Challenges and strategies for generating therapeutic patient-specific hemangioblasts and hematopoietic stem cells from human pluripotent stem cells

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Abstract

Recent characterization of hemangioblasts differentiated from human embryonic stem cells (hESC) has further confirmed evidence from murine, zebrafish and avian experimental systems that hematopoietic and endothelial lineages arise from a common progenitor. Such progenitors may provide a valuable resource for delineating the initial developmental steps of human hemato-endotheliogenesis, which is a process normally difficult to study due to the very limited accessibility of early human embryonic/fetal tissues. Moreover, efficient hemangioblast and hematopoietic stem cell (HSC) generation from patient-specific pluripotent stem cells has enormous potential for regenerative medicine, since it could lead to strategies for treating a multitude of hematologic and vascular disorders. However, significant scientific challenges remain in achieving these goals, and the generation of transplantable hemangioblasts and HSC derived from hESC currently remains elusive. Our previous work has suggested that the failure to derive engraftable HSC from hESC is due to the fact that current methodologies for differentiating hESC produce hematopoietic progenitors developmentally similar to those found in the human yolk sac, and are therefore too immature to provide adult-type hematopoietic reconstitution. Herein, we outline the nature of this challenge and propose targeted strategies for generating engraftable human pluripotent stem cell-derived HSC from primitive hemangioblasts using a developmental approach. We also focus on methods by which reprogrammed somatic cells could be used to derive autologous pluripotent stem cells, which in turn could provide unlimited sources of patient-specific hemangioblasts and HSC.

Keywords

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Prospects for generating patient-specific hemangioblasts and HSC from human pluripotent stem cells

Since Thomson and colleagues first isolated pluripotent human embryonic stem cells (hESC) from the inner cell mass of preimplantation blastocyst embryos (Thomson *et al.*, 1998), hESC have been envisioned as an unlimited source for generating transplantable cell lineages. Furthermore, because hESC can readily be manipulated and genetically altered using transgenesis (Ma *et al.*, 2003; Vallier *et al.*, 2004) or homologous recombination (Zwaka and Thomson, 2003; Urbach *et al.*, 2004), these cell lines have great potential for use in novel cellular therapies for a number of genetic and degenerative diseases, including hematologic disorders. Despite increased efforts to match allogeneic transplant donors and recipients, many patients fail to receive immunocompatible bone marrow, cord blood, or peripheral blood stem cell transplants required for effective, long-term treatment of their hematologic diseases (Grewal *et al.*, 2003). As a result, the survival rate for most potentially transplantable hematologic disorders remains low (Davies *et al.*, 1995), and new sources of transplantable, immunocompatible hematopoietic stemcells (HSC) are needed. A long sought-after goal has been to generate large amounts of autologous, genetically corrected HSC to treat severe hematologic diseases such as sickle cell anemia, thalassemia, and leukemia. The recent scientific achievement of genetically-induced pluripotency of somatic cells (*e.g.,* from skin fibroblasts) has introduced the possibility that HSC generated from patient-specific induced pluripotent stem cells (iPSC) will eventually meet this challenge. The proof of principle for this approach was recently demonstrated in a murine model of sickle cell hemoglobinopathy where iPSC were generated from sickle hemoglobin-affected murine fibroblasts (Hanna *et al.*, 2007). These studies showed that genetically-corrected iPSC could generate normal, autologous transplantable HSC that could cure sickle cell anemia in a murine model. Additionally, the efficient generation of unlimited supplies of hemangioblasts with endothelial progenitor capacity from hESC and iPSC may also lead to novel strategies for treating myocardial infarction, stroke, retinal blindness, and a multitude of other vascular disorders.

The realization of these goals poses great scientific challenges, since the generation of transplantable hemangioblasts and HSC from hESC or iPSC has not yet been efficiently described. We previously showed that hESC differentiation produces blood cells similar to those found in the human yolk sac (YS), which are too immature to provide adult-type hematopoietic reconstitution. A sophisticated developmental biologic approach is necessary for elucidating effective strategies that can differentiate hESC/iPSC into either early mesoderm or YS-like hemangioblastic progenitors that can develop into transplantable HSC that are found in the adult bone marrow.

In this review, we outline the challenges and opportunities for deriving hemangioblasts and HSC from human pluripotent stem cells, and propose developmental strategies that could be employed to generate hESC-derived HSC from primitive hemangioblasts *ex vivo*. We also summarize the methods by which reprogrammed somatic cells can produce patient-specific pluripotent stem cells, which subsequently could provide unlimited sources of autologous, transplantable hemangioblasts and HSC.

Embryonic hemangioblasts sequentially develop into the adult hematopoietic system

Classic anatomic observations of an intimate association between emerging embryonic blood cells and vascular endothelium led to the hypothesis that they share a common developmental precursor, termed the hemangioblast (Sabin, 1920; Murray, 1932). In mice

and humans, two waves of hematopoieses form the blood system: primitive (yolk sac; YS) and definitive (adult-type) hematopoieses. Hematopoietic progenitors derived from YS blood islands are limited in their developmental capacity, and produce primarily primitive erythroblasts. In contrast, the second, definitive wave of hematopoiesis generates long-term engrafting lympho-hematopoietic HSC, which arise *de novo* from presumptive hemangioblasts (via hemogenic endothelium) in the aortagonad-mesonephros (AGM) region of the embryo (Medvinsky and Dzierzak, 1996; Cumano *et al.*, 2001). These HSC migrate from the AGM to fetal liver and bone marrow niches around birth in the mouse (Blazsek *et al.*, 2000), and at the end of the first trimester in the human fetus (Charbord *et al.*, 1996), where they sustain the adult's lifetime blood generation.

In the mouse, hemangioblast formation is initiated by the ingress of pluripotent epiblast cells through the primitive streak resulting in fate-committed mesodermal cells (Lawson *et al.*, 1991; Huber *et al.*, 2004). These mesodermal populations within the primitive streak can be divided into four subpopulations/tissue compartments (axial, paraxial, lateral plate, and extraembryonic). The axial mesoderm, which consists of cells that ingress closest to the most anterior region of the primitive streak, forms the notochord. The paraxial mesoderm, which is created by cells that emerge in the anterior third region of the primitive streak, generates the somites, and subsequently the vertebral column and skeletal muscles. The lateral plate mesoderm, which is composed of cells in the middle of the primitive streak, forms the circulatory system, gut wall, and the aorta-gonad mesonephros (AGM), which is the major source of adult, definitive-type HSC. Finally, the extraembryonic mesoderm, which is produced from cells that progress through the most posterior region of the primitive streak, develops into (primitive) blood cells and the vasculature of the YS (Fig. 1A, B).

To date, there are only limited studies of early human hematopoietic development, and such studies have suggested, but not confirmed, the existence of a similar bipotential human hemangioblast (Cortes *et al.*, 1999; Tavian *et al.*, 1999a; Tavian *et al.*, 2001; Oberlin *et al.*, 2002). Thus, despite substantial differences between human and mouse development (Tam and Beddington, 1987; Beddington and Robertson, 1998), our understanding of the mechanisms for the emergence of HSC from embryonic hemangioblasts is primarily derived from studies of murine hematopoiesis. The ethical and technical difficulties associated with studying human development have precluded the detailed characterization of a similar hemangioblastic origin of human primitive and definitive hematopoiesis. Additionally, a narrow repertoire of available human hemangioblastic surface markers has further complicated the characterization of these rare progenitors in the human YS or AGM tissues, which is currently available only in limited amounts.

Keller and colleagues originally demonstrated that mouse embryonic stem cells (mESC) differentiated in a manner that recapitulated embryonic hematopoiesis through the emergence of the hemangioblast (Wiles and Keller, 1991; Keller *et al.*, 1993; Vittet *et al.*, 1996). mESC were differentiated into multi-lineage clusters of aggregated cells, called embryoid bodies (EB), which contain elements of endoderm, mesoderm, and ectoderm before maturing into more defined cell populations. If cultured in methyl-cellulose containing hematopoietic growth factors, or co-cultured on bone marrow OP9 stromal cells (Nakano *et al.*, 1996), single EB cell progenitors differentiated into embryonic hematopoietic progenitors. In particular, hematopoiesis initiated from a hemangioblastic, clonogenic "blast-colony-forming cell" (BL-CFC), which was demonstrated not only during mouse EB (mEB) differentiation (Kabrun *et al.*, 1997; Kennedy *et al.*, 1997; Choi *et al.*, 1998; Robertson *et al.*, 2000; Lacaud *et al.*, 2002; Fehling *et al.*, 2003), but also *in vivo* (Huber *et al.*, 2004). This transient, VEGF-responsive progenitor normally arises in YS blood island mesoderm, where it initiates vasculogenesis, as well as primitive and definitive hematopoieses. Recent single-cell time-lapse microscopy imaging studies of mESC-derived

mesodermal precursor differentiated on OP9 stromal cells, and their equivalent *in vivo* counterparts from mouse embryos, have further confirmed the ability of hemangioblasts (or hemogenic endothelial cells) to give rise to blood cells in real time (Eilken *et al.*, 2009; Lancrin *et al.*, 2009). The emergence of this transient progenitor population was shown to be regulated by SCL/Tal1, whereas further maturation into definitive hematopoietic cells depended on Runx1/ AML1 (Lancrin *et al.*, 2009).

In general, these YS-like, mEB-derived progenitors, however, have had limited engraftment potential when injected into lethally-irradiated murine recipients (Potocnik *et al.*, 1997; Miyagi *et al.*, 2002), although various degrees of maturation into definitive hematopoietic progenitors could be accomplished by culture on AGM stroma (Byrne *et al.*, 2007), or via direct injection into the murine neonatal liver (Egami *et al.*, 2003). These results indicate that murine YS hemangioblasts can give rise to not only primitive YS hematopoiesis, but may also possess the potential to mature and contribute to the definitive-type HSC population which primarily develops in the AGM. Interestingly, ectopic mEB expression of homeobox, and cell-cycling proto-oncogenes such as *Hoxb4*, *Bcr/Abl*, *Stat5*, and *Cdx4*, can "program" YS-like mEB progenitors into definitive-like HSC capable of long-term engraftment (Perlingeiro *et al.*, 2001; Kyba *et al.*, 2002; Kyba *et al.*, 2003; Wang *et al.*, 2005). Unfortunately, similar experiments utilizing hESC differentiation did not result in long-term engrafting, definitive HSC formation, possibly underscoring fundamentally different developmental potentials between mice and humans (Wang *et al.*, 2005a; Wang *et al.*, 2005).

We, and others, have described various methods for the hematopoietic differentiation of hESC, including the derivation of human embryoid body (hEB) progenitors with hemangioblastic potential (Kaufman *et al.*, 2001; Chadwick *et al.*, 2003; Cerdan *et al.*, 2004; Wang *et al.*, 2004; Vodyanik *et al.*, 2005; Zambidis *et al.*, 2005; Vodyanik *et al.*, 2006; Zambidis *et al.*, 2008). Our group described a hESC model that recapitulates the human YS stages of embryonic hemato-endothelial development (Zambidis *et al.*, 2005) (Fig. 1). Hematopoietic activity was shown to arise in hEB cells from CD45⁻ mesodermal-hemato-endothelial (MHE) progenitors that give rise to hematopoietic blast colonies, followed by two sequential YS-like primitive and definitive hematopoietic waves. We also delineated the kinetics of hEB differentiation in serum-free conditions for the emergence of a putative common hemangioblastic progenitor responsible for these two waves. Hematopoietic colony-forming cell (CFC) potential of hEB cells differentiated in serum-free medium with hematopoietic growth factors showed that 6–9 day old hEB vigorously initiated YS-like, primitive and definitive hematopoieses. Primitive foamy macrophages and large nucleated erythroblasts expressing exclusively embryonic and fetal hemoglobins were observed during days 7–12 of hEB differentiation. This was followed by YS-like definitive erythropoiesis (that had limited amounts of adult hemoglobin; Fig. 2), myelopoiesis, and megakaryopoiesis typical of a late YS stage of development at days 12–20 of hEB development. Progressive expression of *SCL/TAL1*, *RUNX1/AML1*, *GATA1*, and *GATA2* (genes known to play roles in the initiation of hematopoiesis from murine hemangioblasts) during this period indicated that this hEB-derived model recapitulated early embryonic hematopoietic development.

Although these detailed kinetic studies of hEB differentiation predicted the emergence of a hemangioblastic progenitor, they did not demonstrate this stem-progenitor in a clonal manner. In contrast to murine experimental systems, the clonal characterization of human hemangioblasts that give rise to such primitive and definitive hematopoieses has been hindered by several obstacles, including 1) inefficient methods for hematopoietic differentiation of hESC, 2) the lack of an accurate prospective hemangioblast surface marker, and 3) the absence of a quantitative, robust human BL-CFC assay, that was recently

described (Kennedy *et al.*, 2007; Zambidis *et al.*, 2008). Furthermore, although KDR/flk-1 (VEGFR2) is a cell surface marker of populations containing murine hemangioblasts, it appears to be less specific for specifying rare hemangioblasts in human tissues, and is expressed abundantly in a majority of differentiating hEB cells, as well as during normal human embryonic development (Carpenter *et al.*, 2003; Kennedy *et al.*, 2007; Zambidis *et al.*, 2008). The identification of more specific markers for human hemangioblasts, or hemogenic endothelium will greatly facilitate the ultimate experimental goal of efficiently deriving HSC from them.

ACE (CD143) expression marks the emergence of a clonogenic, hESC-derived hemangioblastic progenitor of primitive and definitive YS-like hematopoieses

Using a monoclonal antibody (mAb BB9) specific for the somatic isoform of surface angiotensin-converting enzyme (ACE/ CD143), Simmons and colleagues identified a primitive subset of NOD-SCID mouse-engrafting $CD34^+$ HSC in adult bone marrow, mobilized peripheral, and umbilical cord blood. Moreover, in rare 30-day-old human embryonic tissues, ACE (CD143) was shown to mark emergent HSC in the human YS, intra-embryonic subaortic patches of the AGM, and fetal liver (Ramshaw *et al.*,2001; Jokubaitis *et al.*, 2008). The embryonic pattern of human ACE expression is consistent with a dorsal emigration of ACE^+CD34^- hemangioblasts from the paraaortic splanchnopleura, and subsequent colonization of the ventral aspect of the dorsal aorta to give rise to $CD34^+$ hemogenic endothelial cells.

Our group extended these studies and used the ACE/CD143 marker to specifically identify hESC-derived clonogenic hemangioblasts that can initiate both primitive and definitive YS-like hematopoieses (Zambidis *et al.*, 2008), similar to that observed in mEB differentiation and murine embryos (Kennedy *et al.*, 1997; Huber *et al.*, 2004). Using a serum-free BL-CFC assay supplemented with BMP4, VEGF, FGF2/heparan sulfate and TPO, single $ACE^+CD34^{+/-}$ hEB cells generated blast colonies containing multilineage endothelial-primitive-definitive hematopoietic progenitors (Fig. 3A). On secondary re-culture assays, these multipotent blast colonies generated YS-like $GlyA^+CD71^+CD45^-$ erythroid cells, and endothelium (if re-cultured on stromal-free conditions), or alternatively definitive-type $CD45^+$ erythromyeloid cells (if re-cultured on OP9 bone marrow stroma (Fig. 3A)).

Interestingly, our hEB differentiation system appears to recreate hematopoietic events *in vitro* which normally occur during the first weeks of human embryonic development, and that are likely initiated with mesodermal commitment to a YS hemangioblast (Fig. 1). In murine embryos, hemangioblasts first appear in the posterior region of the primitive streak (Huber *et al.*, 2004), and *in vitro* mEB differentiation is generally considered an experimental surrogate for these post-implantation developmental events. Similarly, the developmental kinetics of our hEB-derived mesodermal-hemato-endothelial (MHE) clusters, and ACE^+ BL-CFC mirror the emergence of hemangioblasts that presumably arise following human primitive streak formation at days 12–13 with a remarkably congruent timeline (FIGURE 1D, E). Similarly, normal YS blood island generation at gestational days 14–16 was recapitulated by an hEB-derived wave of primitive hematopoiesis, followed by a definitive wave of erythromyelopoesis, which normally occurs at gestational days 18–20 (prior to the onset of fetal circulation at day 21). These results suggested that our hEB-based differentiation system serves as an *in vitro* model to study the earliest developmental steps in human hematopoietic genesis.

The renin-angiotensin system may dictate embryonic hemangioblast lineage differentiation

The discovery that angiotensin converting enzyme (ACE/ CD143) marks primitive embryonic hemangioblasts raised the possibility that the versatile renin-angiotensin system plays a critical role in regulating the earliest stages of human hemato-endothelial differentiation, as it does in avian embryos (Savary *et al.*, 2005). In adults, the renin-angiotensin axis regulates blood pressure and water balance via hormonal secretion of angiotensin II (Ang II) peptide. Ang II is the proteolytic product of a series of enzymatic processes that begin with the cleavage of angiotensinogen by renin to yield the catalytic substrate of the ACE, angiotensin I (Ang I), which is ultimately converted to Ang II (Paul *et al.*, 2006). Renin-angiotensin system signaling is subsequently mediated by angiotensin II type 1 and 2 receptors (AGTR1/AGTR2), which are locally expressed in all important sites of emerging angio-hematopoiesis including the YS, liver, kidney, embryonic aorta, and retinal/choroid regions (Schutz *et al.*, 1996). Notably, while AGTR1 is expressed constitutively from development to adulthood where it functions as a key regulator of the cardiovascular system through G-protein-coupled interactions, AGTR2 is rarely expressed in adult tissues (Grady *et al.*, 1991; Akishita *et al.*, 1999), suggesting a key role during early development.

In our studies (Zambidis *et al.*, 2008), we demonstrated a dramatic upregulation of *AGTR2* during expansion of hEB-derived ACE[+] hemangioblasts, which suggests a unique role for the renin-angiotensin axis in guiding the initial developmental phases of human angio-hematopoiesis (Hubert *et al.*, 2006; Heffelfinger, 2007). Indeed, we found that hEB-derived BL-CFC could be directed to differentiate into either hematopoietic or endothelial progeny by manipulating signaling pathways normally mediated by the renin-angiotensin axis. Firstly, enzymatic ACE activity was required for hEB-derived hemangioblast expansion since the specific ACE inhibitor Captopril dramatically blocked BL-CFC formation, suggesting a requirement for Ang II peptide synthesis, and/or downstream AGTR signaling. Moreover, manipulation of angiotensin II signaling with either AGTR1- or AGTR2-specific inhibitors resulted in pronounced deviations of hEB differentiation toward either endothelium, or multipotent hematopoietic progenitors. For example, specific blockade of AGTR1 signaling with Losartan significantly boosted hEB-derived hematopoietic colony-forming cells (CFC) formation, especially the generation of multipotent mixed CFC. To determine if AGTR inhibitors acted either directly on hemangioblast expansion, or via indirect amplification of more committed progenitors, we included these blockers in clonogenic BL-CFC assays. Secondary replating of AGTR1-inhibited (Losartan) blast colonies produced predominately he-matopoietic cell lineages, with limited endothelial differentiation. In contrast, replating of blast colonies treated with an AGTR2-specific inhibitor (PD123–319) resulted in predominantly endothelial progeny, with limited hematopoietic differentiation. Thus, AGTR2 function was necessary for expansion of hemangioblast colonies into multipotent hematopoietic progenitors, and its inhibition with PD123–319 completely abolished hematopoietic differentiation, while imposing an almost exclusive endothelial fate on hemangioblast colonies.

Since AGTR2 signaling is known to antagonize AGTR1 signaling directly (Hubert *et al.*, 2006), our data implicated a general mechanism by which emerging hemangioblasts in the YS or AGM may be directed to differentiate by the hematopoietic stem cell niche. One hypothesis is that antagonistic competition between AGTR2 and AGTR1 for Ang II binding on emerging hemangioblasts directs their development into either hematopoietic progenitors, or alternatively into vascular-endothelial networks. Finally, these studies suggest that manipulation of AGTR2 signaling could be an important strategy, in general, for expanding multipotent hematopoietic progenitors from primitive HSC. We speculated

that AGTR1/ AGTR2-regulated stem cell proliferation may be a generalized phenomenon, since AGTR1 inhibition by Losartan was reported to increase skeletal muscle regeneration in patients with primary muscular dystrophies (Cohn *et al.*, 2007), and the renin-angiotensin axis critically influences normal fetal development, since both ACE and AGTR1 receptor blockers are teratogenic. AGTR2 signaling, in particular, is known to regulate cellular growth and apoptosis during vascular and neural development (Grammatopoulos *et al.*, 2005; Savary *et al.*, 2005). Interestingly, mutations of *AGTR2* have been reported in patients with severe mental retardation and autism (Vervoort *et al.*, 2002), thus further substantiating a role for the renin-angiotensin system in directing the proper development of the nervous system.

Generation of engraftable definitive adult-type HSC may require developmental maturation of mesodermal precursors in specific mesenchymal stromal niches

Although we initially demonstrated that hESC, like mESC, primarily model the developmental steps associated with primitive YS extra-embryonic hematopoiesis, it is not yet clear if hESC differentiation can model the definitive, AGM-like lympho-hematopoiesis that normally arises from the intra-embryonic AGM. EB or stromal-based differentiation protocols described thus far have shown that both mESC and hESC-derived hematopoietic progenitors have a YS molecular and cellular phenotype with limited long-term engrafting potential in adult recipients (Potocnik *et al.*, 1997; Matsuoka *et al.*, 2001; Miyagi *et al.*, 2002), which is a defining feature only of AGM-derived HSC. Since YS progenitors can be conditioned into engrafting, definitive HSC following co-culture on appropriate stromal niches (Matsuoka *et al.*, 2001), these hematopoietic progenitors may be inherently deficient in a "definitive HSC instructive program" that can be provided only by an *in vivo* mesenchymal AGM, fetal liver, or bone marrow niche. We recently demonstrated that hESC-derived CD34^{+} cells committed in culture into hemato-endothelial cell progenitors could be further developed into mature blood and vascular cells following transplantation into chicken embryo yolk sacs (Park *et al.*, 2009). These observations not only demonstrated the utility of the avian embryo as a convenient and reliable host to model the angiohematopoietic development of hESC, but they also underscored the importance of evaluating how *in vivo* niches can mature primitive progenitors into adult hematopoiesis.

Extensive experimental evidence has suggested that mesenchymal stromal niches are regulators of the native adult HSC niche. Bone morphogenetic protein (BMP)-responsive mesenchymal stromal cells (MSC), for example, are found in hematopoietic sites during ontogeny, and are key components of the HSC-supportive micro-environmental niche by activating, or differentiating into supportive cells (*e.g.,* osteoblasts). Osteoblasts, in turn, express Notch ligands (*e.g.* Jagged1) and can increase HSC numbers *via* niche-mediated Notch signaling (Noort *et al.*, 2002; Angelopoulou *et al.*, 2003; Arai *et al.*, 2004; Bensidhoum *et al.*, 2004; Sammons *et al.*, 2004; Koch *et al.*, 2005; Martin and Bhatia, 2005; Mendes *et al.*, 2005; Adams *et al.*, 2006; Francois *et al.*, 2006; Friedman *et al.*, 2006; Muguruma *et al.*, 2006; Suzuki *et al.*, 2006). Notch signal transduction may be critical in determining definitive HSC maturation and expansion, and mediates multiple key cell fate decisions *via* the lateral inhibition of cell fate mechanism, or inductive signaling. NOTCH1 and NOTCH2 are expressed in adult human CD34^{+} HSC, and their ligand JAGGED1 is expressed on bone marrow osteoblasts, suggesting direct involvement in the regulation of HSC by the stromal microenvironment (Calvi *et al.*, 2003; Zhang *et al.*, 2003; Taichman, 2005; Wilson and Trumpp, 2006). Although the Notch pathway plays a major role in lymphopoietic lineage decisions, its role in the genesis and maintenance of HSCs is only beginning to emerge. Embryonic *Notch1*^{−/−} knockout mice have severely impaired

hematopoiesis (Kumano *et al.*, 2003) due to an inability of hemangioblasts in the AGM region to differentiate into definitive HSC. In contrast, mEB-based studies showed that the Notch$^{-/-}$ phenotype does not affect YS hematopoiesis, whereas AGM-type definitive HSC generation is defunct (Hadland *et al.*, 2004; Robert-Moreno *et al.*, 2005), and likely depends on a Notch-Runx1 interaction.

Indeed, Notch-Runx1 signaling may be central to the initial "executive" decision-making process that generates definitive murine HSC at its embryonic AGM origin. There is abundant evidence that Runx1 is a key regulator of definitive HSC emergence in the AGM, where it is indispensable for formation of long-term engrafting CD45$^+$c-kit$^+$ HSC (North *et al.*, 1999; North *et al.*, 2002). Runx1 binds DNA as a heterodimer with CBFβ, and neither *Runx1*$^{-/-}$ nor *CBFβ*$^{-/-}$ mutant embryos can develop definitive HSCs (Okuda *et al.*, 1996; Wang *et al.*, 1996). In contrast, targeted deletion of Runx1 in adult mice leads to hematopoietic abnormalities but preservation of HSC (North *et al.*, 1999; Nakagawa *et al.*, 2006). While other factors associated with hematopoietic emergence, such as the bHLH factor SCL (Robb *et al.*, 1995; Shivdasani *et al.*, 1995), play critical roles in both primitive and definitive hematopoieses, only Runx1 is uniquely required for the emergence of definitive, long-term engrafting HSCs. In human embryos, control of a NOTCH-RUNX1 axis may simply be mediated by regulating the expression of Notch proteins *per se*. NOTCH-1, 2, 4, and ligands DELTA1–4, and JAGGED1, for example, are not expressed in human YS sites, but expression appears later in the fetal liver when fetal definitive HSCs first expand (Dando *et al.*, 2005).

Multiple lines of evidence now support the concept that primitive hematopoietic progenitors, such as those generated from ESC can develop into an adult definitive phenotype with appropriate stromal contact. For example, co-culture of hESC on primary stromal cells derived from murine embryonic day E10.5 AGM, or E12.5 fetal liver stromal cell lines significantly increased definitive CFC activity (Krassowska *et al.*, 2006; Ledran *et al.*, 2008). AGM stromal cell lines, in particular enhanced hematopoietic mesoderm differentiation with subsequently increased hematopoietic progenitor cell formation. Direct cell-cell contact and paracrine signaling with stroma was necessary to mature hESC into definitive hematopoietic cell lineages since conditioned media and extracellular matrix from these stromal cell lines alone abolished or significant reduced definitive CFC activity. These studies suggested a role for TGFβ1 and TGFβ3 in definitive hematopoietic induction. Nonetheless, evidence for generation of a true adult AGM-type definitive HSC has not yet been conclusively demonstrated in these recently published methodologies (Tian *et al.*, 2006; Ledran *et al.*, 2008). Similar to previous reports (Wang *et al.*, 2005a), erythropoiesis generated from engrafted cells in these latter studies was still at an embryonic/fetal stage. Furthermore, primary engraftment in immunodeficient murine recipients remained low, short-term (<3 months), displayed limited lymphopoiesis, and had low subsequent secondary engraftment frequencies. These characteristics of poor lympho-hematopoietic engraftment are consistent with hESC-derived progenitors possessing an embryonic YS, and not adult-type definitive phenotype.

Another study, using preimmune fetal sheep recipients of hEB-derived CD34$^+$CD38$^-$ Lin38$^-$ cells demonstrated low levels of human hematopoietic cell long-term primary and secondary transplant engraftment potential (0.05%–0.2%; evaluated at 10–39 months post injections) (Narayan *et al.*, 2006). Since the bone marrow of these fetal recipients was not conditioned (*e.g.,* with irradiation or busulfan) prior to cell transplantation, however, it is unclear whether the low engraftment frequency observed would be sufficient to expand or ensure their survival postnatally.

To further elucidate the role of mesenchymal stromal niches in generation of definitive HSC, our group has begun related experiments, as those described above. We co-cultured FACS-purified ACE$^+$CD34$^+$-derived hemangioblasts from differentiated day 9–10 hEB onto E12.5–E14.5 murine fetal liver (FL) stroma (Fig. 3B–D), or human MSC (hMSC), and supplemented these cultures with human hematopoietic growth factors. Under these conditions, purified YS-like ACE$^+$CD34$^+$ hemangioblasts differentiated into "cobblestone"-appearing CD34$^+$CD45$^+$CD38$^-$ cells that produced abundant definitive-type CFU (GM-CFC, G-CFC, M-CFC, GEMM-CFC, BFU-e, CFU-e) in methylcellulose assays. These hematopoietic colonies were indistinguishable from cord blood CD34$^+$ cell colony controls (Fig. 3B, C). hEB-derived erythroid CFU-e, for example, were indistinguishable from CB CFU-e in both morphology, and in their expression patterns of adult hemoglobin with low intracytoplasmic embryonic globin (epsilon), and high amounts of neonatal/adult gamma and beta globin expression, in comparison to hEB-derived CFU-e that persist in their expression of embryonic globins (Fig. 3C). More importantly, these stroma-differentiated hEB cells are only capable of short-term (evaluated at 12 weeks), multilineage lympho-erythromyeloid engraftment following injection into the neonatal livers of highly immunodeficient, irradiated NOD/SCID/IL2Rg^{null} (NOG) mice (Fig. 3C). These pilot experiments strongly suggest that a proper hematopoietic microenvironment (*e.g.,* as provided by hMSC osteoblastic or FL-type stroma), can indeed pattern YS-like hEB progenitors into at least short-term (12 weeks) engraftable, adult-type progenitors.

In summary, although unclear at this time, *in vitro* generation of engraftable definitive HSC may require a recreation of their *in vivo* stem cell niche milieu. As illustrated above, a better understanding of the relevant growth factors and signaling molecules that activate definitive HSC-specific genes from primitive progenitors is needed. Experiments that define the critical components of the definitive HSC niche will probably also rely on additional methods for enhancing the homing of these *ex vivo*-generated HSC following transplantation and engraftment into conditioned recipients.

The role of homeobox (HOX)-regulating factors (CDX, MLL) in determining definitive HSC genesis and self-renewal

Although multiple factors such as BMP4, activin A, VEGF, FGF2, Hedgehog, TPO, and Wnt factors are implicated in the generation and downstream self renewal of HSC from mesoderm (Johansson and Wiles, 1995; Bhatia *et al.*, 1999; Faloon *et al.*, 2000; Baron, 2001; Dyer *et al.*, 2001; Lako *et al.*, 2001; Li *et al.*, 2001a; Adelman *et al.*, 2002; Byrd *et al.*, 2002; Murdoch *et al.*, 2002; Perlingeiro *et al.*, 2003; Reya *et al.*, 2003; Willert *et al.*, 2003), the upstream molecular "switch" for patterning hematopoietic mesoderm into HSC remains obscure, yet vital for understanding how to differentiate hESC toward adult-type definitive HSC. Multiple studies have suggested that the regulated control of *HOX* genes may be the effectors for this upstream "definitive HSC-genesis" switch. Thus, in addition to patterning hESC-derived cells into definitive HSC by recapitulating hematopoietic stem cell niches, manipulation of *HOX* gene expression may also serve a role in generating long-term engraftable HSC from pluripotent stem cells.

HOX proteins are a highly-conserved group of transcription factors characterized by a 60-amino acid DNA-binding motif termed the homeodomain. *HOX* genes are organized into four major genomic clusters (A, B, C, D), and play crucial roles in embryonic organogenesis. Clusters A-C are expressed at high levels in normal HSC, and have been widely implicated in HSC self-renewal and regulation. Notably, HOXB4 has been exploited for its ability to expand engraftable human HSC (Antonchuk *et al.*, 2002). Additionally, multiple *HOX* genes participate as leukemic translocation partners, or are dysregulated in

leukemias with normal karyotypes (Antonchuk *et al.*, 2002; Owens and Hawley, 2002; Grier *et al.*, 2005), further implicating their role in HSC self-renewal.

Loss-of-function studies have shown that most *HOX* genes have redundant functions, whereas gene disruption of upstream HOX-regulators such as *MEIS1*, *PBX1*, *MLL*, and *CDX genes* results in severe hematopoietic phenotypes (Yu *et al.*, 1995; Antonchuk *et al.*, 2002; Owens and Hawley, 2002; Davidson *et al.*, 2003; Krosl *et al.*, 2003; Abramovich and Humphries, 2005; Davidson and Zon, 2006), suggesting that these genes directly regulate HSC genesis or expansion. *HOX* regulation by the highly conserved *CAUDAL* family of genes (*CDX1*, *CDX2*, and *CDX4*) (Chawengsaksophak *et al.*, 2004; Shimizu *et al.*, 2006) was recently established. *CDX* genes encode a family of homeodomain transcription factors that regulate anterior-posterior embryonic patterning by orchestrating the specific expression of *HOX* genes during ectodermal and mesodermal development (Epstein *et al.*, 1997; Charite *et al.*, 1998; Reece-Hoyes *et al.*, 2005). The normal embryonic expression pattern of *cdx4*, for example, appears to be tightly regulated in *Xenopus* (Reece-Hoyes *et al.*, 2005), zebrafish (Rawat *et al.*, 2004), and mouse development (Gamer and Wright, 1993; Horn and Ashworth, 1995; Epstein *et al.*, 1997; Charite *et al.*, 1998; Rawat *et al.*, 2004). *Cdx4* is first detected in the posterior embryo during gastrulation and somitogenesis, and its expression domains in lateral plate mesoderm include the hematopoietic-initiating tissues of the allantois and early YS wall cells (which also co-express *scl*).

C*dx2* and *cdx4* function are likely redundant, yet critical for patterning mesoderm to primitive hematopoietic lineages. Zebrafish "kugelig*"* *cdx4*-mutants (Davidson *et al.*, 2003), showed that cdx4 makes posterior mesoderm competent for subsequent hematopoietic commitment (possibly at the hemangioblast stage) by upregulating target *Hox* genes *HoxA6*, *HoxB3*, *HoxB4*, *HoxB8*, and *HoxC6*. Additionally, cdx1a and cdx4 (redundantly) regulate the genesis of downstream runx1a+ definitive HSC derived from the AGM region (Davidson and Zon, 2006), thus implicating a role of *cdx* genes in both primitive and definitive hematopoieses. These zebrafish studies reveal a novel vertebrate cdx-hox axis that may act as the long-sought-after instructive signal for HSC genesis from a hemangioblast. Indeed, Daley's group recently strengthened this hypothesis by generating transplantable, multi-lineage HSC via retroviral co-expression of both *Cdx4* and *Hoxb4* in mEB progenitors (Wang *et al.*, 2005). In contrast*,* dysregulated expression of *Cdx* genes results in various malignancies (Chawengsaksophak *et al.*, 1997; Rawat *et al.*, 2004) over-expression of C*dx2/ Cdx4* is leukemogenic either alone (Bansal *et al.*, 2006), with fusion partners (Rawat *et al.*, 2004), or most potently when co-expressed with *HOX* cofactors (Bansal *et al.*, 2006). Although our group reported *CDX4* expression in primitive hEB-derived hematopoietic progenitors (Zambidis *et al.*, 2005), human *CDX* function remains technically difficult to study, since *CDX* expression is restricted to embryonic development. Interestingly, a recent report described *CDX4* overexpression in human acute leukemias with both normal karyotypes or t(9;11) MLL fusions (Bansal *et al.*, 2006). The mixed lineage leukemia (MLL) proto-oncogene is a better-characterized *HOX*-regulator than *CDX*. MLL is a histone methyltransferase involved in recurrent (11q23) translocations found in ~80% of infant and 10% of adult leukemias (Tkachuk *et al.*, 1992; Corral *et al.*, 1993; Yu *et al.*, 1998; Hess, 2004; Eguchi *et al.*, 2005). These aggressive leukemias often have biphenotypic myeloid-lymphoid markers, and a very poor prognosis (Poirel *et al.*, 1996; Pui *et al.*, 2002). The N-terminal portion of MLL is known to be fused in-frame to over 40 known (apparently disparate) leukemogenic fusion partners that all elaborate a common pathologic motif: aberrant activation of *HOX* genes via direct transcriptional activation (Joh *et al.*, 1996; Dobson *et al.*, 1999; Joh *et al.*, 1999; Ayton and Cleary, 2003; Cozzio *et al.*, 2003; Ferrando *et al.*, 2003; Forster *et al.*, 2003; Johnson *et al.*, 2003; Martin *et al.*, 2003; Rozovskaia *et al.*, 2003; Zeisig *et al.*, 2004; Milne *et al.*, 2005a; Milne *et al.*, 2005b; Chen *et al.*, 2006; Kong *et al.*, 2006; Krivtsov *et al.*, 2006; Meyer *et al.*, 2006; Somervaille and Cleary, 2006). The

normal wildtype *MLL* gene encodes a Trithorax-group chromatin regulator essential in Hox-mediated body specification (Yu *et al.*, 1995), but also specifically necessary for definitive lympho-hematopoietic stem cell genesis (Fidanza *et al.*, 1996; Hess *et al.*, 1997). *Mll$^{-/-}$* mouse embryos and mEB have revealed a crucial role for Mll in definitive HSC development in the AGM (Ernst *et al.*, 2004a; Ernst *et al.*, 2004b). Although primitive hemangioblast development appears intact in differentiating *Mll$^{-/-}$* mEB, downstream definitive-type progenitors from mEB or AGM regions are deficient, and correlate to a paucity of *Hox* gene upregulation. Engraftable AGM-derived HSC, for example, could not be generated from Mll$^{-/-}$ murine embryos, although ectopic expression of Cdx4 in *Mll$^{-/-}$* mEB partially rescues their hematopoietic deficiency, thus implicating a potential Cdx-Mll-Hox axis. A better understanding of a CDX4-MLL-HOX axis in humans may reveal efficient strategies for expanding definitive HSC from primitive hEB-derived progenitors.

Manipulation of *HOX* gene expression to derive engraftable hESC-derived hematopoietic progenitors has already been reported, but with limited success compared to murine ESC systems. Generation of long-term engraftable hematopoietic cells from mouse ESC-derived cells using ectopic *Hoxb4/Cdx4* expression (Kyba *et al.*, 2002; Wang *et al.*, 2005a), for example, raised the possibility that similar studies could result in the derivation of definitive HSC from hESC (Wang *et al.*, 2005b). While *HOXB4* vector-transduced hEB-derived progenitors generated a ~2.5 fold increase in hematopoietic cell expansion, it did not lead to the maturation of hESC-derived hematopoietic cells into long-term engraftable HSC as was, in contrast, shown in mice. This discrepancy between mESC and hESC systems remains unresolved, although it remains possible that other *HOX*-regulating factors such as MLL or CDX are more potent than HOXB4 for maturing hESC-derived cells into definitive HSC. Moreover, there are likely many other undiscovered HOX-related factors that might play important roles for patterning definitive HSC from primitive hemangioblasts, although the search for such factors should be tempered by the concomitant oncogenicity that ectopic HOX factor expression inevitably results in. In fact, expression analysis of other genes important for HSC physiology, behavior, self-renewal, and *in vivo* maintenance has already revealed major differences between hESC-derived and adult hematopoietic progenitors. hESC-derived hematopoietic progenitors, for example, express high levels of cell-cycle proteins, and reduced levels of expression of genes important in stem cell self-renewal. In contrast, adult somatic HSC show upregulation of multiple genes involved in quiescence and HSC renewal such as cyclin G2, p53 binding protein, p21, and p57 (Wang *et al.*, 2005b).

Definitive, long-term engraftable HSC may arise from distinct mesodermal precursors of a different origin than the YS hemangioblast

Although it was originally suggested that the same population of HSC found in the embryonic YS also supplied the fetus and adult (Weissman *et al.*, 1978), it is now generally accepted that the *majority* of adult-type blood-forming stem cells likely arise independently from endothelium-associated cell clusters localized on the ventral aspect of the dorsal aorta and vitelline artery in the AGM (*e.g.,* between days 27–40 for human development; (Tavian *et al.*, 1999a)). These CD34$^+$CD31$^+$CD45$^+$CD38$^-$ endothelium-adherent cells yield long-term hematopoietic, myeloid and lymphoid cells, while their counterparts in the YS only produce erythro-myeloid lineages (Tavian *et al.*, 1996; Tavian *et al.*, 1999b; Tavian *et al.*, 2001).

Nonetheless, the developmental origins of adult HSC that arise from the AGM currently remains elusive. The majority of YS hematopoietic progenitors likely survive only long enough to support organogenesis in the rapidly developing embryo. In contrast, HSC generated within the aortic floor (*e.g.,* via hemogenic endothelium), or from mesenchymal cells within the subaortic patches, or perhaps directly from a non-mesenchymal lateral plate

mesoderm-derived hemangioblastic progenitor (Fig. 1A) likely provide the long-term source of adult hematopoiesis (Jaffredo *et al.*, 2005).

Although murine AGM-derived progenitors show long-term engraftment properties and recapitulation of the entire adult blood system when injected into the bone marrow of lethally-irradiated adult mice (Medvinsky and Dzierzak, 1996; Cumano *et al.*, 2001), it has not been excluded that the primitive hemangioblast (derived from extraembryonic mesoderm, and which dictates ephemeral YS hematopoiesis) could also contribute to the definitive HSC population found in the adult to some extent. Several studies have suggested the possibility that blood-forming stem cell clusters originating in the YS subsequently mature as they transverse through the embryo to the AGM, where they initiate definitive HSC formation. Recent studies, for example, using an inducible *lacZ* cell-labeling system which genetically marked YS-derived Runx1/ Aml1^{+} cells, proposed that adult HSC in murine bone marrow originate from a *combination* of progenitors derived from ancestral extraembryonic mesoderm-derived YS blood islands, *and* lateral-plate mesoderm-derived AGM blood stem cell clusters (Samokhvalov *et al.*, 2007; Ueno and Weissman, 2007). At E7.5 of mouse development, the expression of *Runx1* (which is crucial for hematopoietic emergence) is limited in expression only to cells found in the YS, since vascular cell trafficking connections between the YS and the embryo have not yet been established. Intriguingly, the genetic tracking of these Runx1^{+} cells for up to a year in the adult, demonstrated that these YS-derived cells could contribute to the adult HSC population found in the bone marrow (albeit in limited amounts (~1–10%)). These results indicate that YS blood progenitors can, indeed, play a role in contributing to the pool of adult, long-term reconstituting lympho-hematopoietic stem cells.

In summary, the spatial separation of the extraembryonic mesoderm and lateral plate mesoderm that form primitive and definitive hematopoiesis respectively, raises the possibility that two types of mesoderm may produce qualitatively different hemangioblasts with differing cell lineage potentials, gene expression patterns, and perhaps epigenetic changes (Fig. 1A, B). It remains to be seen whether the hESC-derived YS-like hemangioblasts we have described can be further matured into definitive HSC by means of recreating their natural *in vivo* microenvironments. An alternative approach is to devise hESC differentiation strategies that generate lateral plate/AGM-type mesoderm, rather than YS-type extraembryonic mesoderm. Toward this end, the mouse model has provided numerous insights into methods for improving lateral plate mesoderm differentiation based on a complex signalling network that directs epiblast patterning and primitive streak formation (Tam and Beddington, 1987; Ang and Constam, 2004). In particular, the key regulators of *in vivo* mesodermal induction, which we review below, would have to be recapitulated *in vitro*.

Targeted use of mesodermal morphogens may enhance the efficiency of hemangioblast and HSC generation from human pluripotent stem cells

A better understanding of the signaling pathways and growth factor environments that differentiating hESC are exposed to may guide enhanced mesodermal differentiation, and subsequent hemangioblast-HSC generation. Specifically, a detailed map of mesodermal transcriptional control could lead to the formation of an *in vitro* system that can specifically direct cellular differentiation into either extraembryonic (YS), or lateral plate (AGM) mesoderm, and thus give rise to distinct types of hematopoietic progenitors. For example, the duration, location and concentration of key selected signaling pathways (*e.g.,* NODAL, TGFβ, WNT, and FGF) are thought to play critical roles in the development of distinct mesodermal populations during primitive streak formation (Ben-Haim *et al.*, 2006). Below, we briefly review the known mechanisms of some of these key morphogens, which we

suggest will have to be properly manipulated *in vitro* for proper hESC mesodermal-hemangioblast differentiation.

NODAL establishes the proximal-distal axis and mesoendoderm specification of the embryo

NODAL expression is first detected in the inner cell mass and primitive endoderm at mouse embryonic day E4.5 (Varlet *et al.*, 1997), where it maintains the expression of the pluripotency markers *Pou5f1(Oct4)* and *Nanog* in the epiblast (Mesnard *et al.*, 2006). In addition to a role in pluripotency, NODAL is also required for primitive streak formation (Conlon *et al.*, 1994). Around E5.0, a subset of visceral endoderm at the distal tip of the embryo responds to NODAL signalling by expressing a specific repertoire of genes (Brennan *et al.*, 2001) including the NODAL antagonists, *Lefty1* (Perea-Gomez *et al.*, 1999) and *Cer1*(Belo *et al.*, 1997). The combination of a proximally located source of NODAL and a distally located source of NODAL antagonists creates a gradient of NODAL signalling with highest expression at the extraembryonic/epiblast boundary (Lu *et al.*, 2004). Around E5.5 the *Cer1* and *Lefty1*-expressing cells migrate anteriorly, rotating the area of NODAL inhibition, which results in higher NODAL signalling in the posterior part of the embryo (Kimura *et al.*, 2000; Perea-Gomez *et al.*, 2002; Yamamoto *et al.*, 2004). Loss of NODAL signalling leads to the absence of anterior primitive streak mesoderm derivatives and definitive endoderm (Beck *et al.*, 2002; Yamamoto *et al.*, 2004). In contrast, elevation of NODAL signaling due to transcriptional loss of a co-repressor or expression of a NODAL antagonist, results in the over-expression of mesodermal markers (Meno *et al.*, 1999; Perea-Gomez *et al.*, 2002). In hESCs, a role for NODAL in maintaining pluripotency, and inhibiting neuroectodermal differentiation (Vallier *et al.*, 2004; Smith *et al.*, 2008; Wu *et al.*, 2008) has been described, but its role in mesoendoderm differentiation has not yet been deciphered, but will likely be dependent on the duration and magnitude of NODAL signaling (Vincent *et al.*, 2003).

Activin A, although dispensable for mouse gastrulation (Matzuk, *et al.*, 1995), binds to the same receptors as NODAL, and is thought to mimic some of its activities. In *Xenopus,* exogenous activin A is well-characterized as a mesoderm inducer (Ariizumi *et al.*, 1991). Indeed, activin A has been used to improve hESC differentiation into mesodermal lineages (Schuldiner *et al.*, 2000; Burridge *et al.*, 2007; Yang *et al.*, 2008), although its addition to hESCs also conversely induced endodermal, rather than mesodermal lineages (Levenberg *et al.*, 2003; D'Amour *et al.*, 2005; Hay *et al.*, 2008; Ishii *et al.*, 2008), requiring the suppression of PI3K signaling through insulin/IGF inhibition (McLean *et al.*, 2007). This apparent paradox in activin A activity may likely be explained by dose dependent effect, as seen in *Xenopus* animal caps, where lower concentrations promote mesodermal lineages, and higher concentrations promote endodermal lineages (Okabayashi and Asashima, 2003). As with NODAL, the use of activin A to maintain hESC pluripotency has also been described (Beattie *et al.*, 2005; James *et al.*, 2005; Xiao *et al.*, 2006).

BMP4 signals from the extra embryonic ectoderm induce mesoderm formation

The high levels of NODAL expression in the proximal epiblast at E5.0 also maintain *BMP4* expression in extraembryonic ectoderm adjacent to the epiblast (Brennan *et al.*, 2001; Ben-Haim *et al.*, 2006). BMP4 is required for mesoderm formation and patterning (Winnier *et al.*, 1995) and acts by inducing WNT3 expression in the proximal epiblast (Liu *et al.*, 1999). The distal visceral endoderm that expresses NODAL inhibitors also expresses WNT3 inhibitors such as DKK1, which similarly results in the movement of WNT3 expression towards the posterior epiblast (Glinka *et al.*, 1998; Kemp *et al.*, 2005). It is at this pregastrulation stage that the expression of mesoendodermal markers such as *T* (Wilkinson *et al.*, 1990), *Mixl1*

((Pearce and Evans, 1999), *Eomes* (Ciruna and Rossant, 1999), and *Gsc* (Blum *et al.*, 1992) is detected.

Fibroblast Growth Factors (FGFs) control mesoderm pattering and maintenance

In contrast to NODAL, FGFs control the specification of and maintenance of mesoderm rather than primary mesoderm induction (Burdsal *et al.*, 1998; Ciruna and Rossant, 1999). Mutational analyses of the known *Fgf* genes have demonstrated that only *Fgf4* and *Fgf8* are required for early embryonic development (Niswander and Martin, 1992; Crossley and Martin, 1995; Ciruna and Rossant, 1999; Sun *et al.*, 1999). Although FGF2 has a well established role in maintaining hESC pluripotency, when hESC are differentiated and FGF receptor signalling is inhibited by SU5402, hEBs demonstrate a significant reduction in expansion by day 4 and <1% cell survival by day 8 (Vallier *et al.*, 2005). Additionally, a dominant negative FGFR2 abrogated the formation of the primitive ectoderm layer in mEBs by affecting basement membrane formation, and mEBs became necrotic after 6–8 days (Li *et al.*, 2001b). These results suggest that FGF2 signalling is essential for EB growth and development.

Unpatterned mesoderm expresses KDR/flk-1 (VEGFR2) (Ema *et al.*, 2006), and PDGFRA, the alpha receptor of platelet derived growth factor (Takakura *et al.*, 1997). Lateral plate mesoderm, which forms the hematopoietic lineages, subsequently downregulates PDGFRA, whereas paraxial mesoderm downregulates KDR (Sakurai *et al.*, 2006). In addition, the expression of KDR/flk-1 is maintained in hemangioblasts and HSC (Ziegler *et al.*, 1999). Furthermore, the addition of VEGFA during mESC hematopoietic differentiation has been shown to enhance the effects of BMP4 (Nakayama *et al.*, 2000; Park *et al.*, 2004). Similarly, abundant insulin growth factor 2 (IGF2) expression in gastrulating mouse embryo mesoderm, and an impairment of *in vitro* mesoderm differentiation of *Igf2*−/− mESC, suggested that IGF2 plays an important role in the induction of mesoderm during development. Addition of exogenous IGF2 to *in vitro* cultures of *Igf2*−/− differentiating mESC rescued mesoderm formation and expression of mesodermal markers (Morali *et al.*, 2000)

Our group has already begun to test the effects of these critical morphogens on hEB hematopoietic lineage generation using our novel serum-free hEB differentiation system. We recently described that, in the absence of serum, the minimal combination of VEGFA, BMP4, and FGF2/heparan (VBF2) was necessary and sufficient for efficiently inducing hematopoiesis in differentiating hESC (Zambidis *et al.*, 2008). Further supplementation of mesodermal morphogens such as NODAL, WNT3A, IGF2, or activin A to developing hEB appeared to mimic some of the *in vivo* mesoderm-enhancing effects described above (Fig. 4). In particular, the addition of NODAL to the minimal VBF2 combination led to a significant increase of total hematopoietic CFC, multipotent CFC, and primitive and definitive erythrocytes, in comparison to VBF2 alone. Addition of WNT3A, a downstream effector of NODAL, to VBF2 also modestly increased the same populations of hematopoietic cells with the exception of definitive erythrocytes, suggesting that this factor primarily enhances the formation of primitive YS cells. Likewise, addition of IGF2 to VBF2 augmented only modestly the total formation of hematopoietic CFC, multipotent CFC, and primitive and definitive erythroid CFC, although this combination appeared to greatly enhance myeloid lineage differentiation. Our current efforts are focused on isolating distinct mesodermal progenitors from these optimized morphogen-treated hEB cultures, and assaying their primitive and definitive hematopoietic potential.

Role of microRNAs in pluripotency and directed differentiation of hESC into hemangioblasts and HSC

MicroRNAs (miRNAs) have recently been found to play critical roles in embryonic differentiation and developmental timing of cellular differentiation, including hematopoietic lineage specification. MiRNAs are small, approximately 22 nucleotide-long noncoding RNAs that post-transcriptionally regulate gene expression (Lee *et al.*, 1993; Lagos-Quintana *et al.*, 2001; Lau *et al.*, 2001; Lee and Ambros, 2001; Ruvkun, 2001). MiRNAs are transcribed by POL II as loop-structured pre-miRNAs, processed by nuclear Drosha, exported to the cytoplasm by exportin V, and cleaved into their mature forms by the Dicer/Argonaute complex (Chang and Mendell, 2007). The Dicer complex allows miRNA to recognize target mRNA by base-pairing with sequences in the 3′ untranslated regions of the mRNAs. Complementary base-pairing results in target mRNA degradation, whereas incomplete base-pairing results in target mRNA translational repression by physical inhibition of the mRNA ribosomal protein translational machinery (Kloosterman and Plasterk, 2006). A single miRNA can interact with and affect translation of hundreds of target mRNAs, thus potentially producing dramatic effects on cellular expression and in a tissue-specific manner (Lim *et al.*, 2005; Chang and Mendell, 2007; Landgraf *et al.*, 2007).

MiRNAs have also recently been shown to play a crucial role in nuclear reprogramming (Lin *et al.*, 2008), and normal ESC self-renewal and differentiation (Houbaviy *et al.*, 2003; Suh *et al.*, 2004; Wang *et al.*, 2007). In particular, key pluripotency-associated transcription factors bind to promoters and enhance transcription of miRNA preferentially expressed in ESC while silencing miRNA genes upregulated in differentiating cells (Marson *et al.*, 2008). Moreover, miRNAs are believed to orchestrate the developmental timing of cellular differentiation (Giraldez *et al.*, 2005; Brown *et al.*, 2007; Chang and Mendell, 2007; Martello *et al.*, 2007; Neilson *et al.*, 2007), including hematopoietic lineage specification (Chen *et al.*, 2004; Felli *et al.*, 2005; Costinean *et al.*, 2006; Garzon *et al.*, 2006; Ramkissoon *et al.*, 2006; Bruchova *et al.*, 2007; Choong *et al.*, 2007; Fontana *et al.*, 2007; Georgantas *et al.*, 2007; Thai *et al.*, 2007; Xiao *et al.*, 2007; Zhan *et al.*, 2007). For example, regulation of *HOX* genes is regulated by several miRNAs (Yekta *et al.*, 2004). Aberrant miRNA expression has also been established as an important pathway of tumorigenesis, including in hematopoietic and germ cell malignancies (Eis *et al.*, 2005; Voorhoeve *et al.*, 2006). In mice, several hematopoietic-specific miRNAs have been reported, including mir-223, mir-142, and mir-181 (Chen *et al.*, 2004). Additionally, ectopic expression of mir-155 in adult murine hematopoietic progenitors results in profound effects on lymphopoietic function (Thai *et al.*, 2007). In human CD34^{+} cells, down-regulation of miRNA 221 and 222 occurs during erythropoietic differentiation (Felli *et al.*, 2005).

Several groups have shown that miRNAs may play instructive roles in mesodermal commitment of the embryo by directly controlling *Nodal* expression (Martello *et al.*, 2007), which plays a pivotal role in the induction and patterning of mesoderm, as reviewed above. MiRNA regulation in epithelial to mesenchymal transition, which is key in gastrulation, has also been identified (Gregory *et al.*, 2008). In hESCs, two miRNAs, miR-1 and miR-133, have been demonstrated to promote mesoderm formation by repressing non-muscle genes but have opposing functions in subsequent differentiation to cardiac progenitors (Ivey *et al.*, 2008).

Although miRNAs clearly regulate developmental decisions, the regulatory role of miRNAs during normal human embryonic development of HSC is currently impossible to study, and the hESC differentiation system provides a valuable system for understanding their function. The miRNA signature of human hemangioblasts and HSC has not yet been reported. A clear role for miRNAs in hemangioblast development and differentiation should enlighten the

developmental role of miRNAs in specifying the hematopoietic lineage. Furthermore, the ectopic expression of miRNAs acting as mimics (Voorhoeve *et al.*, 2006) or antagomirs (anti-sense miRNA oligonucleotides) (Krutzfeldt *et al.*, 2005) may augment or efficiently direct differentiation of hESC to mesodermal-hematopoietic lineages.

The utility of hESC-derived hemangioblasts for generating therapeutically-useful hematopoietic lineages

Understanding and mimicking the *in vivo* stem cell niches that generate hemangioblasts during development, as outlined above, may eventually allow powerful methodologies for generating therapeutically useful, patient-specific hemangioblasts from hESC/ iPSC. *In vitro* hemangioblast differentiation may provide unique opportunities for regenerative medicine by generating transplantable endothelial cells, HSC, megakaryocytes, lymphocytes, and transfusable granulocytes and erythrocytes (Olsen *et al.*, 2006). Endothelial progenitor cells (EPCs), in particular, have received increased attention in recent years as the number of adults affected by cardiovascular diseases continues to rise (Rosamond *et al.*, 2008). Researchers and clinicians hope to use EPCs to repair ischemic tissues, enhance blood vessels and heart valves that could replace the need for coronary angioplasty or coronary artery bypass graft surgery, which are currently used to repair damaged circulatory tissue (Niklason, 1999; Liew *et al.*, 2006). The derivation of patient-specific transfusable erythrocytes, lymphocytes, and platelet-forming progenitors (megakaryocytes) from pluripotent stem cells would also have enormous utility. However, the derivation of all these therapeutically important lineages from human pluripotent stem cells currently all face unique technical challenges.

Efficient generation of hESC-derived B- and T-lymphocytes, for example, could play an important role in replenishing the immune system of patients suffering from severe combined immunodeficiency or AIDS. Although they are derived from a common progenitor, B- and T-lymphocytes develop in distinct sites *in vivo* and pose unique challenges in deriving them from hESC. By recapitulating their *in vivo* development within the bone marrow, for example, the growth of $CD45R^+CD19^+$ mature functional B cells from both mESCs and hESCs has been achieved (Cho *et al.*, 1999; Cho and Zuniga-Pflucker, 2003) via co-culture on bone marrow stromal cell lines such as S17, MS5, and OP9 (Collins and Dorshkind, 1987; Landreth and Dorshkind, 1988; Itoh *et al.*, 1989; Cumano *et al.*, 1990). The addition of FLT3L and IL-7 are crucial for this system's efficient production of B lymphocytes, although other cytokines, such as IL-4, have also been shown to augment this process.

Cord blood (CB), fetal liver, bone marrow (BM), and peripheral blood $CD34^+$ cells have all been shown to be capable of generating T cells *in vitro*. Various methodologies, including the use of thymic stroma, have been described toward this end (Hare *et al.*, 1999; Plum *et al.*, 2000; LA MOTTE-MOHS *et al.*, 2007). Clustering of human $CD34^+$ CB cells with murine fetal thymic epithelial cells in reaggregated thymic organ cultures (RTOC) implanted into NOD/SCID mice gave rise to $CD4^+CD8^+$ immature double positive (DP) as well as some functionally mature $CD4^+$ and $CD8^+$ single positive (SP) T-cells (Saito *et al.*, 2002). Murine thymic stromal cells have been used to seed matrices in a tissue-engineering approach to produce T cells from human bone marrow derived hematopoietic progenitors (Poznansky *et al.*, 2000). Thymic stromal culture from rhesus macaques has also been used for coculture with human CD34+ BM derived progenitor cells (Rosenzweig *et al.*, 1996). Fetal thymic organ culture (FTOC), in which human HPC are cocultured with fetal thymic lobes from NOD-SCID mice, is a favored approach which, like all these systems, is useful but produces only a very limited number of T cells (Jenkinson and Anderson, 1994; Tavian *et al.*, 2001).

In vitro production of T-cells from ESC, however, has been more challenging due to difficulties in recreating the unique thymic microenvironment (which plays a central role in the sequential, temporal, and spatial *in vivo* development of these cells) which guides primitive HSC toward T-cell differentiation. The discovery of the key role of Notch-Delta signaling in the thymic stromal environment of the developing T cell led to the advent of the OP9-DL1 ESC co-culture system. The ability of the OP9-DL1 system to expand and maintain progenitor T cells allows it to be uniquely utilized for large-scale generation of human T cells from various sources. OP9-DL1 cells support the generation of DP immature T cells, as well as some CD8$^+$ SP (but not as many CD4$^+$) cells from mouse HSC or differentiating mESC (from KDR/flk-1 populations, which contain murine hemangioblasts). The system has also been used successfully with human CD34$^+$ CB and bone marrow HSC (Schmitt and Zuniga-Pflucker, 2002; Schmitt *et al.*, 2004; La Motte-Mohs *et al.*, 2005). Recent attempts to generate lymphocytes from hESC-derived hematopoietic progenitor cells using both the OP9-DL1 co-culture system as well as FTOC have met with limited success (Martin *et al.*, 2008). Since definitive HSC with lymphoid potential have been detected only from human AGM, and not YS embryonic tissues (Tavian *et al.*, 2001), these results are not entirely surprising. They do, nonetheless, further highlight the necessity for methods that can generate adult-type definitive HSC from hESC before large-scale production of T cells for cell therapy will be possible.

One of the most promising applications of hESC hematopoietic differentiation technology is the efficient large-scale production of red blood cells (RBC) for clinical transfusion (Olivier *et al.*, 2006). hESC-derived erythroid progenitors, which we and others have demonstrated can arise directly from hESC-derived hemangioblasts, may provide a clinically useful and standardized source of transplantable RBC for urgent surgeries, or for treatment of hemoglobinopathies such as sickle cell disease and alpha/beta thalassemias. In general, a simple transfusion requires ~1–2.5×10^{12} red blood cells (500 ml of whole blood at 5×10^9 red blood cells per ml) (Ma *et al.*, 2008b). Since blood donations are often in short supply, a blood bank with hESC-derived erythroid progenitor cells (for example from a pluripotent line expressing the universal blood type O antigen) may provide an unlimited source of transfusable erythrocytes. This goal would require hESC differentiation and subsequent mass production of erythroid cells expressing definitive-type fetal- or adult beta globin-expressing RBC, which has not yet been reported. Murine erythropoietic differentiation systems have thus far proven more successful in the massive production of definitive, transfusable RBC. mESC-derived erythroid progenitors (ES-EPs) with cell morphology, growth and terminal differentiation characteristics similar to those of immature erythroid progenitors found during *in vivo* stress erythropoiesis, were generated efficiently in the presence of Epo, SCF, and glucocorticoids (Carotta *et al.*, 2004). ES-EPs (Ter119$^+$ cKit$^+$CD71$^+$ erythroblasts) could be expanded for over 70 days, and terminally differentiated into enucleated erythrocytes. In a similar study, ES-EPs could ameliorate acute anemia upon injection into affected mice, suggesting that EP from hESC could similarly function in anemic disorders (Hiroyama *et al.*, 2008).

Recent reports have confirmed the feasibility of massively expanding enucleated, human RBC from hESC differentiated in suspension liquid cultures (*e.g.* ~10^9–10^{11} erythrocytes from one six-well starting plate with ~3×10^6 cells hESC) (Lu *et al.*, 2008). In these studies, hEB-derived hemangioblasts were differentiated into mature, enucleated red blood cells with oxygen equilibrium, pH and 1,3-diphosphoglycerate depletion responses analogous to those of transfusable RBC. Although these erythroid cells expressed low levels of adult beta-globin (~16%), and high levels of embryonic/fetal hemoglobins (thus indicating that they are sill at a YS-like developmental phase), this large-scale production could potentially be optimized for establishing and expanding erythroid progenitor lines with long-term proliferation capacity. Although enucleated fetal-type definitive erythroid cells expressing

the universal blood group O were generated in these studies, before such methodologies could be used more practically for transfusions in clinical or medical research, efficient protocols for the derivation of more mature adult type, beta-globin-expressing RBC will need to be developed (as they have been for CB CD34^{+} cells (Neildez-Nguyen *et al.*, 2002; Giarratana *et al.*, 2005; Miharada *et al.*, 2006). The generation of such adult-type RBC from ESC has generally depended on the recapitulation of the *in vivo* microenvironment found in the FL and BM using cytokines, growth factors, and stromal cells. For example, liquid culture of hESC-derived CD34^{+} cells in the presence of IL-3, BMP4, FLT3L, SCF and EPO in a stage-specific manner during hematopoietic differentiation, and subsequent co-culture on MS-5 feeder layers resulted in the formation of megaloblastic, nucleated erythroid cells (Olivier *et al.*, 2006). These cultures initially formed pro- and basophilic erythroblasts expressing embryonic Gower I ($\zeta_2 \epsilon_2$ and Gower II $\alpha_2 \mu_2$. In another system, co-culture of hESC-derived CD34^{+} cells on immortalized fetal liver stroma resulted in similar erythroblast generation with expression of embryonic hemoglobins (Qiu *et al.*, 2008). Extended culture to 35 days produced enucleated erythrocytes with hemoglobin switching to fetal hemoglobins ($\alpha_2 \epsilon_2$), and minimal (~2%) adult β-hemoglobin expression. In contrast, formation of definitive erythroid colonies, bursts, and mixed colonies from hESC was recently described using a mouse fetal liver stromal cell co-culture system (Ma *et al.*, 2008a). CFC assays at various developing timepoints from these co-cultures revealed erythrocytes with rapidly increasing beta-globin expression (from 26% to ~100%), and decreasing epsilon-globin expressions (from 100% to 50%). Comparison of hESC-derived erythroid cells to definitive CB erythroid cells confirmed a similarity in their G6PD and oxygen dissociation curves. These studies collectively demonstrate that transplantable adult-typeRBC expressing adult hemoglobins could eventually be produced on a large scale from human pluripotent stem cells.

Mass production of platelets using hESC derived-megakaryocytes (the platelet producing cell of the human body) could alleviate the need for donors and allow the production of unlimited supplies for treating the thrombocytopenia of chronic chemotherapy, or from blood clotting disorders (*e.g.,* Bernard-Souliér syndrome and Glanzmann thrombasthenia). The OP9 stromal system may be useful for generating hESC-derived, functional megakaryocytes. Several groups have already shown that ESC can readily be induced to differentiate into such megakaryocytic progenitors using TPO and OP9 stroma to give rise to functional cells secreting proplatelets and fibrinogen after exposure to platelet agonists (Era *et al.*, 2000; Eto *et al.*, 2002; Eto *et al.*, 2003; Fujimoto *et al.*, 2003)

Furthermore, mass production of transfusable granulocytes could also supplement host-deficient neutrophil production in congenital leukocyte function deficiencies, or chemotherapy-induced myelosuppression (Saeki *et al.*, 2008). 75% efficient neutrophil production from mESC was first accomplished using a 3-step differentiation protocol. In this system, formation of mEBs (8 days) followed by sequential co-culture of mEBs on OP9 stroma in medium containing a broad cocktail of hematopoietic growth factors, resulted in the production of 10^7 neutrophils from 80,000 mESC (Lieber *et al.*, 2003; Lieber *et al.*, 2004). Differentiation of hESC into neutrophils was similarly accomplished using a 2-step feeder-free hEB-based differentiation method supplemented with hematopoietic growth factors (Saeki *et al.*, 2008), albeit at much lower efficiencies (4×10^6 neutrophils from ~4×10^6 hESC). These neutrophils expressed CD66b, CD16b, and GPI-80, had morphological and cytochemical (myeloperoxidase and neutrophil alkaline phosphatase) characteristics of mature neutrophils. hESC-derived granulocytes also exhibited neutrophil characteristics of chemotactic activity, chemoattractant stimulation, production of reactive oxygen species upon phagocytosis, and *in vivo* IL-1b-enhanced migration

Patient-specific pluripotent stem cells can be generated via various methods for the ultimate production of autologous hemangioblasts

A crucial step that remains to be elucidated before hESC can becomeclinically useful for generating therapeutic hemangioblasts and HSC is the avoidance of the immune-mediated rejection that will result upon transfer of allogeneic, hESC-derived cells into recipients. The best way to circumvent this technical, yet critical caveat is to generate autologous, patient-specific pluripotent stem cells that could subsequently be used for generating HSC. Currently, three methodologies have been proposed to generate patient-specific pluripotent cells: 1) parthenogenesis (PG), 2) somatic cell nuclear transfer (SCNT), and 3) induced pluripotent stem cells (iPSC) (Fig. 5).

Parthenogenesis (PG)

This alternative asexual reproductive mechanism, which occurs in insects and reptiles, can be exploited experimentally to produce a diploid embryo from an unfertilized, artificially-activated oocyte (Cibelli *et al.*, 2002; Cibelli *et al.*, 2006). In mammals, PG-derived embryos arrest at the limb bud stage due to the lack of essential paternal genomic imprinting of critical genes required for proper development (Kono *et al.*, 1996). Although limited in their potential to undergo complete organogenesis, PG-derived pre-implantation embryos at the blastocyst stage represent a valuable source of parthenogenetic ESC (pgESC) for research purposes. These embryos can be used to produce pgESC lines that are substantially (but not completely) genetically identical to the oocyte donor. Experimentally, oocytes can retain diploidy via retention of a polar body using cytokinesis inhibitors and calcium ionophores (Revazova *et al.*, 2007; Revazova *et al.*, 2008), or rarely via spontaneous duplication of the haploid genome during subsequent development. pgESC from mice, monkeys, and humans have been shown to retain characteristics of ESC including the ability to self-renew, to contribute to chimeric animals, and to give rise to all three germ layers, including hematopoietic mesoderm (Kaufman *et al.*, 1983; Cibelli *et al.*, 2002; Vrana *et al.*, 2003; Eckardt *et al.*, 2007; Kim *et al.*, 2007).

Since PG embryos are inherently defective in developing to full term, human PG-derived hESC (pg-hESC) from donated oocytes arguably avoid many of the ethical and political hurdles associated with the derivation of new hESC lines. Because PG embryos are derived from an artificially activated oocyte in the absence of sperm, the resulting embryo and pg-hESC line derived from it are remarkably similar to the oocyte donor, with a few exceptions in distal loci from the centromeres where crossing-over occurred during meiosis I. It has been shown that pg-hESC are as much as 90% identical, including all human leukocyte antigens (HLA) loci, thus representing a source of autologous transplantable cells for female oocyte donors, as well as their offspring. The derivation of pg-hESC with both heterozygous and homozygous HLA alleles has recently been shown (Kim *et al.*, 2007; Lin *et al.*, 2007; Mai *et al.*, 2007; Revazova *et al.*, 2007; Revazova *et al.*, 2008). Thus, autologous transplantable cells from pg-hESC have become are a potential resource for therapeutic strategies for a number of diseases, including blood disorders.

HLA-homozygous genomes generated via PG will result in only three HLA markers for tissue transplantation matching, in contrast to six HLA markers in heterozygous diploid genomes. This has resulted in the proposal for the creation of a universal pg-hESC stem cell that may ultimately serve the transplantation needs of large populations. Computational models have predicted that a small number of such pg-hESC lines could provide a beneficial match for the majority of the population (Taylor *et al.*, 2005). A stem cell bank could include HLA-homozygous pg-hESC lines, but also HLA-homozygous human adult fibroblast cells (for the generation of iPSC; see below) derived from donors previously

tested for a desired haplotype through the National Marrow Donor Program. However, such banks will not be suitable for minority populations such as African-Americans (primarily affected by sickle cell anemia), or other ethnic minorities (*e.g.* affected by beta-thalassemia) with rare haplotypes.

However, the scarce availability of human oocytes, makes PG technically unpractical, although PG embryos could potentially be generated from surplus mature metaphase II oocytes donated in rare numbers as a by-product of *in vitro* fertilization (IVF). Alternatively, pg-hESC could also be derived from excess immature germinal vesicle and metaphase I oocytes, which are not suitable for reproductive technologies such as intracytoplasmic sperm injection or IVF. Frozen, surplus oocytes, which are voluntarily donated by couples routinely for research, could also potentially be utilized (Polak *et al.*, 2007). Overall, other alternative methods will likely ultimately prove more practical than pG for generating autologous hESC for patients.

Somatic cell nuclear transfer (SCNT)

SCNT, which was first described ~sixty years ago by Briggs and King in classic frog developmental studies, demonstrated that somatic cell nuclei transferred to enucleated oocytes can be reprogrammed back into a pluripotent state, indicating that somatic differentiation is a reversible process (Briggs and King, 1952). During the SCNT process, nuclei extracted from adult somatic cells (*e.g.,* fibroblasts) can reactivate pluripotency genes that were previously silenced during cellular specification and differentiation following exposure to oocyte cytoplasmic factors. Subsequent culture of these artificially-activated pseudo-zygotes leads to the formation of a blastocyst, from which an inner cell mass can be isolated to generate ESC that are genetically identical to the donor of the somatic cell nucleus.

SCNT has now been demonstrated in a number of mammals since a cloned sheep ("Dolly") was first reported (Campbell *et al.*, 1996). Although SCNT has a long history, it was only recently demonstrated that SCNT-derived ESC could be generated from nonhuman primates using adult male rhesus macaque fibroblasts as a donor nuclei source (Byrne *et al.*, 2007). Although these studies open avenues for generating patient-specific SCNT-derived hESC for therapeutic cell therapies, a goal that yet remains to be achieved, SCNT inefficiency (<1% in monkeys compared to 16% in parthenogenesis) requires a large number of oocytes for this technique to be clinically applicable. Additionally, SCNT requires costly and complex equipment making therapeutic cloning via SCNT an unlikely source to generate autologous patient-specific hESC in the near future.

Induced pluripotent stem cells (iPSC)

The technical and ethical challenges associated with deriving hESC using SCNT and PG have sparked alternative efforts for generating autologous pluripotent stem cells. One of the most promising approaches has been the emergence of genetically-induced pluripotency. During this process, somatic cells can be reprogrammed to a pluripotent-like state using defined groups of retrovirally-expressed pluripotency-associated transcription factors (*e.g.,* SOX2, OCT-3/4, KLF4, C-MYC, LIN28, and NANOG; SOKMLN), as first demonstrated by Takahashi and Yamanaka using murine embryonic fibroblasts and SOKM (Takahashi and Yamanaka, 2006; Okita *et al.*, 2007; Takahashi *et al.*, 2007a). These iPSC readily form multi-lineage teratomas in NOD/SCID immunodeficient hosts, and contribute efficiently to the development of fertile chimeric mice, thus demonstrating comparability to *bona fide* ESC derived from blastocysts.

This methodology quickly translated to human somatic cells including fibroblasts, MSC, and keratinocytes, resulting in the first human iPSC, albeit at significantly lower frequencies than in murine cells (Takahashi *et al.*, 2007b; Yu *et al.*, 2007; Nakagawa *et al.*, 2008; Park *et al.*, 2008b). Derivation of iPSC lines from patients' diseased somatic cells has recently confirmed the feasibility of generating autologous iPSC lines for transplantation purposes and disease modeling. Several diseased iPSC lines have already been generated, including ones affected with Down syndrome, Parkinson's disease, Huntington's disease, and Shwachman-Bodian-Diamond syndrome (Park *et al.*, 2008a). Diseased iPSC have also been derived from fibroblasts of an 82-year-old woman diagnosed with amyotrophic lateral sclerosis. These iPSC could successfully be differentiated into motor neurons, which are destroyed in ALS (Dimos *et al.*, 2008).

Although the generation of patient-specific iPSC appears to resolve the problem of immune rejection, a major problem in this technology is the serious potential for malignant transformation of these pluripotent lines. This risk is considerable with the current use of retro- and lentiviruses, since such vectors are prone to random insertional mutagenesis. In particular, the use of the proto-oncogenes KLF4 and C-MYC in current iPSC cocktails is problematic, since they have known potentials for malignant transformation. Indeed, chimeric mice made with iPSC that were generated using these pluripotency factors as transgenes eventually formed malignant tumors (Nakagawa *et al.*, 2008). The oncogenicity of iPSC can be reduced by using only OCT4, and either KLF4 or C-MYC (Kim *et al.*, 2008), or alternatively OCT4 and SOX2 transgenes alone, thus avoiding KLF4 and C-MYC altogether (Huangfu *et al.*, 2008b), but insertional mutagenic potential inherent in the use of retroviral constructs will still remain a grave concern for ultimate clinical utility (Hacein-Bey-Abina *et al.*, 2003; McCormack and Rabbitts, 2004).

Recently published studies have already begun to address the caveats of iPSC derived by first-generation methods. Using Cre-LoxP excisable lentiviral and transposon–inserted transgenes, recent studies have demonstrated that persistent viral-integrated transgene expression is not required for reprogramming somatic cells, and that vector-free iPSC can be generated, albeit at lower efficiencies (Soldner *et al.*, 2009; Woltjen *et al.*, 2009). Additionally, generation of human iPSC using nonintegrating constructs was also recently reported (e.g., using recombinant adenoviruses (Stadtfeld *et al.*, 2008), Epstein-Barr virus-based episomal constructs (Yu *et al.*, 2009), SOKM proteins fused with cell penetrating peptides (CPPs) (Kim *et al.*, 2009; Zhou *et al.*, 2009), or repeated transfections of plasmids harboring SOKM cDNAs (Okita *et al.*, 2008)), thus confirming that reprogramming does not rely on genomic integration or insertional mutagenesis. Although this field is now rapidly progressing and holds great potential for ultimately producing clinically safe iPSC, methodologies for further optimizing the currently low reprogramming process will still need to be developed (Huangfu *et al.*, 2008a).

Future Directions

Although the existence of the hemangioblast was first recognized almost a century ago (Sabin, 1920; Murray, 1932), *in vitro* strategies for isolating, characterizing, or generating human hemangioblasts, and adult-type, definitive hematopoietic cell lineages from them, has thus far been elusive. The recent isolation and characterization of the human hemangioblast from hESC has created new potential to further evaluate mechanisms by which hemangioblasts and HSC could be generated, maintained, and expanded. It has become apparent that complex *in vivo* developmental signaling pathways and environments will need to be recapitulated to further mature these hESC-derived primitive progenitors into adult blood cells before they can be practically used in regenerative medicine for a number of hematological diseases.

Moreover, before hESC-derived hemangioblast and HSC can be used for clinical applications, patient-specific pluripotent stem cells will first need to be generated with more efficient, and ethically non-controversial methodologies than the current approaches that PG or SCNT provide. Human iPSC, which solve some of these obstacles, will have to be generated via methods that lack malignant potential, as was recently described using nonintegrating episomal vectors (Okita *et al.*, 2008; Stadtfeld *et al.*, 2008; Yu *et al.*, 2009) or reprogramming proteins fused with CPPs (Kim *et al.*, 2009; Zhou *et al.*, 2009). Moreover, despite their great similarities to hESC, it remains to be seen whether iPSC will hold comparable, or perhaps even greater differentiating potential than hESC for generating hematopoietic lineages (Choi *et al.*, 2009; Loh*etal.*, 2009). Finally, the differentiation of hemangioblasts and HSC from diseased patient-specific iPSC will shed important biologic insight into the developmental pathways and molecular nature of a number of hematologic disorders.

Acknowledgments

Abbreviations used in this paper

hEB	human embryoid body
hESC	human embryonic stem cell
HSC	hematopoietic stem cell
iPSC	induced pluripotent stem cell
PG	parthenogenesis
SCNT	somatic cell nuclear transplant
YS	yolk sac

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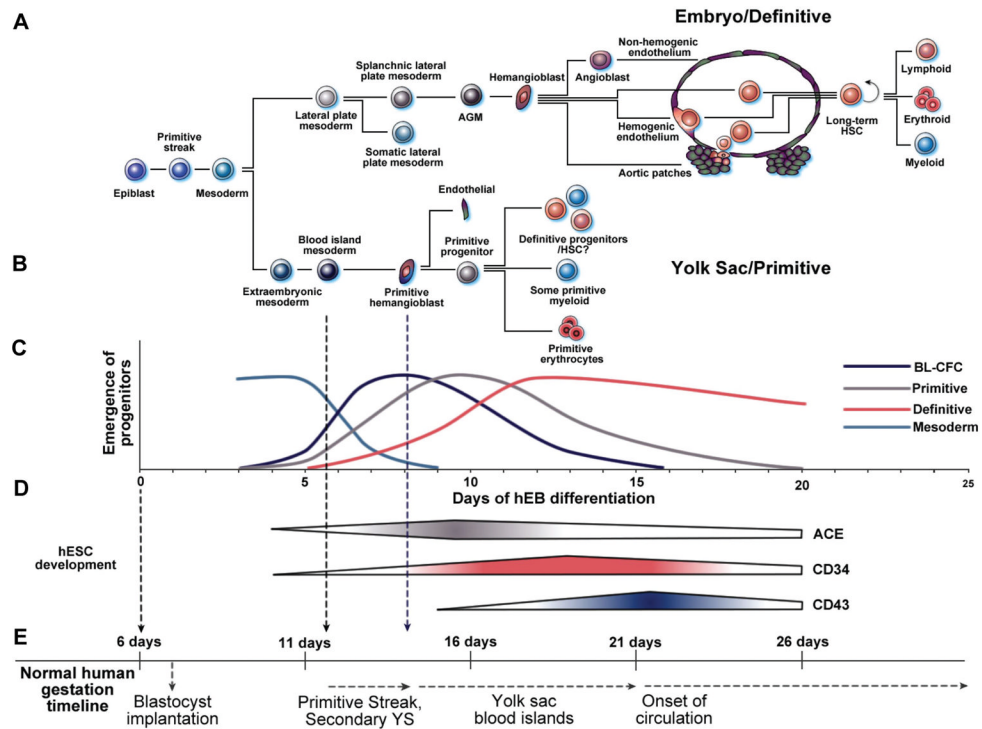
**Fig. 1. Comparative schematic of normal human embryonic hematopoiesis and *in vitro* human embryonic stem cell (hESC) differentiation**

(A) Aorta-gonad-mesonephros (AGM) embryonic definitive hematopoiesis originates from the lateral plate mesoderm, and gives rise to adult definitive-type hematopoietic stem cells (HSC) capable of long-term lympho-hematopoietic engraftment in adult recipients. **(B)** In contrast, primitive yolk sac (YS) hematopoiesis initiates from extra-embryonic mesoderm, and is characterized by the formation of primitive hemangioblasts within YS blood islands that provide ephemeral hematopoiesis for the developing embryo. **(C)** Human embryoid body (hEB) differentiation recapitulates human YS development and gives rise to mesodermal progenitors, hemangioblasts (BL-CFC), and YS-like primitive and definitive blood cells in two sequential waves. **(D)** Angiotensin converting enzyme (ACE; CD143), CD34, and CD43 gene expression are upregulated during hemangioblast development, emerging hematopoietic stem cells, and embryonic hematopoietic cells, respectively (Zambidis *et al.*, 2008). **(E)** Normal human YS development appears to parallel the timeline of our *in vitro* hEB hematopoietic differentiation system. hESC lines are derived from the inner cell mass of a ~day 6 human blastocyst, and are maintained in an undifferentiated state prior to hematopoietic hEB differentiation. During normal gestation, human YS development directly follows formation of the primitive streak, and the extraembryonic secondary YS layers at gestational days 12–13. The formation of primordial YS blood islands first occurs at days 14–16, with subsequent generation of primitive erythro-myelopoietic cell lines starting after 18–20 days. MHE clusters and ACE+ BL-CFC similarly arise in our system at a corrected gestational timeline of ~12–13 days (6d of blastocyst age + days 6–7 of hEB differentiation), which is when the human primitive streak first develops. Subsequently, primitive erythropoiesis arises at a corrected timeline of ~14–16 days (6d of blastocyst age + 8–10 days of hEB differentiation), which is the milestone for YS blood island generation. Finally, definitive erythro-myelopoiesis arises in our system at a corrected timeline of >18–20 days (6d of blastocyst age + 12–20 days of hEB differentiation), when similar events occur in the human YS, prior to the onset of fetal circulation at about 21 days.
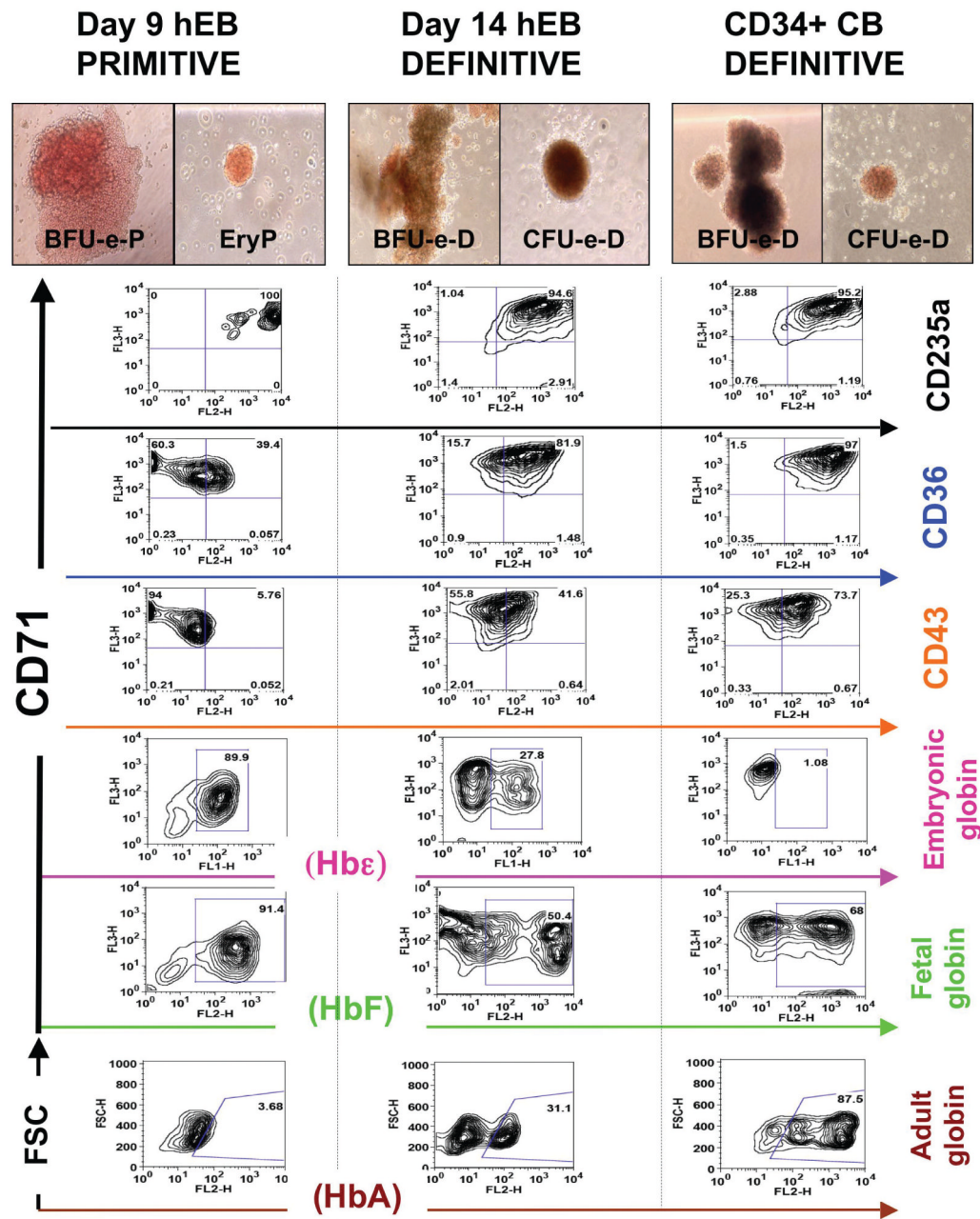
Fig. 2. Recapitulation of normal embryonic erythropoietic development using *in vitro* human embryonic stem cell (hESC) differentiation

Line H1 (WA01) hESC-derived hEB were differentiated into hematopoietic cells using SF culture conditions in the presence of VEGF, BMP4, FGF2, and heparan sulfate (VBF2 as above). Single-cell suspensions from day 9 and day 14 hEBs, as well as CD34+-enriched neonatal cord blood (CB) cells (positive control for adult-type definitive colonies) were re-cultured for colony-forming-cell (CFC) potential in methylcellulose medium. Brownish, "Salmon-red" hemoglobinization was observed on primitive erythroid colonies (EryP, BFU-e-P; left column) generated from day 7 to 12 hEBs, while day 12 to 20 hEBs erythroid colonies (EryD, BFU-e-D; middle column) resembled definitive CD34+ CB blood cells (BFU-e-D, CFU-e-D; right column). Surface marker expression by FACS analysis revealed

that all types of erythoid CFCs had similar surface expression of CD71 and CD235a (GlycophorinA) erythoid progenitor markers, but CD36, an erytho-myeloid lineage marker, was only moderately expressed in EryP/ BFu-e-D, and the embryonic pan-hematopoietic marker CD43 was mostly absent in EryP/ BFU-e-P, and only moderately expressed in BFU-e-D/ CFU-e-D. Hemoglobin detection showed high levels of embryonic Hbε and fetal HbF, but little adult HbA in EryP/ BFU-e-P. In contrast, BFU-e-D/ CFU-e-D express moderate levels of all hemoglobins, while CB erythroid hemoglobin status is limited mostly to adult HbA and HbF. Figure adapted from Zambidis *et al.*, 2008, with permission.
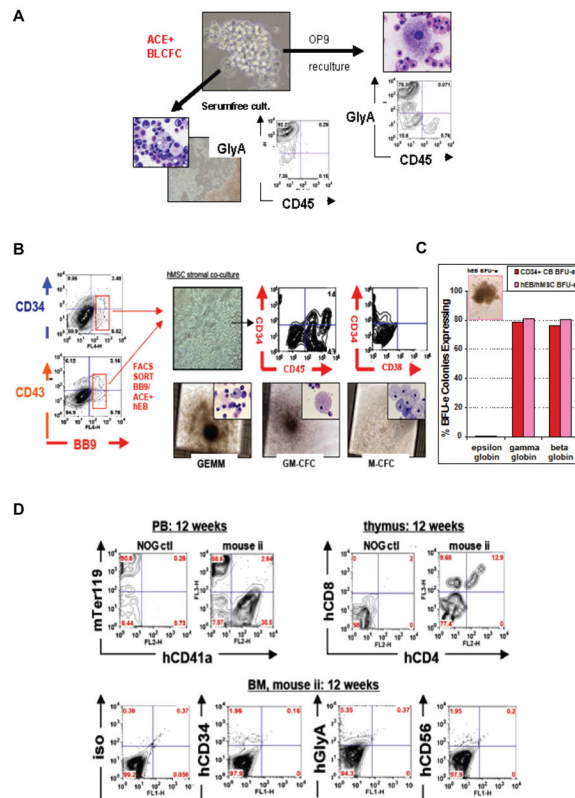
Fig. 3. Generation of short-term engrafting he-matopoietic progenitors from hESC-derived hemangioblasts

(A) Clonogenic human blast colony-forming cell (BL-CFC) assays of hEB-derived ACE$^+$ hemangioblasts. Clonal blast colony formation (BL-CFC assays) from single Line H1 (WA01) hEB cells was tested and optimized in serum-free (SF) methylcellullose medium using growth factors known to expand mouse BL-CFC. Human blast colonies expanded from day 6–10 hEB, and minimally required VEGF, FGF2, and TPO to proliferate. Mature blast colonies differentiated into clusters of multipotent primitive-definitive CD43$^+$CD45$^+$CD41$^+$ progenitors, and contained clonal progenitors for primitive YS-like GlyA$^+$ CD45$^-$ erythroid cells and endothelium following reculture into serumfree and stromal-free culture conditions; or definitive GlyA$^+$ CD45$^+$ erythro-my-eloid cells if alternatively cultured on OP9 bone marrow stromal layers. Figure adapted from Zambidis *et al.*, 2008, with permission. **(B–D)** Generation of engraftable CD34$^+$CD45$^+$CD38$^-$ adult-type definitive-type he-matopoietic progenitors from ACE$^+$ hEB-derived human hemangioblasts following *in vitro* maturation on mesenchymal stro-mal niches, or *in vivo* injection into mouse neonatal livers. (B) Day 9 to 10 FACS-sorted ACE$^+$ CD34$^+$ and ACE$^+$ CD43$^+$ hEB populations (from Line H1 (WA01)) containing hemangioblasts were cultured on human mesenchymal stromal cells (hMSC), and supplemented with human hematopoietic growth factors. This co-culture system differentiated YS-like ACE$^+$CD34$^+$ hEB cells into " cobblestone " - appearing CD34$^+$CD45$^+$CD38$^-$ cells that generated abundant definitive CFU in meth-ylcellulose assays (GEMM-CFC, GM-CFC, and M-CFC shown, as well as G-CFC, BFU-e, CFU-e) that were indistinguishable from CD34$^+$ cord blood (CB) control colonies. **(C)** These hEB-derived erythroid CFU resembled CB BFU-e in morphology and adult hemoglobin expression with low intracyto-plasmic embryonic globin (epsilon), and high amounts of fetal (gamma) and adult globin (beta) expressions. **(D)** Analysis of in vivo maturation of hEB-derived cells following injection of stroma-differentiated hEB into the neonatal liver of highly immunodeficient, irradiated (150 Gy) *NOD/SCID/IL2Rg^{null}(NOG)*

mice (n=4). At 12 weeks, there was evidence for multi-lineage lympho-erythro-myeloid engraftment; peripheral blood (PB) FACS analysis detected high levels of mouse erythroid marker Ter119 and human CD41a (hCD41a), a megakaryocyte marker. Double positive hCD4$^+$CD8$^+$ hematopoietic cells in the thymus demonstrated mature T cell maturation/ engraftment, while low levels of detectable hCD34, hGlycophorin A (CD235a), and hCD56 were found in the bone marrow of transplanted NOG mice.
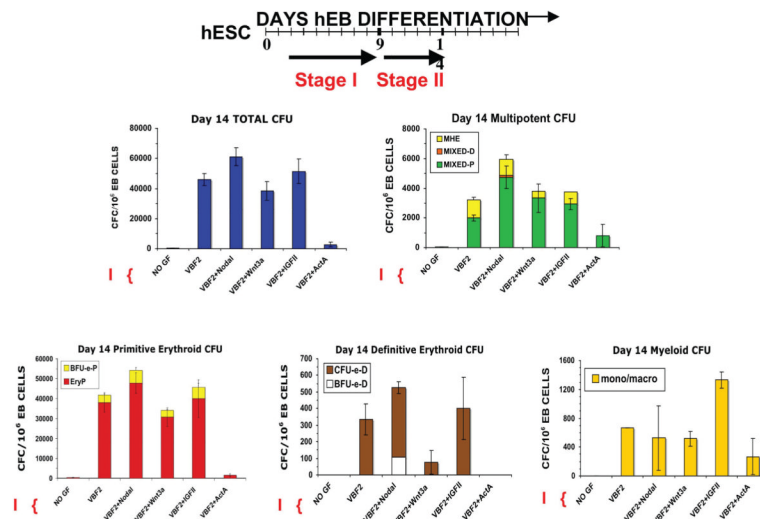
**Fig. 4. Mesodermal morphogens and hematopoietic growth factors mediate agonistic and antagonistic effects on hEB hematopoietic differentiation potential**
Line 1 (WA01) hEB were cultured in non-adherent serum-free (SF) suspension cultures in the presence of absence of VEGF, BMP4, and FGF2 (VBF2) starting on day 2 until day 9 (Stage I). On day 9, hEB were recultured as single cells onto gelatinized tissue culture plates in SF medium and supplemented with 50 ng/ml of the inductive or inhibitory hematopoietic growth factors Nodal, Wnt3a, IGFII, or Activin A (ActA) for 5 days (Stage II). Single cell suspensions of Day 14 hEB were evaluated for Colony-Forming-Cell (CFC) potential in serumfree H4436 SF methylcellulose containing hematopoietic growth factors. Total CFU, mutipotent CFU (MHE, Mixed-P, and Mixed-D), primitive erythroid (BFU-e-P, EryP), definitive erythroid (CFU-e-D, BFu-e-D), and myeloid (macrophage/monocyte) CFU formation was recorded. In comparison to basal VBF2, further addition of Nodal, a mesoderm inducer, enhanced the generation of all CFC except monocyte/ macrophage CFU. Wnt3a, a downstream effector of Nodal, acted as a moderate agonist during mutlipotent CFU formation, but drastically reduced definitive erythropoiesis. IGFII, another key player in mesodermal differentiation, moderately augmented multipotent CFU and primitive and definitive erythropoiesis. On the other hand, Activin A (ActA) dramacially abolished hematopoietic CFU.
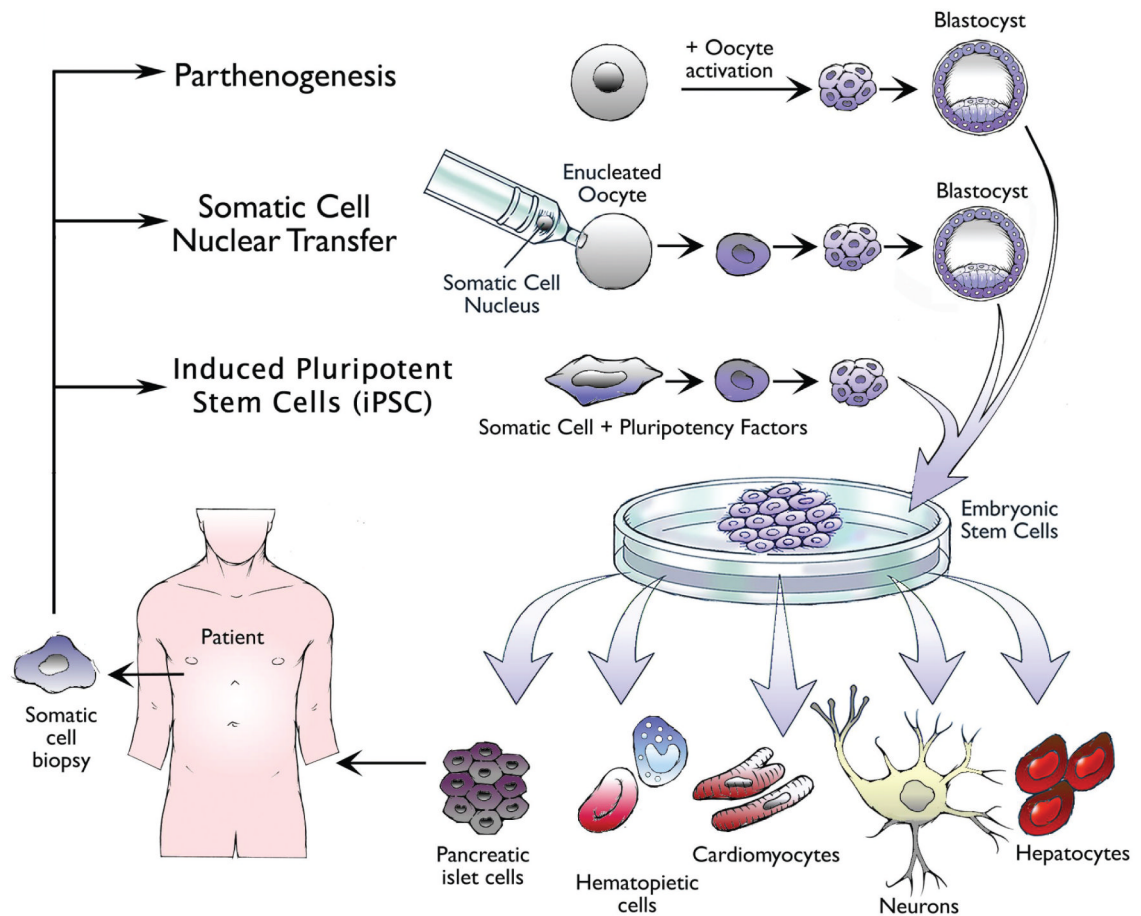
Fig. 5. Methods for generating autologous, patient-specific pluripotent stem cells for therapeutic cell transplantation

Parthenogenesis (PG), somatic cell nuclear transfer (SCNT), and induced pluripotent stem cells (iPSC) have been proposed as the three technologies by which patient-specific pluripotent stem cells could be generated for the generation of transplantable patient-specific progenitors, including hemangioblasts. The process of PG requires chemical activation of a patient's isolated oocyte to proceed to the blastocyst stage, at which point embryonic stem cells can be isolated for subsequent therapeutic generation of somatic cells. Comparatively, SCNT also requires the use of an oocyte, but in an enucleated state. Transfer of a patient's somatic cell nucleus into the cytoplasm of the nucleus, resets the engineered cell to a pluripotent state. Induced pluripotency reprograms a somatic cell into a pluripotent state in the absence of an oocyte or a blastocyst intermediate by genetically transmitting defined pluripotency factors that can reprogram somatic cells to an embryonic stem cell-like state. Pluripotent stem cells generated by any of these methods can be differentiated into all cell types of the body. Progenitor cells from various lineages (e.g. blood, pancreas, heart, neurons, and liver) generated from patient-specific pluripotent stem cells can be transplanted back into the donor as autologous cell grafts without fear of immune rejection.