be derived from human ESCs, they would enable cell transplants from the same human ESCs to survive in the host without broad spectrum immunosuppression.

Three groups recently reported the derivation of induced pluripotent stem (iPS) cells from human somatic cells. These studies demonstrated reprogramming of either the differentiated progeny of stem cells (Park et al., 2008), fetal and neonatal fibroblasts (Yu et al., 2007), or adult fibroblasts (Takahashi et al., 2007). The groups used a cocktail of four retrovirally encoded transcription factors—either Oct4, Sox2, c-*myc* and Klf-4 or Oct4, Sox2, Nanog, and Lin28—to reprogram fibroblasts to cells that closely resemble human ESCs. These iPS cells had gene-expression profiles and DNA-methylation patterns closely resembling human ESCs, grew vigorously while expressing telomerase, maintained a normal karyotype, and formed teratomas after transplantation into immunocompromised mice. Importantly, Takahashi et al. (2007) showed that the human iPS cells could be directed down the cardiomyocyte lineage using activin/BMP4 and toward neurons using PA6 stromal cell coculture. Most recently, Nakagawa et al. (2008) demonstrated that iPS cells could be derived without c-*myc*, removing a potential oncogenic factor from the reprogramming cocktail.

If these results hold up as anticipated there are important implications for the field. The ability to generate pluripotent cells from readily available fibroblasts should obviate ethical concerns surrounding human ESCs as no human embryos are harmed in the process. Furthermore, iPS cells offer the potential to generate patient-specific cells that would be recognized as "self" by the immune system, thus preventing rejection of cell transplants. In addition, iPS cells could be generated from patients with specific diseases having genetic components and allowed to differentiate into relevant populations (such as dopamine neurons from Parkinson's patients) enabling study of disease pathogenesis and development of new treatment strategies. There are, however, two drawbacks to the clinical use of iPS cells. The first is the current need to use integrating retroviruses to deliver the reprogramming factors; this may be solved with transient gene transfer or through delivery of the protein factors in cell-permeant forms. The second is that iPS cells are not an "off-the-shelf" product and would likely only be produced after the patient becomes ill, precluding their use in the acute phase of the disease. Quality control is also likely to be difficult and expensive, because a separate batch of iPS cells would have to be made for each patient.

Summary and Conclusions

Insights gained from developmental biology have facilitated our understanding of the control of ESC growth and lineage-specific differentiation. These insights enable the reproducible generation of highly enriched cell populations from a number of different lineages. The demonstration that somatic cells can be reprogrammed to an ESC-like cell suggests it will be possible to generate such populations from patients in the near future. With these advances, we can now to begin testing the function of these cell types through the transplantation of highly enriched, well-characterized populations into different preclinical models of disease. Using protocols with defined reagents ensures that different groups will be able to reproducibly isolate comparable cell populations for such studies. Access to lineage-specific progenitors for transplantation will allow comparison to more mature populations to determine which stage integrates best into the adult tissue and which ultimately provides the most benefit. The availability of highly enriched cell populations from different lineages also provides an opportunity for cell biologists to interact with tissue engineers to generate culture systems that will more accurately mimic important three-dimensional aspects of organogenesis. Such engineered tissues may be more effective following transplantation and may also support more efficient maturation of the different cell types in culture. With these tools at hand, the therapeutic potential of ESCs is now ready to be tested.

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