

## Hematopoietic Specification from Human Pluripotent Stem Cells: Current Advances and Challenges Toward de novo Generation of Hematopoietic Stem Cells

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## **Abstract**

Significant advances in cellular reprogramming technologies and hematopoietic differentiation from human pluripotent stem cells (hPSCs) have already enabled the routine production of multiple lineages of blood cells *in vitro* and opened novel opportunities to study hematopoietic development, model genetic blood diseases and manufacture immunologically matched cells for transfusion and cancer immunotherapy. However, the generation of hematopoietic cells with robust and sustained multilineage engraftment has not been achieved. Here, we highlight the recent advances in understanding the molecular and cellular pathways leading to blood development from hPSCs and discuss potential approaches that can be taken to facilitate the development of technologies for de novo production of HSCs.

## Introduction

Pluripotent stem cells (PSCs) are defined as cells capable of self-renewal and differentiation into derivatives of all three germ layers. The first successful derivation of human PSCs (hPSCs), embryonic stem cells (hESCs), by James Thomson in 1998<sup>1</sup> dramatically elevated the interest in PSC biology because many viewed hESCs as a novel unlimited source of human cells for cell replacement therapies, drug screening and developmental studies. In 2006, advances in understanding of the core transcriptional regulatory circuitry in mouse and human ESCs led to another crucial discovery by Shinya Yamanaka,<sup>2</sup> who identified the set of reprogramming factors capable of inducing ESC-like cells (induced PSCs; iPSCs) from mouse somatic fibroblasts. One year later iPSCs were obtained from human somatic cells.<sup>3-5</sup> Human iPSCs (hiPSCs) offer a novel tool to study and treat diseases because they capture the entire genome of a particular patient and provide an inexhaustible supply of immunologically compatible cells for experimentation and transplantation. Although initially iPSCs were generated from fibroblasts using retroviral vectors, multiple strategies for generating transgene-free iPSCs from fibroblasts and other cell types, including blood, have been developed within a short period of time (reviewed in <sup>6,7</sup>). With the iPSC field progressing very rapidly, the next challenge will be to demonstrate the functional utility of iPSC-derived cells in preclinical models of various human diseases and eventually move this technology into the clinic.

Hematopoietic stem cell (HSC) transplantation has become the standard of care for otherwise incurable blood cancers and deadly genetic diseases. The expansion of HSC donor registries, along with the development of alternative sources for HSC

transplantation, including cord blood and haploidentical donors, and the use of novel conditioning regimens have significantly improved access to transplantation for patients with hematologic diseases.<sup>8,9</sup> However, transplant engraftment failure, graft-versus-host disease (GVHD) and delayed reconstitution still remain significant causes of morbidity and mortality following bone marrow transplantation<sup>8,9</sup> leaving about 50% of patients with a permanent disability or without a cure.<sup>10</sup> Because iPSCs can be expanded indefinitely *ex vivo* and potentially differentiated into hematopoietic cells with blood-reconstituting capability,<sup>11,12</sup> they open a unique opportunity to improve the outcomes of bone marrow transplantation by providing a supply of unlimited number of immunologically matched HSCs.<sup>13,14</sup> Patients with monogenic hematologic and immune diseases would benefit the most from a iPSC-based bone marrow transplantation procedure. Currently, a lack of methodology for efficient expansion and genetic modifications of somatic HSCs and the risk of insertional mutagenesis with viral vectors remain the major limitations for HSC-based gene therapy.<sup>15</sup> As shown in Figure 1, autologous iPSC lines can be generated from patients with genetic defects, precisely corrected with the wild type gene by homologous recombination and then used to produce healthy hematopoietic cells for transplantation without the risk of GVHD. The successful treatment of sickle cell anemia in a mouse model using gene-corrected iPSCs provided proof-of-principle that the clinical application of iPSCs to treat genetic blood diseases is feasible.<sup>16</sup> In the setting of leukemia, iPSCs can be used to produce immunologically matched HSCs as well as T cells targeted to leukemia antigens and antigen-loaded dendritic cells to induce an anti-leukemia immune response.<sup>17,18</sup> In addition, autologous pan-myeloid progenitors can be generated from iPSCs<sup>19</sup> for the management of cytopenias in patients with delayed engraftment.

In recent years, major progress has been made in developing systems for hematopoietic differentiation and producing major types of blood cells from hPSCs (reviewed in <sup>14</sup>). However, the generation of hematopoietic cells with robust long-term reconstitution potential from hPSCs remains a significant challenge. The identification of sequential progenitors and molecular mechanisms leading to formation of various blood lineages from hPSCs is critical in overcoming this limitation. In this review, we focus on recent progress made in understanding cellular and molecular pathways leading to hematopoietic specification from hPSCs and discuss key approaches that could be undertaken to induce the formation of engraftable blood cells from hPSCs.

## **Translating Embryonic Hematopoiesis to PSC Differentiation**

### *Mesodermal development and HSC specification in the embryo*

The knowledge gained from studies of embryogenesis and mouse ESC differentiation provided major insights into key pathways that regulate the sequential commitment of PSCs to blood cells and laid the foundation for the development of hematopoietic differentiation protocols for hPSCs. During embryogenesis, gastrulation is the first critical step in specification of pluripotent embryonic cells into blood. The beginning of gastrulation is marked by formation of primitive streak (PS). Epiblast cells ingress into the PS to give rise to the mesoderm and definitive endoderm.<sup>20</sup> Although the entire PS expresses the *T* gene (also known as *Brachyury*),<sup>21</sup> the subset of cells with hematoendothelial potential within the PS can be identified by the expression of KDR (Flk1 or VEGFR2).<sup>22,23</sup> KDR<sup>+</sup> cells migrate into the yolk sac, where they form vascular plexus and

blood islands. Nodal, BMP4, WNT3, and FGF2 are the most critical factors required for PS and mesoderm induction.<sup>24-27</sup> Importantly, FGF2 upregulates expression of KDR on mesodermal precursors and makes them sensitive to VEGF.<sup>28</sup> At this stage, the interaction of VEGF-A produced by the visceral endoderm with KDR becomes essential for the normal development of endothelial and blood lineages.<sup>23,29</sup> Manipulation of these pathways in mouse ESC cultures helped to optimize mesodermal differentiation and provided additional knowledge regarding molecular regulation of hematopoietic mesoderm (reviewed in <sup>30,31</sup>).

In the mouse embryo, the first blood progenitors are formed in the yolk sac where they can be identified using hematopoietic colony-forming assays as early as embryonic day 7.25 (E7.25). The yolk sac initially generates primitive hematopoietic cells, including nucleated red blood cells expressing embryonic hemoglobin, macrophages, and megakaryocytes.<sup>32,33</sup> The second wave of yolk sac hematopoiesis, termed definitive erythromyeloid hematopoiesis, emerges when distinct blood islands can be recognized morphologically at E8.25.<sup>32,34</sup> This wave is associated with an expansion of erythroid precursors expressing adult  $\beta$ -globins and uni- and multilineage myeloid precursors.<sup>32</sup> The first HSCs capable of reconstituting the entire hematopoietic system of wild type adult animals are observed in the aorta-gonado-mesonephros (AGM) region, vitelline and umbilical arteries and placenta by E10.5 -11.<sup>35-40</sup> At E11.5, it is estimated that the AGM region contains approximately one HSC.<sup>41</sup> Emerging HSCs undergo expansion in the fetal liver and subsequently migrate to bone marrow which becomes the predominant site of hematopoiesis in postnatal life.<sup>42</sup>

*Hemogenic specification: Concept of hemangioblast and hemogenic endothelium*

During embryogenesis endothelial and hematopoietic cells develop in parallel. The close spatial and temporal relationships between these two lineages was first noted by early embryologists in the late 19<sup>th</sup> century.<sup>43</sup> In 1917, Florence Sabin postulated the existence of a common precursor for blood and endothelial cells based on her observation of blood development within the yolk sac of chicken embryos.<sup>44</sup> This common precursor was later named hemangioblast by Murray, who defined it as aggregates of yolk sac mesenchyme (mesoderm) from which endothelial and hematopoietic cells develop.<sup>45</sup> Although the term hemangioblast initially designated the mesodermal precursor, the modern literature applies it very broadly to describe any type of cell that can produce both endothelial and blood cells after culture in specific conditions *in vitro*. It became clear however, that cells with hemangioblastic activity represent a very heterogeneous group of progenitors, which include cells at the mesodermal stage of development in the yolk sac and cells with typical endothelial characteristics in the AGM region.<sup>46-48</sup>

Within the AGM, hematopoietic cells were found to bud off from the endothelium lining the wall of the aorta.<sup>49</sup> Recent studies in mice have provided direct evidence that this process represents the formation of definitive blood cells and HSCs from a unique population of endothelial cells on the ventral wall of the dorsal aorta defined as hemogenic endothelium (HE), through an endothelial-hematopoietic transition.<sup>50-52</sup> Dynamic tracing and imaging studies conducted *in vivo* have demonstrated that endothelial-hematopoietic transition represents a continuous process in which endothelial cells gradually acquire hematopoietic morphology and phenotype.<sup>50,53,54</sup> Although the concept of HE was initially developed

based on AGM studies, it became clear that endothelium in other embryonic and extraembryonic sites possess hemogenic potential. Among these sites are the vitelline and umbilical arteries,<sup>55</sup> placenta,<sup>38</sup> head vasculature,<sup>56</sup> endocardium,<sup>57</sup> and nascent yolk sac capillaries.<sup>58</sup>

### **Hematopoietic Development from Human PSCs**

In general, the approaches for hESC differentiation into blood cells are similar to those employed for mouse ESCs. The first successful differentiation of hESCs was achieved by Kaufman et al. using coculture with the S17 mouse stromal cell line.<sup>59</sup> Since then, several embryoid body formation and 2D culture protocols, including serum- and feeder-free, have been developed for hematopoietic differentiation of hESCs.<sup>60-66</sup> After successful reprogramming of human somatic cells to pluripotency, hESC protocols have also been applied to differentiate hiPSCs. It has been shown that the patterns of hematopoietic differentiation from hESCs and iPSCs are very similar.<sup>66-69</sup> Many factors that are important for hematopoietic specification of mouse PSCs appear to play critical roles in induction of mesoderm and hematopoietic commitment in the hPSCs as well. As expected from murine studies, BMP4, WNT, FGF2, and VEGF have been shown to promote hematopoiesis from hESCs (Figure 2A).<sup>60,62,65,70-74</sup> Hematopoietic cytokines are important components of hPSC differentiation systems and are required for amplification of emerging hematopoietic CFCs and specification toward lymphoid lineages. The protocols for hematopoietic differentiation of hPSCs are extensively reviewed by Kardel and Eaves.<sup>75</sup> Overall, hESC studies have revealed many similarities in hematopoietic differentiation with mouse ESCs, including requirements for intrinsic and extrinsic signaling and hierarchical organization of



hematopoietic precursors. However, several differences have also been noted and will be discussed below.

### *Mesoderm induction from hPSCs*

Understanding the mechanisms regulating induction and specification of mesoderm to hematovascular progenitors is essential in tracing the development of pre-HSCs and in defining factors required for their specification. Similar to mouse ESCs, the early stages of mesodermal development from hESCs can be monitored by expression of KDR (FLK1) and PDGFR $\alpha$ .<sup>62,70,71,76,77</sup> However, in contrast to mouse ESCs, low levels of KDR can be detected in undifferentiated hESCs.<sup>77-79</sup> The successful targeting of GFP reporter to the locus of *MIXL1*, a gene transiently expressed in the PS, enabled the more accurate identification and isolation of the mesodermal populations at the PS stage in hESC cultures.<sup>76</sup> Molecular profiling of *MIXL1-GFP* cells or wild type hESCs at early stages of differentiation revealed apelin receptor (APLNR) as a novel marker of the PS population in hPSC cultures.<sup>77,80</sup> APLNR was found to be expressed in cells with features of posterior mesoderm and anterior mesendoderm.<sup>77,80</sup> In contrast to KDR, undifferentiated hESCs do not express APLNR and because APLNR expression is homogenous, positive and negative populations can be clearly separated by flow cytometry.

Using the *MIXL1-GFP* cell line, Davis et al.,<sup>76</sup> demonstrated the essential role of BMP4 in induction of PS mesoderm from hESCs. The formation of mesoderm is also dependent on Activin A and FGF signaling.<sup>81-83</sup> Inhibition of these pathways using small molecules completely blocks mesoderm development from hESCs.<sup>70</sup> Several studies have documented

the important role of canonical WNT signaling in blood development from hESC,<sup>71,72</sup> which can be at least partially attributed to the enhancement of mesoderm formation following WNT signaling activation. Canonical WNT/ $\beta$ -catenin signaling is required for establishing nascent PS/mesendoderm from hESCs, while specification of nascent PS to anterior and posterior PS is regulated by the balance between BMP and Activin/Nodal signaling.<sup>84</sup> Furthermore, activation of canonical WNT signaling promotes the development of more mature hematovascular mesoderm expressing a high level of KDR (KDR<sup>bright</sup> mesoderm) and hematoendothelial progenitors from hESCs.<sup>71,72</sup> Vijayaragavan et al. reported that mesoderm formation from hESCs is also affected by non-canonical WNT signaling.<sup>85</sup>

#### *Hemangioblastic potential of hPSC-derived mesodermal precursors*

Similar to mouse ESCs, the onset of hematopoiesis in hESC and hiPSC cultures is marked by the emergence of blast CFCs (BL-CFCs).<sup>62,77,80,86</sup> Because BL-CFCs consist of vascular and hematopoietic progenitors they are commonly referred as hemangioblasts. BL-CFCs are detected at the mesodermal stage of development, prior to the appearance of hematopoietic progenitors that form colonies in response to hematopoietic cytokines. Development of BL colonies from mesodermal cells requires FGF2 and VEGF but not hematopoietic cytokines.<sup>62,77</sup> In defined serum-free clonogenic medium, FGF2 alone is sufficient to induce BL colonies from APLNR<sup>+</sup> mesodermal cells.<sup>77,86</sup> The formation of BL-CFCs is also promoted by the addition of apelin peptides to differentiation cultures or clonogenic medium.<sup>80,86</sup> Similar to findings in mouse systems,<sup>87</sup> human hemangioblasts (BL-CFCs) generate hematopoietic colonies through endothelial intermediates.<sup>77,86</sup> Using time-lapse studies, we demonstrated that development of BL colonies in clonogenic

cultures proceed through a core stage at which highly motile mesodermal cells undergo several divisions, upregulate expression of *KDR* and other endothelial genes (including *CDH5*, *PECAM* and *ESAM*), and form immotile tight aggregates composed of approximately 30 VE-cadherin<sup>+</sup> epithelioid cells (cores). The core stage of differentiation is readily identifiable following 3 days of culture of mesodermal cells in semisolid clonogenic medium. Subsequently, VE-cadherin<sup>+</sup> cells undergo endothelial-hematopoietic transition leading to the formation of CD235a<sup>+/−</sup>/CD41<sup>+/−</sup> cells with erythroblast morphology. The hematopoietic potential of BL-CFCs is mostly restricted to primitive erythroid and megakaryocytic cells, and macrophages.<sup>62,86</sup> Interestingly, the development of BL colonies in culture closely recapitulates events leading to blood formation *in vivo*. In chicken embryo, FGF produced by endodermal cells induces the aggregation of migrating PS cells adjacent to the endoderm, upregulation of *KDR* and formation of angioblasts and hemangioblasts.<sup>28,88</sup> In differentiating hPSC cultures (Figure 2A), the BL-CFCs with hemangioblastic activity are highly enriched within the KDR<sup>+</sup> and APLNR<sup>+</sup>PDGFR $\alpha$ <sup>+</sup> nascent mesodermal population expressing *MIXL1* and other PS genes.<sup>62,77,80,86</sup> However, the proportion of BL-CFCs within isolated KDR<sup>+</sup> or APLNR<sup>+</sup> cells remains low at 1.5-4%.<sup>62,77,80</sup>

Step-wise specification toward hematopoietic and endothelial lineages in mouse ESC cultures proceeds through conversion of KDR<sup>+</sup>PDGFR $\alpha$ <sup>+</sup> primitive mesodermal cells into KDR<sup>+</sup>PDGFR $\alpha$ <sup>−</sup> cells with properties of lateral plate mesoderm.<sup>89</sup> To define the mesodermal subsets of differentiating hPSCs, we analyzed the kinetics of expression of APLNR, PDGFR $\alpha$ , and *KDR* mesodermal markers in hPSCs differentiated on OP9 bone marrow stromal cell line.<sup>77</sup> Because these markers could also be found on differentiated cells at

postmesodermal stages, we demarcated mesodermal stage of development as  $^{EMH}lin^-$ , i.e. the stage at which cells lack the expression of endothelial (CD31, VE-cadherin), endothelial/mesenchymal (CD73, CD105) and hematopoietic (CD43, CD45) lineage markers.<sup>86,90</sup> Based on these analyses we identified two distinct phases of mesodermal development.  $^{EMH}lin^-APLNR^+PDGFR\alpha^+$  the most primitive mesodermal cells emerge at day 2-3 of differentiation in OP9 coculture (Figure 2). These cells are reminiscent of the primitive posterior mesoderm (PM) in the embryo and express genes associated with PS (*T*, *MIXL1*, *EOMES*) and lateral plate/extraembryonic mesoderm (*FOXF1*, *WNT5a*, *BMP4*). As discussed above, the day 3  $^{EMH}lin^-APLNR^+PDGFR\alpha^+$  cells have the potential to form BL colonies in response to FGF2.<sup>77,86</sup> The next step of more advanced mesodermal commitment is associated with emergence of hematovascular mesodermal precursors (HVMPs), which can be detected based on high expression of KDR and low to no expression of PDGFR $\alpha$  in  $^{EMH}lin^-APLNR^+$  cells, i.e.  $^{EMH}lin^-KDR^{bright}APLNR^+PDGFR\alpha^{low/-}$  phenotype. Similar to day 3 PM, HVMPs express lateral plate/extraembryonic mesoderm genes. However, they upregulate expression of *TAL1*, *HHEX*, *LMO2*, *GATA2* and *ETV2* genes associated with angiohematopoietic development, and down regulate expression of PS genes (Figure 2). HVMPs lack BL-CFC potential but are highly enriched in bipotential cells that can form hematoendothelial clusters when cocultured on OP9 and produce the entire spectrum of myeloid progenitors.<sup>86</sup> It has been shown that  $KDR^+PDGFR\alpha^{low}$  mesodermal population with angiohematopoietic potential is also present in hESC cultures differentiated using the embryoid body method, and that generation of such cells is significantly elevated by the addition BMP4 and WNT3a.<sup>71</sup> Collectively, these results indicate that at least two distinct mesodermal subsets are formed during hESC specification

to endothelial and hematopoietic lineages. Both of these populations (PM and HVMPs) have endothelial and hematopoietic potentials when cultured *in vitro*, i.e. possess hemangioblastic activity. Primitive hematopoietic potential can be detected within immature PM cells, while more mature HVMPs generate blood cells with definitive features.

*Formation of hemogenic endothelium in hPSC cultures.*

The evidence that HSCs originate from HE with definitive hematopoietic potential underscores the need to identify HE progenitors in hPSC cultures.<sup>91</sup> In embryos, HE can be reliably identified based on morphology and anatomical location.<sup>49</sup> However, identification of HE in hESC differentiation cultures faces significant challenges because of the considerable overlap in expression of surface markers by endothelial and hematopoietic cells. One of the most specific markers for endothelial cells, VE-cadherin,<sup>92-94</sup> is commonly used to isolate endothelial cells from mouse embryo and ESC cultures.<sup>95-97</sup> In mouse systems, VE-cadherin staining is typically combined with blood cell specific antibodies CD41a (early marker of embryonic hematopoietic progenitors) and CD45 (pan-hematopoietic marker) to separate endothelial and hematopoietic phases of development and identify a transient population of HE emerging around the onset of blood cell generation.<sup>87,97,98</sup> Initially, Wang et al.<sup>99</sup> demonstrated that hESC-derived VE-cadherin<sup>+</sup> cells expressing CD31 (PECAM) and KDR (FLK1) but lacking CD45 have hematopoietic and endothelial potentials. Recently major progress has been made in further dissection of this population to enrich towards HE activity and separate HE more precisely from already established blood cells and non-HE (see Supplementary Table S1). Identification of CD43 as a marker that covers the entire population of hematopoietic cells,

including CD45<sup>-</sup> CFCs, in hPSC cultures<sup>67,100</sup> made it possible to accurately segregate endothelial stage from hematopoietic stage. CD31<sup>+</sup>CD43<sup>-</sup> or CD34<sup>+</sup>CD43<sup>-</sup> cells isolated from differentiated hESC entirely lacked of hematopoietic CFC potential but had the capacity to generate blood and endothelial cells after culture with OP9 stromal cells, i.e. displayed HE properties.<sup>100,101</sup> Recently, Rafii et al.<sup>102</sup> engineered hESCs to express fluorescent reporters under the VE-cadherin and CD41a promoter (Pr). This system makes it possible to directly observe the formation of round CD41aPr<sup>+</sup> blood cells from VE-cadherinPr<sup>+</sup>CD41aPr<sup>-</sup> epithelioid precursors. Round cells developed from VE-cadherinPr<sup>+</sup> cells expressed CD43 and possessed broad hematopoietic CFC potential. Although CD43 enables the precise separation of VE-cadherin<sup>+</sup> blood cells from endothelium, further narrowing down the HE phenotype requires the identification of markers that distinguish HE from non-HE. By analyzing the kinetics of VE-cadherin and other endothelial marker expression in hPSC coculture with OP9 we identified two distinct populations of cells within VE-cadherin<sup>+</sup>CD43<sup>-</sup> endothelial cells based on expression of CD73 (5'-nucleotidase; Figure 2B).<sup>86</sup> Both VE-cadherin<sup>+</sup>CD43<sup>-</sup>CD73<sup>+</sup> and CD73<sup>-</sup> populations expressed KDR, CD31, ESAM, CD34 and other typical endothelial molecules and were capable of generating endothelial cells but lacked hematopoietic colony-forming activity (formation of blood colonies in semisolid medium supplemented with hematopoietic cytokines). However, only VE-cadherin<sup>+</sup>CD43<sup>-</sup>CD73<sup>-</sup> cells displayed HE properties, i.e. the ability to generate blood cells after secondary coculture with OP9. In contrast to non-HE, HE expressed high levels of *RUNX1*, which is known to mark HE in the embryo,<sup>103</sup> and were capable of generating the entire spectrum of myeloid progenitors, including erythroid cells expressing adult  $\beta$ -hemoglobin. The segregation of HE from non-HE based on expression of CD73 was also

shown using VE-cadherinPr/CD41Pr dual reporter transgenic hESCs.<sup>102</sup> Kennedy et al.<sup>66</sup> found that cells with HE phenotype possessed T lymphoid potential and demonstrated the critical role of activin/nodal signaling in establishing a lymphoid program in HE. It has also been found, that, in parallel with the acquisition of endothelial markers, differentiated hESCs begin to express angiotensin-converting enzyme (ACE or CD143). When CD143<sup>+</sup>CD43<sup>-</sup>CD45<sup>-</sup>CD41a<sup>-</sup> cells were isolated and analyzed, they showed hemangioblastic properties.<sup>104</sup> ACE activity was found to be important for regulation of hematopoietic vs endothelial differentiation of isolated CD143<sup>+</sup> cells. Inhibition of angiotensin II type 1 receptor enhanced hematopoietic potential of CD143<sup>+</sup> cells, while inhibition of angiotensin II type 2 receptor skewed their differentiation toward endothelial cells.<sup>104</sup>

The mechanisms regulating HE development and endothelial-hematopoietic transition remain largely unknown. Mouse studies have identified Runx1<sup>52</sup> as a positive regulator and HoxA3<sup>105</sup> as a negative regulator, of blood formation from endothelium, and showed the pivotal role of Sox17 in HE expansion<sup>106</sup> and Hes-mediated Notch signaling in HSC formation from HE in AGM region.<sup>107</sup> The critical role of SOX17 in regulation of endothelial-hematopoietic transition has been also demonstrated in human PSCs.<sup>101</sup> Conditional expression of SOX17 in hESC-derived CD34<sup>+</sup>CD43<sup>-</sup> cells promoted the formation of a unique population of VE-cadherin<sup>+</sup>CD43<sup>+</sup>CD45<sup>-</sup> cells with HE properties while knockdown of its expression inhibited blood formation, indicating that SOX17 is essential for induction and maintenance of the hemogenic program in endothelial cells. This conclusion was also supported by findings that overexpressed SOX17 binds to promoters and regulatory regions of a large number of hematopoietic and endothelial genes.<sup>101</sup> Lee et al.<sup>108</sup> found that

formation of hematopoietic progenitors from hPSC-derived CD31<sup>+</sup>CD45<sup>-</sup> cells can be enhanced by HES1-mediated activation of NOTCH signaling using Jag1. The developmental progression from hemogenic endothelium to hematopoietic progenitors depends on TGF $\beta$  signaling. In an embryoid body differentiation system, treatment of hESCs at CD31<sup>+</sup>CD43<sup>-</sup> stage of development with TGF $\beta$ 1 suppressed formation of CD43<sup>+</sup> cells, while TGF $\beta$  inhibitors exerted the opposite effect.<sup>74</sup>

#### *Specification and diversification of hematopoietic lineages from hPSCs*

In hPSC differentiation cultures, the cells that are already committed to a hematopoietic fate and capable of forming various colonies of blood cells in response to hematopoietic cytokines arise within the CD34<sup>+</sup> population before expression of CD45 typical pan-hematopoietic marker.<sup>59,61,78,109</sup> Although CD34 specifically identifies hematopoietic progenitors and stem cells within the somatic hematopoietic compartment, in hPSC cultures, CD34 is expressed by a variety cell types, including endothelial and mesenchymal stem cells.<sup>100,110</sup> Thus, the use of CD34 alone may not be sufficient to select for a pure population of hematopoietic progenitors from hPSCs. By analyzing the kinetics of expression of specific hematopoietic markers in hESCs differentiated on OP9, Vodyanik et al.<sup>100</sup> identified leukosialin (CD43) as a marker that reliably separates CD43<sup>+</sup> colony-forming hematopoietic progenitors from CD43<sup>-</sup>CD31<sup>+</sup> endothelial cells and CD43<sup>-</sup>CD31<sup>-</sup> cells with mesenchymal characteristics within the CD34<sup>+</sup> population generated from hESCs in coculture with OP9. Similarly, selection of CD43<sup>+</sup> cells from hiPSC differentiated on OP9 or in embryoid bodies made it possible to separate hematopoietic CFCs from non-hematopoietic cells.<sup>66,67</sup> Recent time-lapse video recordings of endothelial-hematopoietic



transition demonstrated that the gain of CD43 expression tightly correlated with the transformation of epithelioid endothelial cells into round blood cells.<sup>86</sup> Because CD43 provides a repulsive barrier around the cell,<sup>111</sup> acquisition of CD43 expression may have functional significance for budding and separation of blood cells from endothelium.

In hPSC cultures differentiated on OP9, the first CD43<sup>+</sup> cells were detected within emerging VE-cadherin<sup>+</sup> cells as early as day 4 of differentiation.<sup>86,100</sup> These cells were CD41a<sup>-</sup> and expressed the erythroid marker, CD235a (Glycophorin A), and low levels of CD43, which was best detected using highly sensitive fluorochromes. The emerging VE-cadherin<sup>+</sup>CD43<sup>low</sup>CD235a<sup>+</sup>CD41a<sup>-</sup> cells had primarily hematopoietic characteristics and colony forming potential and lacked the expression of *CLDN5* and *CAV1*, typical endothelial genes, however, they retained the capacity to grow endothelial cells (Figure 2B). Because of these properties, VE-cadherin<sup>+</sup>CD43<sup>low</sup>CD235a<sup>+</sup>CD41a<sup>-</sup> cells were defined as angiogenic hematopoietic progenitors (AHP).<sup>86,90</sup> Even though AHPs expressed the erythroid cell marker CD235a, they had a unique potential to form a broad spectrum of hematopoietic colonies in serum-free medium supplemented with hematopoietic cytokines and FGF2.<sup>86</sup> Gross morphologies of some of these colonies resemble hemangioblast/BL colonies formed by BL-CFCs. However, in contrast to BL colonies, colonies generated by AHPs do not develop through an endothelial core stage. Whether AHPs represent a unique transition stage between HE and blood or a distinct wave of hematopoietic progenitors remains to be determined.

In mouse embryo and ESC cultures CD41a (GPIIb) expression marks the initiation of hematopoiesis and precedes the expression of the erythroid marker, TER119.<sup>112-114</sup> However, in hPSC cultures, the first CD41a<sup>+</sup> cells were detected within the already established CD43<sup>+</sup>CD235a<sup>+</sup> population.<sup>86</sup> Thus, the vast majority of CD41a<sup>+</sup> cells coexpress CD235a. Although early CD41a<sup>+</sup>CD235a<sup>+</sup> cells express high levels of VE-cadherin, they are completely devoid of endothelial potential.<sup>86</sup> Analysis of colony-forming capacity of CD41a<sup>+</sup>CD235a<sup>+</sup> cells revealed that they are highly enriched in erythro-megakaryocytic progenitors,<sup>100,115</sup> but also show some myeloid potential in serum-free clonogenic medium.<sup>86</sup> hESC and hiPSCs-derived CD235a<sup>+</sup>CD41a<sup>+</sup> progenitors with erythro-megakaryocytic potential can be expanded and differentiated into erythroid or megakaryocytic cells.<sup>116,117</sup> The expansion and fate switch of CD235a<sup>+</sup>CD41a<sup>+</sup> cells is regulated by aryl hydrocarbon receptor (AhR).<sup>117</sup> Multiple studies have demonstrated the feasibility of high scale production of red blood cells and platelets in bulk hPSC cultures (reviewed in <sup>118,119</sup>). However, erythroid cells generated in these cultures have primitive and fetal-like characteristics.<sup>120,121</sup> They express mostly embryonic and fetal hemoglobins with low levels of adult hemoglobin and do not enucleate efficiently. Although several studies have demonstrated that hPSC-derived platelet are capable of initiating thrombus formation, the hemostatic properties of these cells remains to be evaluated in animal models of thrombocytopenia.

The progenitors with broad myelolymphoid potential and lin<sup>-</sup>CD34<sup>+</sup>CD43<sup>+</sup>CD45<sup>-</sup> phenotype can be detected in hPSC cultures shortly after emergence of CD235a<sup>+</sup>CD41a<sup>+</sup> cells.

Acquisition of CD45 expression by lin<sup>-</sup> cells is associated with progressive myeloid

commitment.<sup>100</sup> The lin<sup>-</sup>CD34<sup>+</sup>CD43<sup>+</sup>CD45<sup>+/-</sup> hematopoietic progenitors are capable of forming the entire spectrum of hematopoietic colonies in serum-containing CFC medium. The CFC potentials of these cells was comparable or even higher than that of phenotypically similar cord blood cells.<sup>122</sup> The hESC and hiPSC-derived lin<sup>-</sup>CD34<sup>+</sup>CD43<sup>+</sup>CD45<sup>+/-</sup> display many phenotypical features of HSCs, including CD90 and c-kit expression and the lack of CD38 and CD45RA expression.<sup>67,100</sup> These cells have high ALDH activity, the ability to efflux rhodamine-123, and LTC-IC potential.<sup>78,100,123</sup> The lin<sup>-</sup>CD34<sup>+</sup>CD43<sup>+</sup>CD45<sup>+</sup> cells could be expanded with GM-CSF and differentiated into myelomonocytic cells including neutrophils, eosinophils, macrophages, dendritic and Langerhans cells, and osteoclasts.<sup>19</sup> CD34<sup>+</sup> and CD43<sup>+</sup> cells generated from hESCs or hiPSCs also possess NK and B lymphoid potentials.<sup>78,100,124-126</sup> NK cells generated from hPSCs have cytotoxic function and can be generated in large numbers using defined conditions.<sup>125,126</sup> However, B lymphoid cultures produced very low numbers of CD19<sup>+</sup>CD10<sup>+</sup> cells. Although these cells exhibited multiple genomic D-JH rearrangements, they did not express IgM or CD5, indicating that their development failed to progress beyond the pre-B cell stage.<sup>124</sup> The T cell potential of hESCs was initially demonstrated using *in vivo* studies which found that hESC-derived CD34<sup>+</sup> cells transplanted into human thymus/fetal liver grafts in SCID-hu mice generated T cells, including CD4<sup>+</sup>CD8<sup>+</sup> T cell precursors.<sup>127</sup> Later, T cells were generated from hESCs and iPSCs *in vitro* using OP9 cells expressing the NOTCH ligands delta-like 1 (DLL1) or 4 (DLL4).<sup>18,66,128,129</sup> By analyzing T cell potential of CD43<sup>+</sup> populations expressing different levels of CD34 and CD43, Timmermans et al. found that T cells were derived exclusively from the CD34<sup>high</sup>CD43<sup>low</sup> population of hematopoietic progenitors.<sup>128</sup> T cell potential could also be detected when NOTCH signaling is activated in

CD34<sup>+</sup>CD43<sup>-</sup>CD45<sup>-</sup> cells expressing endothelial markers.<sup>66</sup> Collectively, these studies indicate that cells with pan-myeloid and lymphoid potentials (i.e. definitive hematopoietic cells) can be generated from hPSCs.

*Hematopoietic engraftment potential of hPSC-derived cells.*

Following the initial studies demonstrating the presence of CD34<sup>+</sup> cells with hematopoietic colony-forming potential in hESC cultures, there was significant interest in evaluating hematopoietic repopulation potential of these cells. Several reports have detected human hematopoietic cells in bone marrow of immunocompromised mice and sheep many months after intravenous or intrafemoral injection of an entire population of differentiated hESCs or hESC-derived CD34<sup>+</sup> cells.<sup>130-134</sup> However, bone marrow engraftment observed in these studies was low (within 0.1-2%) and mostly restricted to the myeloid lineage. Similarly low levels of engraftment were reported after intrafemoral transplantation of differentiated hiPSCs.<sup>135</sup> It was suggested that hPSC-derived cells have limited migratory potential and seem unable to complete maturation within the adult bone marrow environment.<sup>131,135</sup> To find out whether the neonatal environment provides a better support for hematopoietic engraftment of differentiating hESCs, Tian et al. transplanted CD34<sup>+</sup> cells obtained from luciferase (luc)-expressing hESCs into newborn NSG mice.<sup>136</sup> Although the authors detected luc<sup>+</sup> cells several months after transplantation, they found that they were mostly endothelial and not hematopoietic cells. Recently, two studies have demonstrated that hematopoietic differentiation could be achieved *in vivo* during teratoma formation from hiPSCs.<sup>137,138</sup> The generation of blood cells within teratomas was enhanced by injecting hiPSCs together with OP9 stromal cells ectopically expressing DLL1 and WNT3a. When

CD34<sup>+</sup>CD45<sup>+</sup> cells were isolated from these teratomas and transplanted into NSG mice, the pattern of hematopoietic engraftment was very similar to that observed following transplantation of *in vitro* differentiated hPSCs, i.e. very limited 0.1-2% hematopoietic chimerism in the bone marrow with predominant myeloid engraftment.<sup>137</sup> In another study, hematopoietic differentiation within teratomas was amplified by administration of human SCF and TPO via micro-osmotic pump. However, only ~4% NOD/SCID and ~ 30% of NOD/SCID/JAK3<sup>null</sup> mice showed a low level of hematopoietic chimerism after transplantation of 600 CD34<sup>+</sup>CD45<sup>+</sup> teratoma-derived cells.<sup>138</sup> Although these studies demonstrated the feasibility of generating hematopoietic cells with limited engraftment potential from hPSCs they also indicate that current differentiation conditions do not reproduce the complexity of embryonic hematopoietic development that leads to HSC specification and expansion.

### **Defining conditions which permits engineering HSCs**

Why do *in vitro* hPSC cultures fail to yield cells with robust hematopoietic engraftment potential? Because HSC specification in the AGM region of an embryo is a very rare event (1 HSC per AGM at E11.5)<sup>41</sup> followed by HSC expansion in fetal liver, one can argue that hPSC differentiation cultures produce rare HSCs but fail to expand them. However, this situation is probably unlikely. During embryogenesis, the hematopoietic hierarchy appears in reverse sequence as compared to an adult one,<sup>139</sup> i.e the formation of progenitors with more restricted hematopoietic potential precedes emergence of HSCs (Figure 3). In mice, pan-myeloid progenitors and T and B lymphoid precursors were first identified in the yolk sac before HSC potential could be detected in the embryo proper.<sup>139-141</sup> In addition, clusters

of multipotential blood cells formed at many sites within the aorta, however, HSCs are restricted to the ventral wall of the dorsal aorta,<sup>55,142</sup> this indicating that finely tuned local signaling is required for multipotential blood cells to acquire repopulation potential. Therefore, it is more likely that multipotential blood cells generated in hPSC cultures represent a pre-HSC stage of development and that blood cells arising in hPSC cultures simply fail to complete the HSC specification following transition from endothelial to hematopoietic stage.

Currently, two major models exist to explain the relationship between adult and embryonic hematopoietic hierarchies.<sup>139</sup> According to the one model, the embryonic and adult hierarchies arise from a common hematopoietic ancestor that gives rise to both primitive yolk sac and definitive embryonic hematopoiesis. The other model suggests that primitive and definitive hierarchies arise independently. Studies in *Xenopus* found that separation of adult and embryonic hematopoiesis occurs as early as the 32-cell blastomere stage.<sup>143</sup> Independent origins of yolk and definitive embryonic hematopoiesis is also supported by most of the mouse studies.<sup>35-37</sup> Nevertheless, the contribution of the yolk sac precursors to definitive adult hematopoiesis cannot be entirely excluded.<sup>144,145</sup> Regardless of which model more accurately predicts the *in vivo* process, the most critical question in relation to hPSC differentiation studies is whether the more primitive yolk sac precursors can acquire adult HSC properties following exposure to particular signaling events. Studies by Yoder et al. strongly support the hypothesis that local environment can induce HSC potential in yolk sac cells.<sup>146,147</sup> In contrast to AGM, yolk sac organ cultures are unable to initiate and support HSC development *ex vivo* or repopulate adult recipients.<sup>41</sup> However, CD34<sup>+</sup>c-kit<sup>+</sup> or

CD34<sup>+</sup>c-kit<sup>+</sup>CD41<sup>+</sup> cells isolated from the yolk sac of E9-9.5 embryos are capable of reconstituting multilineage long term hematopoiesis when transplanted into busulfan conditioned neonates.<sup>112,146,147</sup> Similarly, hematopoietic progenitors with multilineage potential that engraft in neonates can be isolated from the para-aortic splanchnopleura (P-Sp; the AGM primordium) of an E9 mouse embryo, before definitive HSC potential can be detected in adult recipient repopulation assay.<sup>146</sup> Thus, embryonic cells with detectable myeloid and lymphoid potentials *in vitro*, but lacking adult repopulation potential, can mature into HSCs in the neonatal environment. Based on these studies, induction of the equivalent of yolk sac/P-Sp multilineage newborn repopulating cells in hPSC cultures is considered a critical step toward establishing protocols for generating HSCs *ex vivo*.<sup>30</sup> By using T cell potential as a major criterion for the identification of such cells in hPSC cultures, Kennedy et al. found that progenitors with T lymphoid potential reside within the CD34<sup>+</sup>CD43<sup>-</sup>CD45<sup>-</sup> population of cells expressing CD34 and other endothelial markers.<sup>66</sup> Importantly, the production of these T lymphoid progenitors could be enhanced by manipulation of Activin/Nodal signaling pathways during the first 4 days of hPSC differentiation,<sup>66</sup> indicating that the definitive hematopoietic program can be enhanced during the mesodermal stage. Further engraftment studies will be required to determine whether hPSC-derived cells with lymphoid potential can repopulate newborn mice, similar to cells produced *in vivo* during embryogenesis.

In order to produce HSCs from hPSCs, it is also necessary to better understand the mechanisms regulating the formation of and specification of blood cells from HE. The acquisition of arterial identity through activation of NOTCH signaling and activation of

hematopoietic programming by RUNX1 are the most critical factors for the establishment of HE *in vivo* (reviewed in <sup>148</sup>). However it has become clear that HE are heterogeneous and not all HE can produce HSCs. Recent evidence indicates that at least two distinct populations of HE with erythromyeloid and HSC potentials exist in the mouse embryo, and that Ly6a (Sca1) specifically marks HSC-producing HE.<sup>149</sup> It is already known that at least two distinct types of VE-cadherin<sup>+</sup> cells with primitive and broad erythromyeloid hematopoietic potentials can be produced from hPSCs.<sup>66,86,91,102</sup> However, the specification of cells with broad lymphomyeloid potential directly from HE remains to be shown using clonal studies. The recent identification of HE progenitors in hPSC cultures<sup>66,86</sup> and the generation of genetically-engineered cell lines to trace endothelial-hematopoietic transition<sup>102</sup> have already provided a platform for assaying upstream factors required for HE formation with HSC potential and downstream factors that promote HSC expansion in the human system.

The reprogramming studies provided strong evidence that cellular identities are defined by gene regulatory networks controlled by few master regulatory factors. Identification of the master factors required for the specification of definitive/adult type HSCs from embryonic precursors would be one of the essential prerequisites to establishing a protocol for generating HSCs *ex vivo*. Mouse studies discovered HoxB4 or its upstream regulator Cdx4 as chief factors capable of inducing a self-renewal program in ESC-derived hematopoietic progenitors.<sup>12,150</sup> The rationale for selecting HoxB4 to engineer HSCs from ESCs came from embryonic studies which demonstrated the lack of expression of several critical homeobox regulators of definitive HSCs, including HoxB4, in the yolk sac.<sup>151</sup> Nevertheless, HoxB4-



induced cells do not entirely mimic the function and phenotype of somatic HSCs and produce mostly myeloid engraftment. Recently, the Daley group performed comprehensive molecular profiling studies to compare *in vitro* generated hematopoietic cells from mouse ESCs with cells from embryonic hematopoietic sites. These studies revealed that HoxB4-induced HSCs lack *HoxA9* expression and a Notch signaling signature,<sup>152</sup> which may explain their limited lymphoid potential. Although attempts have been made to induce HSCs from human PSCs using HOXB4, it was found that hESC-derived hematopoietic cells already express HOXB4, and its forced expression does not enhance engraftment of human cells.<sup>131,153</sup> In a search for the intrinsic determinants required for HSC specification from human PSCs, several groups performed molecular profiling studies of ESC-derived and *in vivo* produced human primitive hematopoietic cells. These studies revealed an apparent similarity between the transcriptomes of phenotypically identical fetal liver and hESC-derived primitive hematopoietic populations,<sup>122,154</sup> although distinctive differences in the expression of genes regulating HSC self-renewal, homing and chromatin remodeling were also noted.<sup>122</sup> Interestingly, in comparison to the fetal liver primitive hematopoietic cells, hESC-derived hematopoietic progenitors showed much higher levels of expression of genes from the *HOXB* cluster, but significantly lower levels of genes from the *HOXA* cluster.<sup>122,154</sup> Differences in genes involved in NOTCH signaling pathways,<sup>155</sup> polycomb and thiritorax complexes were also noted.<sup>122,156</sup> Capacity to home to bone marrow is one of the critical features of HSCs. However, hESC-derived lin-CD34<sup>+</sup>CD43<sup>+</sup>CD45<sup>+/-</sup> hematopoietic progenitors express very low levels of HSC homing molecule CXCR4, which may indicate that these cells have yet to acquire competent homing capability.<sup>122</sup> Overall, molecular profiling studies have identified several unique features of the transcriptome of human and

mouse PSC-derived hematopoietic precursors. Insights gained from these studies can be further explored to define novel molecular targets capable of activating an adult type HSC program during hPSC differentiation.

### **Concluding Remarks**

The de novo generation of HSCs remains a significant challenge. Achieving this goal requires collaborative efforts of developmental, stem cell, and molecular biologists. Embryonic studies have identified the essential role of Notch, Wnt, Hedgehog and TGF $\beta$ /Smad signaling pathways in HSC development and the maintenance of HSC program. Further understanding the molecular mechanisms and niche factors critical for specifying HE vs non-HE and HSCs vs non-HSC precursors from HE in the embryo will be essential prerequisite to the development of optimal conditions for HSC production *in vitro*. The reconstruction of gene regulatory networks and identification of master regulatory factors that control HSC identity and drive HSC precursors to adopt HSC fate will ultimately enable conversion of hPSC-derived cells into adult type HSCs (Figure 4). Recently, a hemogenic program was successfully induced in mouse and human fibroblasts by forced expression of Gata2, Gfi1b, cFos, and Etv6<sup>157</sup> and OCT4, respectively.<sup>158</sup> Although these reports demonstrated the feasibility of generating hematopoietic hierarchy for clinical application by direct transformation of somatic cells and bypassing the pluripotency stage, the next challenging step is to determine factors capable of inducing the hematopoietic self-renewal program in directly transformed somatic cells and achieve the scalability of the direct reprogramming process. Nevertheless, both approaches, PSC-based and direct reprogramming of somatic cells to HSCs, have to be pursued to better understand the

genetic and epigenetic factors governing hematopoietic specification and the self-renewal program. No doubt these studies will continue to provide fascinating insights into fundamental questions of HSC biology, and eventually will lead to the development of novel stem cell therapies.

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### **Authorship Contribution**

I.S. wrote the paper.

### **Conflict of Interest Disclosure**

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## Figure Legends

**Figure 1. Therapeutic potential of hPSCs for blood diseases.** iPSCs can be potentially used to treat patients with monogenic genetic blood diseases such as sickle cell anemia,  $\beta$ -thalassemia, Fanconi anemia, or SCID (upper panel). Autologous skin or blood cells from these patients can be reprogrammed into iPSCs. The defective gene in iPSCs can be repaired using homologous recombination. *De novo* generation of HSCs from gene-corrected iPSCs would provide immunologically matched cells for bone marrow transplantation. For cancer therapy, autologous iPSCs could be generated from skin fibroblasts or other somatic cells lacking leukemia mutation and used to generate HSCs for bone marrow transplantation as well immune cells to induce anti-leukemia immune response (lower panel).

**Figure 2. A model of hematopoietic development from hPSCs.** (A) The most critical factors involved in specification of hematovascular precursors from PSCs and regulation of blood formation from HE. (B) Stages of hematopoietic development from hPSCs. Mesodermal stage of development is defined by expression of the mesodermal markers, APLNR and KDR.<sup>62,77,80</sup> The lack of expression of typical endothelial (CD31, VE-cadherin), endothelial/mesenchymal (CD73, CD105), and hematopoietic (CD43, CD45) markers, i.e.  $^{EMH}lin^{-}$  phenotype, separates mesoderm from lineage-committed cells.<sup>86,90</sup> The most primitive mesodermal precursors with hematopoietic potential arise in coculture with OP9 or embryoid body system on day 3 of differentiation. These cells have features of posterior primitive streak, coexpress KDR, APLNR and PDGFR $\alpha$  and capable of forming BL (hemangioblast) colonies in presence of FGF2 and VEGF.<sup>62,77,80,86</sup> The formation of BL

colonies in clonogenic medium proceeds through VE-cadherin<sup>+</sup> endothelial intermediates, which generate primitive hematopoietic cells with erythroid, megakaryocytic, and macrophage potentials.<sup>77,86</sup> Progressive mesodermal commitment to endothelial and hematopoietic cells is associated with downregulation of PDGFR $\alpha$ <sup>71,86</sup> and primitive streak genes, and upregulation of KDR and *TAL1*, *GATA2*, and *ETV2* genes associated with angiohematopoietic development leading to formation of <sup>EMH</sup>lin<sup>-</sup> KDR<sup>bright</sup>APLN<sup>R</sup>+PDGFR $\alpha$ <sup>low/-</sup> hematovascular mesodermal precursors (HVMPs).<sup>86</sup> HVMPs lack BL-CFC potential, but are highly enriched in cells that form hematoendothelial clusters on OP9.<sup>86</sup> Endothelial stage of development was defined by expression of the typical endothelial markers VE-cadherin, CD31 and CD34 and the absence of the pan-hematopoietic marker CD43 (see also Supplementary Table S1).<sup>66,86,90,101,102</sup> Within the VE-cadherin<sup>+</sup>CD43<sup>-</sup> population, HE cells, i.e. cells lacking hematopoietic CFC potential but capable of forming blood cells after culture with stromal cells, were discriminated from non-HE cells based on lack of CD73 expression.<sup>86,102</sup> The first hematopoietic progenitors emerging from the VE-cadherin<sup>+</sup> population express CD235a, low levels of CD43 and lack CD41a expression. These cells have a unique potential to form hematopoietic colonies in the presence of FGF2 and hematopoietic cytokines, but also retain endothelial potential, and therefore were designated as angiogenic hematopoietic progenitors.<sup>86</sup> Advanced hematopoietic development is associated with upregulation of CD43 expression, segregation of all hematopoietic CFCs to the CD43<sup>+</sup> fraction<sup>66,100,128</sup> and establishment of distinct subsets of CD43<sup>+</sup> hematopoietic cells, including CD41a<sup>+</sup>CD235a<sup>+</sup> erythro-megakaryocytic progenitors<sup>100,115,117</sup> and lin<sup>-</sup>CD34<sup>+</sup>CD43<sup>+</sup>CD45<sup>+/-</sup> multipotent myelolymphoid progenitors.<sup>66,100,128</sup> Progressive acquisition of angiogenic and

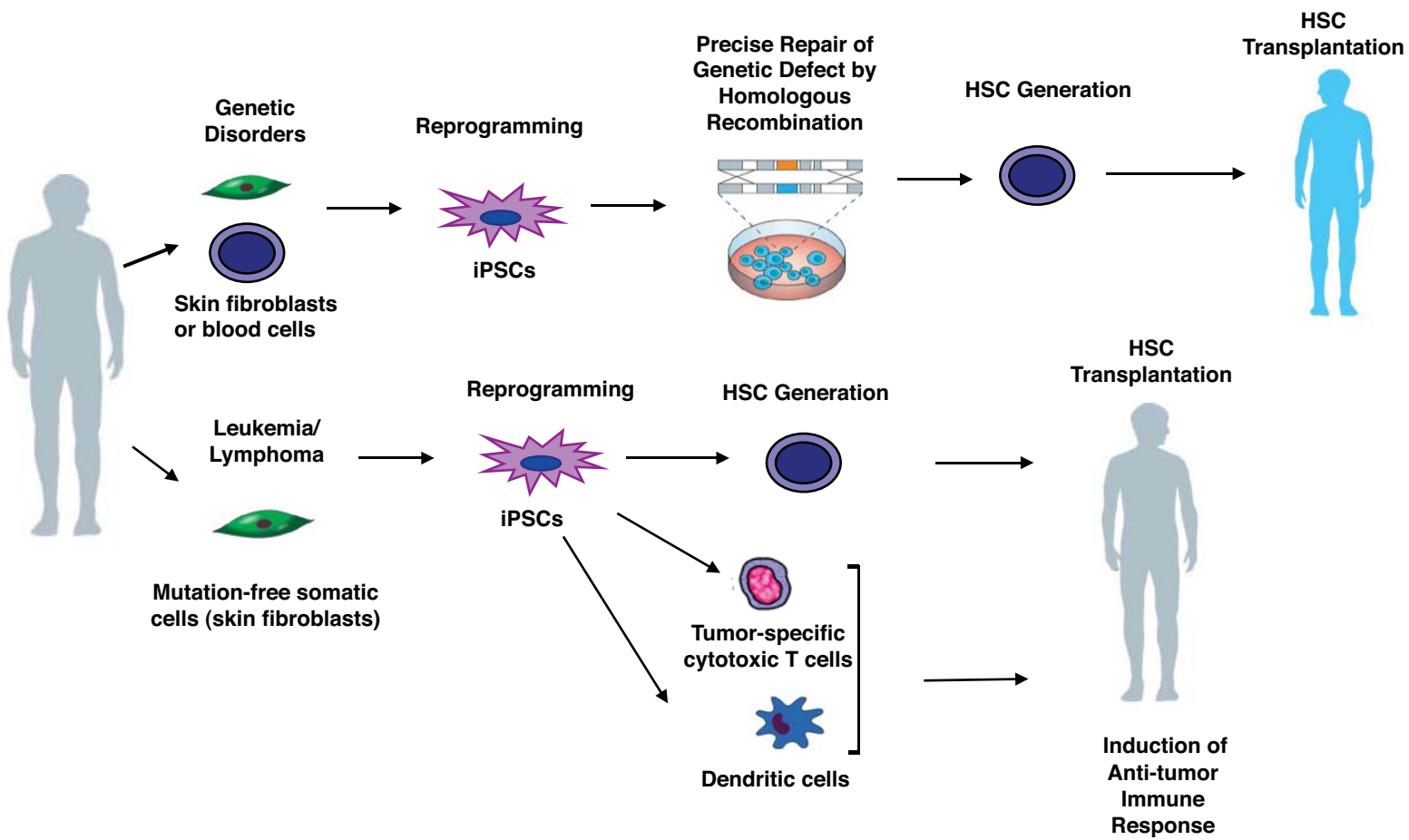
hematopoietic program by differentiated cells is emphasized by green and red color, respectively.

**Figure 3. Schematic diagram demonstrating the opposite sequence of blood cell development between embryo and adults.** In embryos, cells with restricted hematopoietic potential appear prior to HSC specification. In adults, hematopoiesis proceeds through gradual maturation of HSCs leading to formation of progenitors with more restricted potential.

**Figure 4. Potential approaches for *de novo* induction of HSCs.**

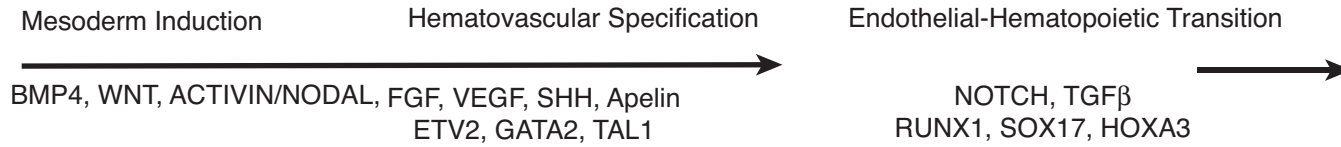


Figure 1

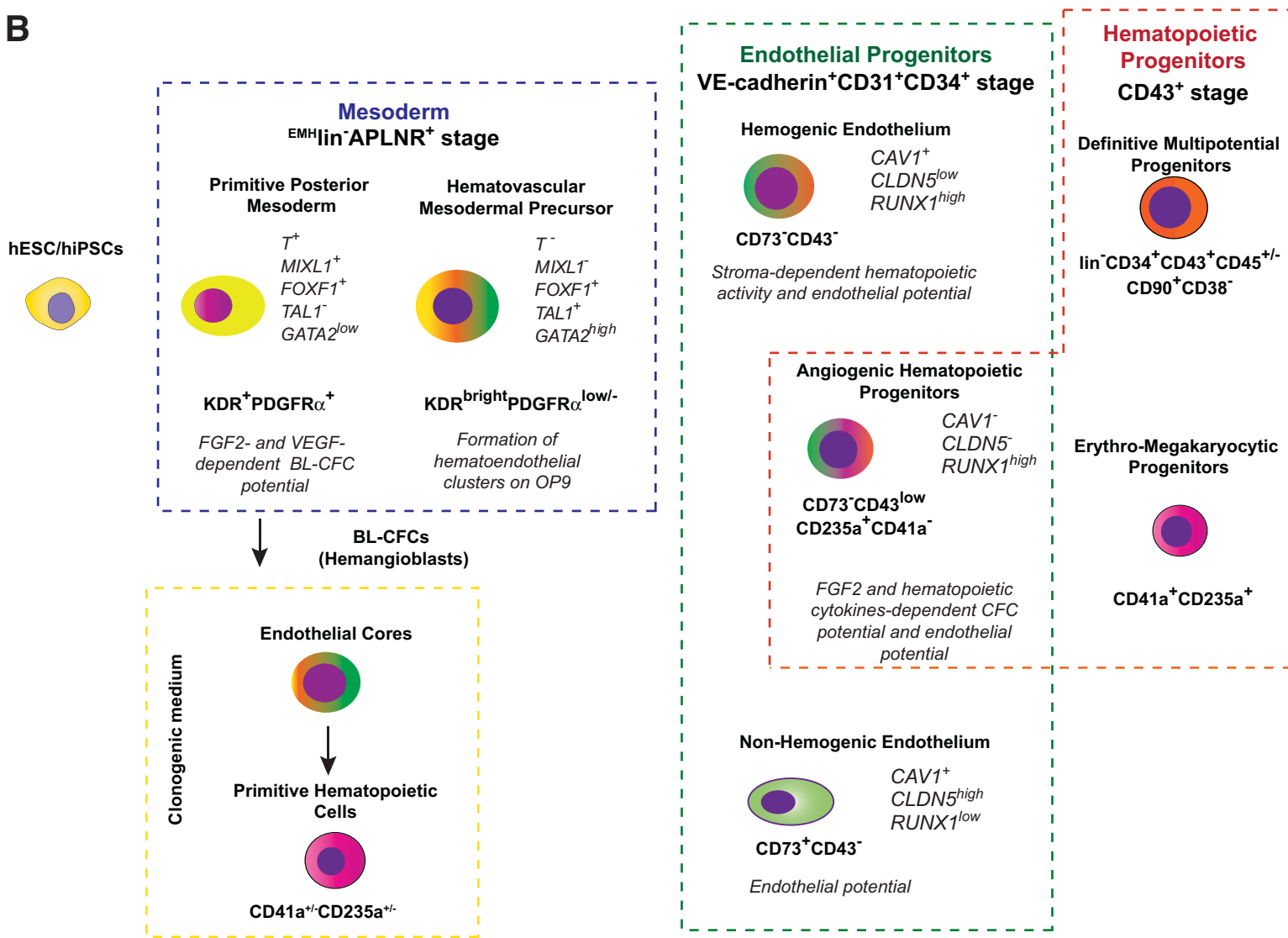


**Figure 2**

**A**



**B**



# Embryonic Hematopoiesis



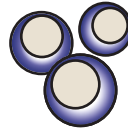
Erythrocyte  
Megakaryocyte  
Macrophage  
Progenitor



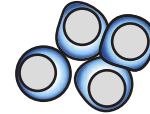
Erythromyeloid  
progenitor



Lymphomyeloid  
progenitor



HSC



Megakaryocyte-  
Erythroid Progenitor

Common Myeloid  
Progenitor

Multipotent  
progenitor

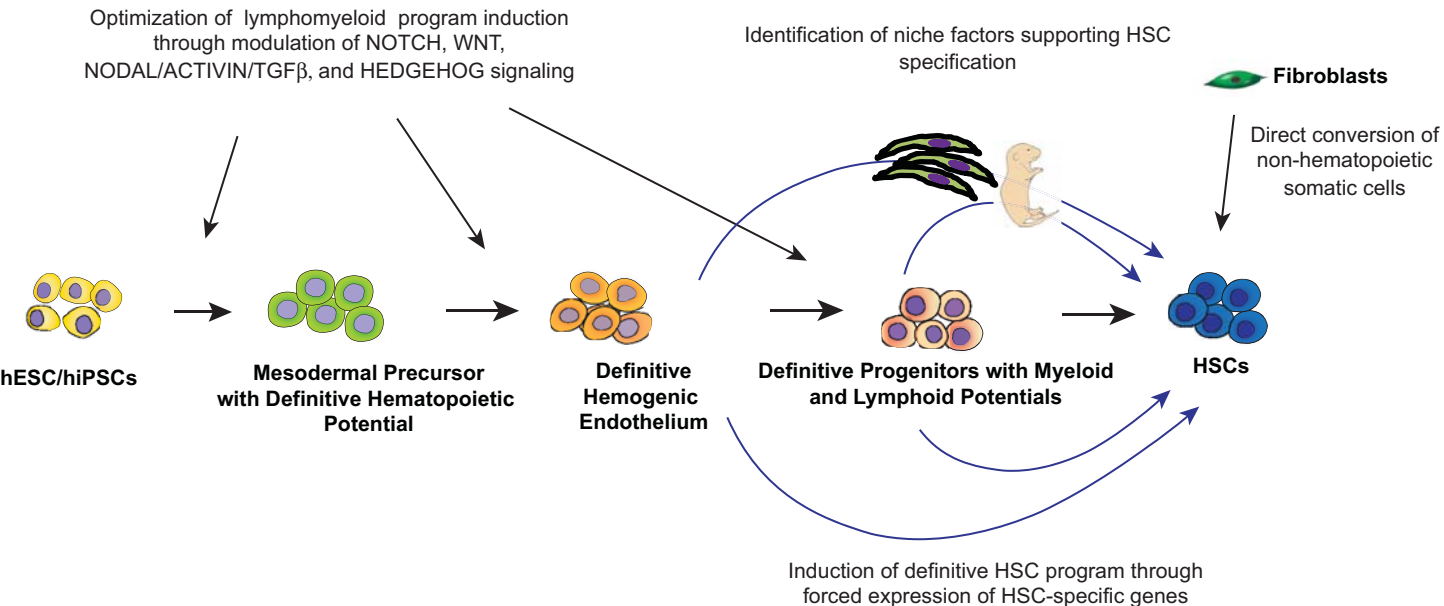
HSC

Common Lymphoid  
Progenitor



# Adult Hematopoiesis

**Figure 4**





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## **Hematopoietic specification from human pluripotent stem cells: current advances and challenges toward de novo generation of hematopoietic stem cells**

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