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Deciphering the hierarchy of angiohematopoietic progenitors from human pluripotent stem cells

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Identification of sequential progenitors leading to blood formation from pluripotent stem cells (PSCs) will be essential for understanding the molecular mechanisms of hematopoietic lineage specification and for development of technologies for in vitro production of hematopoietic stem cells (HSCs). It is well established that during development, blood and endothelial cells in the extraembryonic and embryonic compartments are formed in parallel from precursors with angiogenic and hematopoietic potentials. However, the identity and hierarchy of these precursors in human PSC (hPSC) cultures remain obscure. Using developmental stage-specific mesodermal and endothelial markers and functional assays, we recently identified discrete populations of angiohematopoietic progenitors from hPSCs, including mesodermal precursors and hemogenic endothelial cells with primitive and definitive hematopoietic potentials. In addition, we discovered a novel population of multipotent hematopoietic progenitors with an erythroid phenotype, which retain angiogenic potential. Here we introduce our recent findings and discuss their implication for defining putative HSC precursor and factors required for activation of self-renewal potential in hematopoietic cells emerging from endothelium.

Introduction

The discovery of human pluripotent stem cells (hPSCs), both embryonic (ESCs)[1] and induced (iPSCs),[2,3] have opened novel opportunities to model human diseases, screen drugs and manufacture cells for transplantation therapy in vitro. ESCs and iPSCs can be propagated almost indefinitely in culture and differentiated into any cell type in the human body. The pattern of differentiation from ESCs and iPSCs toward particular cell lineage is very similar. Recently, significant progress has been made in differentiating PSCs toward hematopoietic progenitors and diverse types of blood cells.[4-8] However, the generation of hematopoietic stem cells (HSCs) with bone marrow reconstitution potential from PSCs remains a significant challenge.[9] The identification of immediate blood precursors and mechanisms guiding specification and diversification of hematopoietic cells from PSCs is essential for overcoming this major limitation of current PSC differentiation systems.

During embryogenesis, the first hematopoietic cells are generated in parallel with endothelial cells in the yolk sac. The close spatial and temporal relationship between blood and endothelial cells was noted by early embryologists in the late 19th century.[10] In 1917, Florence Sabin postulated the existence of a bipotential precursor for blood and endothelial cells[11] based on her observations of blood development within the yolk sac in chicken embryos. This bipotential precursor was later named hemangioblast by Murray.[12] At embryonic day 7.5 (E7.5), the mouse yolk sac initially gives rise to primitive hematopoietic cells, including nucleated erythrocytes, macrophages and megakaryocytes.[13,14] The primitive hematopoiesis is

eventually supplanted by definitive hematopoiesis that give rise to HSCs with the potential to reconstitute the wild type adult mice. The first definitive HSCs are detected in the aorta-gonad-mesonephros (AGM) region at E10.5.[15] In this region, HSCs arise from endothelial cells with blood-forming potential or hemogenic endothelium (HE).[15-20] The budding of hematopoietic cells from endothelial cells lining the ventral wall of dorsal aorta in the AGM region was originally described by Dantschakoff in 1907.[21] Recent studies have provided direct evidence that this process represents the formation of definitive blood cells and HSCs from HE through an endothelial-to-hematopoietic transition (EHT).[19,22-24] 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 vitelline and umbilical arteries,[25] placenta,[26] head vasculature[27] and yolk sac nascent capillaries.[28] The evidence that HSCs originate from HE with definitive hematopoietic potential underscores the need to identify HE progenitors in PSC cultures and discriminate them from primitive angiohematopoietic progenitors.

In the embryo, distinct waves of primitive vs. definitive hematopoiesis, and HE vs. non-HE cells can be discriminated reliably based on anatomical location and morphology. However, these criteria cannot be applied to in vitro cultures. In addition, significant overlap in expression of surface markers by endothelial and emerging blood cells, and the lack of knowledge about distinct features of mesodermal progenitors and HE cells with primitive and definitive hematopoietic potential, dictate the need to identify novel stage-specific cellular markers and assays to distinguish the discrete stages of hematopoiesis in PSC cultures.

Identification of Hierarchy of Angiohematopoietic Progenitors in Human PSC Differentiation Cultures

To induce hematopoietic differentiation, we co-cultured hPSCs on OP9 mouse stromal cells.[29,30] Co-culture of PSCs on OP9 induces their differentiation toward mesoderm and endoderm with no detectable ectoderm.[31] To define the distinct stages of mesodermal commitment, we analyzed the expression of the typical mesodermal markers APLNR,[31-33] KDR[34] and PDGFRα (CD140a)[35] on differentiated hPSCs before endothelial, mesenchymal and hematopoietic lineage-commitment markers became detectable. We found that differentiated PSCs upregulate APLNR after 2 d of co-culture on OP9. During days 2–3 of differentiation, APLNR$^+$ cells upregulate PDGFRα, KDR and genes associated with primitive streak and lateral plate/extraembryonic mesoderm,[31] but lack endothelial (CD31, VE-cadherin), endothelial/mesenchymal stem cell (CD73, CD105) and hematopoietic (CD43, CD45) lineage-specific markers (**Fig. 1** and **Table 1**), hereafter referred to as ^{EMH}lin$^-$.[31,36,37] In the OP9 co-culture, the day 3 ^{EMH}lin$^-$APLNR$^+$PDGFRα$^+$ mesodermal cells represent a transient population of mesoderm reminiscent of the primitive posterior mesoderm (PM) in the embryo with the potential to form blast (BL) or hemangioblast (HB) colonies in response to FGF2.[31,37] The BL colony-forming cells (BL-CFCs) reflect the first wave of hematopoiesis in PSC cultures.[31,37,38] The development of BL colonies in clonogenic cultures proceed through a core stage at which PM cells form clusters of tightly-packed cells (cores) composed of VE-cadherin$^+$ cells with epithelioid morphology, endothelial gene expression profile and endothelial potential.[31,37,39] The core stage of differentiation is readily identifiable after 3 d of culture of PM cells in semi-solid clonogenic medium. Subsequently, VE-cadherin$^+$ core-forming cells transform into blood cells with VE-cadherin$^-$ CD235a$^{+/-}$CD41a$^{+/-}$ phenotype and erythroblast morphology. BL-CFC hematopoiesis is mostly restricted to erythroid cells producing embryonic ε- but no adult β-hemoglobin, megakaryocytic cells and macrophages, indicating that in response to FGF2, PM cells generate an endothelial intermediate that undergoes transition into primitive hematopoietic cells. Although PM cells generate mostly primitive blood cells in serum-free semisolid medium in response to FGF2, further culture of these cells on OP9 produces cells with definitive hematopoietic potential.

The next, more advanced stage of mesodermal commitment toward hematopoietic cells is associated with emergence of hematovascular mesodermal precursors (HVMPs), which can be discriminated at day 3.5 of differentiation based on increased KDR and decreased PDGFRα expression by APLNR$^+$ cells (see **Fig. 1** and **Table 1**).[37] The emerging KDR^{bright}APLNR$^+$PDGFRα$^{low/-}$ HVMPs have large blast morphology and remain ^{EMH}lin$^-$, i.e., do not express VE-cadherin, CD31, CD105, CD73, CD43 or CD45. Similar to PM, the HVMPs express genes associated with lateral plate/extraembryonic mesoderm. However, they downregulate primitive streak genes and upregulate *TAL1, HHEX, LMO2, GATA2* and *ETV2,* genes associated with angiohematopoietic development (**Figs. 1 and 2A**). Functional analysis revealed that HVMPs lost FGF2-dependent BL colony-forming potential. However, single cell deposition assay demonstrated that this population was highly enriched in cells capable of forming hematoendothelial clusters on OP9 (**Fig. 2C**). Dynamic imaging studies of hematoendothelial clusters revealed that endothelial cells within these clusters gradually acquired hematopoietic morphology and phenotype, i.e., underwent endothelial-to-hematopoietic transition.[37]

VE-cadherin is considered one of the earliest markers of endothelial-lineage cells in the embryo.[40,41] In hPSC co-cultures with OP9, the first VE-cadherin$^+$ cells arise from HVMPs by day 4 of differentiation.[37] The expression of VE-cadherin coincided with upregulation of the endothelial markers CD31 (PECAM) and ESAM and other typical endothelial genes including *PROCR, SCARF1, ECSR* and *NOS3,* signifying the endothelial commitment following acquisition of VE-cadherin expression by cells arising from HVMPs. Although emerging VE-cadherin$^+$ cells were homogenous in expression of CD31, ESAM and other typical endothelial markers such as TEK, KDR, CD34, CD141, CD146 and CD201, we were able to discriminate the following distinct subsets within this population based on expression of the endothelial/mesenchymal marker CD73 (5'-nucleotidase) and the hematopoietic markers, CD235a and CD43 (Glycophorin A

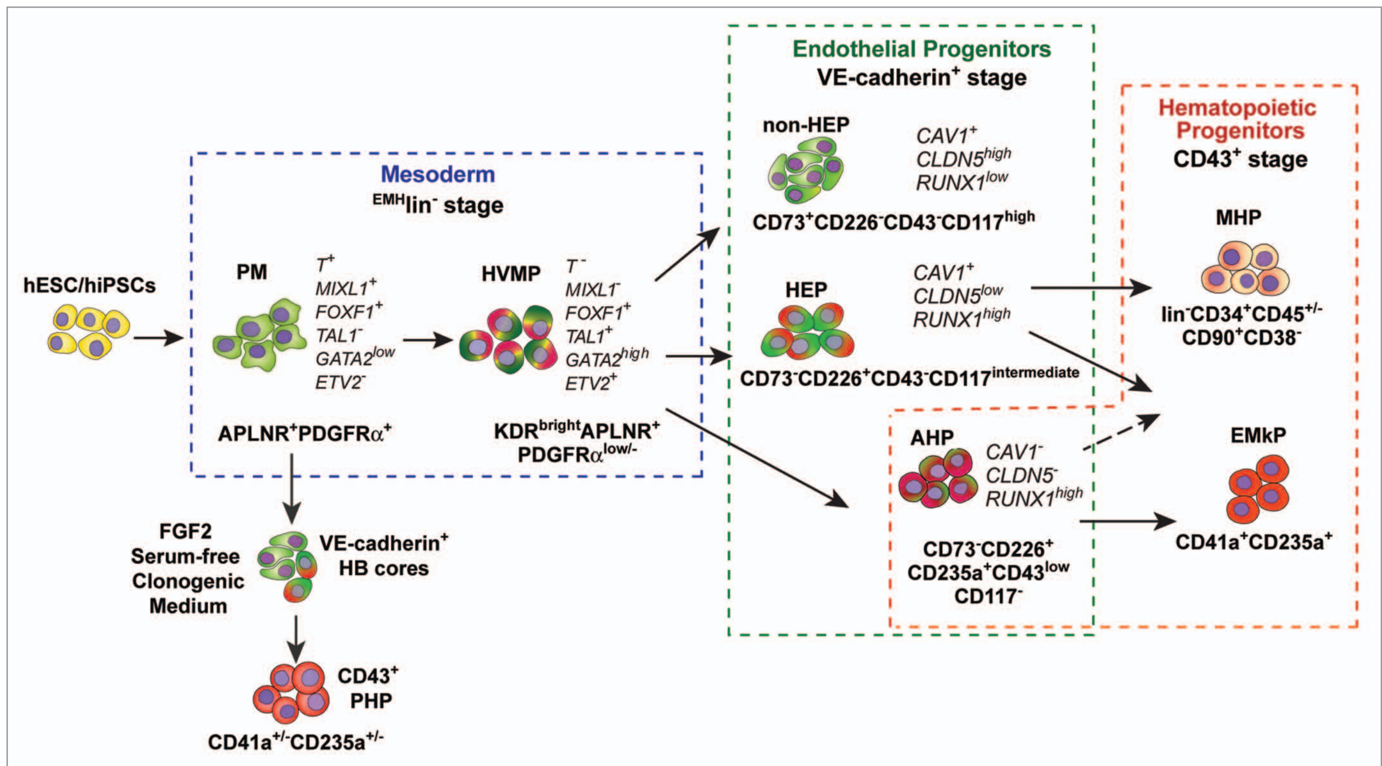

Figure 1. A model of angiohematopoietic differentiation of hPSCs in co-culture with OP9. The specific phenotypes and key stage defining genes are shown. APLNR^{+}PDGFRα^{+} primitive mesoderm (PM) formed during first 3 d of differentiation possess the potential to form hemangioblast (HB) colonies. These colonies can be specifically detected in serum-free medium supplemented with FGF2. HB colonies develop through VE-cadherin^{+} intermediates (cores), which generate primitive hematopoietic progenitors (PHPs). Progressive commitment to endothelial and hematopoietic fate is associated with downregulation of PDGFRα expression and primitive streak genes and upregulation of genes associated with angiohematopoietic development (*ETV2, TAL1, GATA2*) leading to formation of hematovascular mesodermal precursors (HVMPs). HVMPs are highly enriched in cells forming hemato-endothelial clusters on OP9. After gaining VE-cadherin expression, cells gradually acquire endothelial or hematopoietic cell morphology and gene expression profile. The earliest hematopoietic progenitors that emerge from within the VE-cadherin^{+} population display CD235a^{+}CD43^{low} phenotype and possess FGF2/hematopoietic cytokine-dependent erythromyeloid potential. Although these cells have primary hematopoietic characteristics, they retain endothelial potential and are therefore designated as angiogenic hematopoietic progenitors (AHPs). Expression of CD73 within VE-cadherin^{+}CD235a/CD43$^{–}$ population discriminates non-hemogenic endothelial progenitors (non-HEP) and HEPs. HEPs do not form hematopoietic CFCs in semisolid medium but are capable of generating definitive multipotential hematopoietic progenitors (MHP) and hematoendothelial clusters when cultured on OP9. Progressive hematopoietic differentiation is associated with significant upregulation of CD43 expression, acquisition of CD41a and/or CD45 markers and loss of endothelial potential. Also see **Table 1**.

and Leukosialin): (1) CD73^{-}CD235a/CD43^{-}; (2) CD73^{+} CD235a/CD43^{-}; and (3) CD73^{-}CD235a^{+}CD43^{low}CD41a^{-} cells. Although all of these subsets were capable of generating endothelial cells after culture on fibronectin in endothelial media, analysis of their hematopoietic and endothelial potentials using hematopoietic CFC assay and secondary co-culture with OP9 revealed that these newly identified VE-cadherin^{+} subsets have distinct functional properties. Freshly isolated VE-cadherin^{+}CD73^{-}CD235a/CD43^{-} cells did not form colonies in hematopoietic CFC medium but were capable of generating endothelial cells and lin^{-}CD34^{+}CD43^{+}CD45$^{+/-}$CD38$^{–}$ multipotent definitive hematopoietic progenitors after co-culture with OP9. Based on these findings, we designated these cells as hemogenic endothelial progenitors (HEPs). Because VE-cadherin^{+}CD73^{+}CD235a/CD43$^{–}$ formed endothelial colonies but not hematopoietic cells after secondary culture with OP9, we designated these cells as non-HEPs. Molecular profiling and analysis of surface marker expression revealed that HEPs and non-HEPs could be also discriminated based on expression of CD226 and CD117 (c-kit). CD73$^{–}$ HEPs expressed CD226 or DNAM-1, a cell surface marker typically found on hematopoietic cells (NK and T cells, myeloid and megakaryocytic cells). They also expressed CD117 at an intermediate level.[42,43] In contrast, CD73^{+} non-HEPs lacked CD226 expression but expressed CD117 at a very high level. In vivo studies demonstrated that expression of *Runx1* distinguishes hemogenic and non-hemogenic endothelium within mouse AGM.[44] Similarly, HEPs isolated from hPSCs had a much higher expression of *RUNX1* as compared with non-HEPs (**Fig. 2B**).

Previously we demonstrated that expression of CD43 (leukosialin) during PSC differentiation defines hematopoietic progenitors with colony-forming potential, detectable using standard serum-containing semisolid medium supplemented with hematopoietic cytokines.[36] Expression of CD43 reliably separates hematopoietic progenitors from CD43^{-}CD31^{+} endothelial cells and CD43^{-}CD31^{-} mesenchymal

Table 1. Phenotypic and functional properties of angiohematopoietic and hematopoietic progenitors from hPSCs

Abbreviation and phenotype	Day	Definition	Endothelial potential	BL-CFC potential	Blood formation on OP9	Hematopoietic CFCs potential	
						SF FGF2+HC	Serum HC
PM $^{EMH}lin^{-}APLNR^{+}PDGFR\alpha^{+}$	3	Primitive posterior mesoderm enriched in cells expressing typical primitive streak and lateral plate/extraembryonic mesoderm genes	+	+	+	–	–
HVMP $^{EMH}lin^{-}KDR^{bright}APLNR^{+}$ $PDGFR\alpha^{low/-}$	4	Hematovascular mesodermal precursor expressing genes associated with lateral plate/extraembryonic mesoderm and angiohematopoietic commitment, but lacking the expression of primitive streak genes	+	–	+	–	–
HEP VE-cadherin^{+}CD73^{-} CD226^{+}CD235a/CD43^{-} CD117^{intermediate}	5	Hemogenic endothelial progenitors	+	–	+	–	–
non-HEP VE-cadherin^{+}CD73^{+}CD226^{-} CD235a/CD43^{-}CD117^{high}	5	Non-hemogenic endothelial progenitors	+	–	–	–	–
AHP VE-cadherin^{+}CD73^{-}CD43^{low} CD235a^{+}CD41a^{-}CD117^{-}	5	Angiogenic hematopoietic progenitors	+	–	NA	+++	Limited
EMkP VE-cadherin^{+}CD73^{-}CD43^{+} CD235a^{+}CD41a^{+}	5–8	Erythromegakaryocytic progenitors	–	–	NA	++	Limited
MHP lin^{-} CD34^{+}CD43^{+}CD45^{+}CD38^{-}	7–8	Multipotential hematopoietic progenitors that lack expression of hematopoietic lineage-specific hematopoietic markers (lin^{-})	–	–	NA	NT	+++

^{EMH}lin^{-}, lack of the expression of CD31, VE-cadherin endothelial, CD73 and CD105 mesenchymal/endothelial cell markers CD43 and CD45 hematopoietic cell markers; lin^{-}, lack of the expression of mature blood lineages markers; SF, serum-free; HC, hematopoietic cytokines; NA, not applicable; NT, not tested. Day indicates the day of differentiation at which progenitors are detected.

cells within hPSC-derived CD34^{+} population.[30,36] In our recent study, we found that cells expressing CD43 can be identified within an emerging VE-cadherin^{+} population as early as day 4 of differentiation. However, the expression of CD43 at this stage was low and best detectable using antibodies conjugated with high-resolution sensitivity fluorochromes.[37] The first CD43^{low} cells expressed the erythroid marker CD235a but lacked CD41a. CD41a^{+} cells were detected 1 d later within the CD235a^{+} population. The sequence of appearance of erythroid markers and CD41a in differentiating human ESCs was different from differentiating mouse ESCs, where expression of CD41a preceded the expression of TER119 erythroid marker.[45,46] The first cells expressing hematopoietic markers, i.e., VE-cadherin^{+}CD73^{-} CD235a^{+}CD43^{low}CD41a^{-} phenotype, had unique functional properties (see **Table 1**). They were capable of forming a broad spectrum of hematopoietic colonies in serum-free clonogenic medium containing FGF2 and hematopoietic cytokines, but not in serum-containing CFC medium. Interestingly, they also retained endothelial potential. Because VE-cadherin^{+}CD73^{-} CD235a^{+}CD43^{low}CD41a^{-} cells have primary hematopoietic CFC potential and phenotype, we designated these cells as angiogenic hematopoietic progenitors (AHP) rather than HEPs. This designation was also supported by molecular profiling data that revealed the lack of critical endothelial gene expression such as *CLDN5, CAV1, APOLD1, EMCN* and *SOX17* by AHPs (**Fig. 2B**).

Although AHPs expressed erythroid markers, they formed erythroid, myeloid and mixed colonies, indicating that they are multipotent hematopoietic cells, which include CFCs capable of forming: (1) FGF2 and erythropoietin (EPO)-dependent large erythroid colonies; (2) FGF2, EPO and stem cell factor (SCF)-dependent mixed colonies composed of erythroid and megakaryocytic cells and macrophages; (3) FGF2 and IL3-dependent myeloid colonies; and (4) megakaryocytic colonies, which preferentially develop in cultures lacking EPO.

Defining Hemangioblasts and Mesodermal Stage of Development in PSC Differentiation Cultures

HBs are considered as multipotent progenitors with endothelial and hematopoietic potential. HBs were defined by Murray as

yolk sac mesenchyme (mesoderm) aggregates from which endothelial and blood cells develop.[12] However, in modern literature, the term "hemangioblast" is applied very broadly to describe any type of embryonic or adult cells with endothelial and hematopoietic potentials, including hemogenic endothelium. After the discovery of BL-CFCs by the Keller group,[47,48] the term "hemangioblast" has come to be used for bipotential cells generated within the posterior primitive streak in the mouse embryo or its equivalent during ESC differentiation.[49,50] The broad definition of HBs often lead to confusion regarding the type of cells studied and make it difficult to discriminate between the distinct stages of angiohematopoietic development during hPSC differentiation. For example, multiple cell populations identified in our studies have both endothelial and hematopoietic potential, and therefore could be defined as HBs. Therefore, we suggest to limit the use of the term "hemangioblast" to describe multipotential mesodermal rather than endothelial cells, as originally proposed by Murray.[12] During hPSC differentiation, mesoderm can be defined as a stage of development when cells express the mesodermal marker, APLNR, but lack the expression of typical endothelial, mesenchymal and hematopoietic markers ($^{EMH}lin^{-}$ cells; **Fig. 1**). Since BL-CFCs represent the best-characterized clonogenic mesodermal cells, with endothelial and hematopoietic potentials, and have a defined in vivo equivalent,[49] it would be wise to further restrict the use of the term "hemangioblast" to mesoderm with BL-CFC potential, as originally described by the Keller group.[47,48] As we showed, the potential to form BL colonies in serum-free medium in response to FGF2 discriminates PM from more mature mesodermal cells (HVMPs), which are also capable of generating blood and endothelial cells, but require serum and hematopoietic cytokines for differentiation.[37]

We identified FGF2 as the most critical factor required for HB development in vitro,[31] which is consistent with in vivo studies demonstrating the essential role of FGF2 in HB induction.[51] Interestingly, the sequence of events leading to HB-colony development in vitro highly resembles the events observed in vivo during embryonic

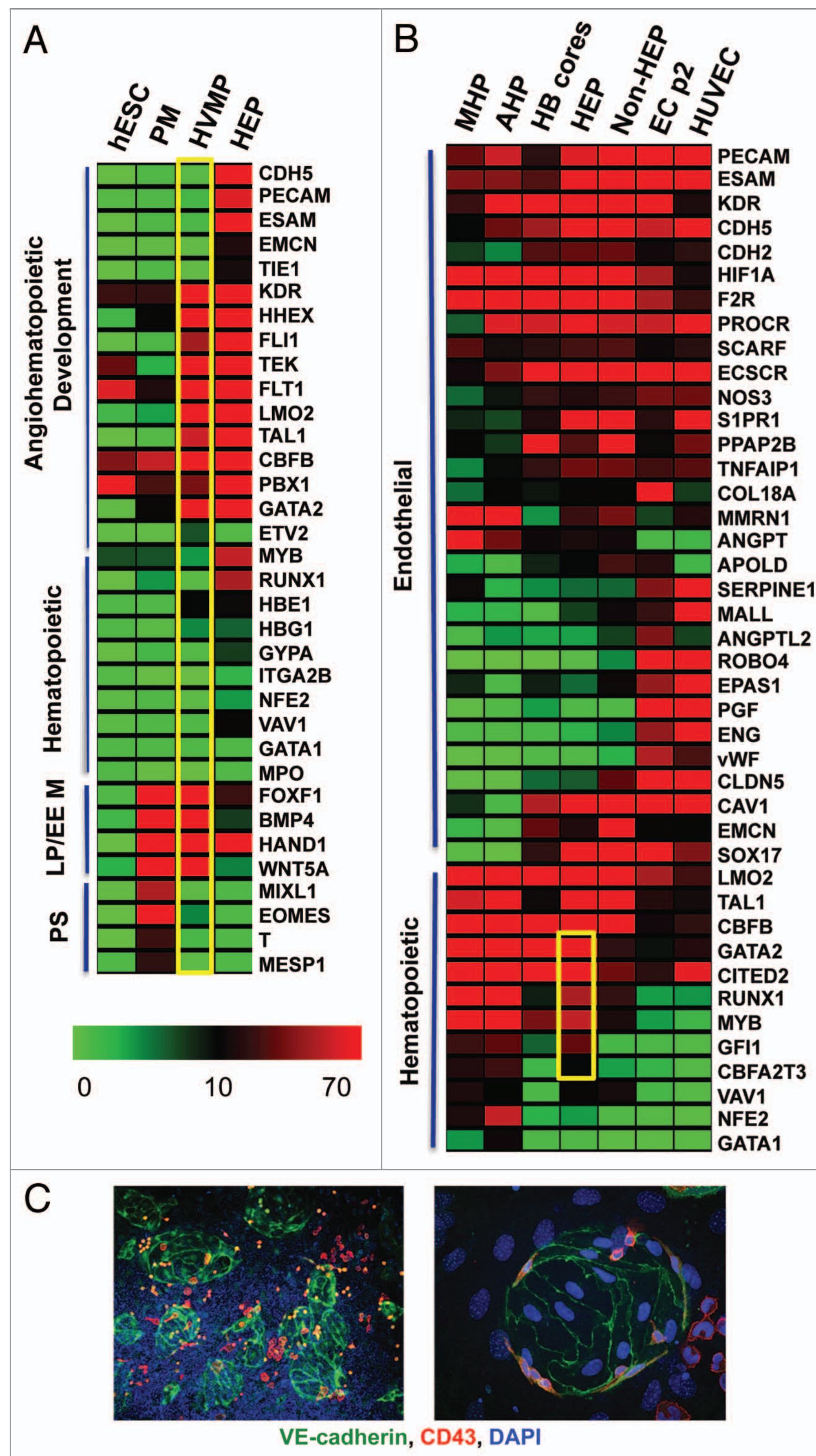

Figure 2. Characterization of hPSC-derived angiohematopoietic progenitors. (**A**) Heat maps of selected genes to demonstrate the key transcriptional features of PM and HVMPs. LP/EE M is lateral plate/extraembryonic mesoderm. PS is posterior streak. (**B**) Heat maps of selected genes to demonstrate the key transcriptional features of HEPs and non-HEPs. Outlined yellow box emphasizes the most critical genes differentially expressed in HEPs and non-HEPs. EC p2 is second passage of endothelial cells obtained from day 8 $CD31^{+}CD43^{-}$ differentiated H1 hESCs. HUVEC human umbilical vein endothelial cells. See **Table 1** for other abbreviations. The gene expression levels are estimated in terms of "transcripts per million." (**C**) Formation of hematoendothelial clusters by HVMPs on OP9. Immunoflourescent staining with VE-cadherin and CD43 antibodies is shown.

development. In the chicken embryo, activation of FGF-signaling leads to the aggregation of migrating mesodermal cells adjacent to the endoderm, upregulation of VEGFR2 (KDR) expression and subsequent formation of angioblasts and hemangioblasts.[52-54] Similarly, our in vitro studies demonstrated that PM cells display high motility when placed in FGF2-containing clonogenic medium. In response to FGF2, PM 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$^+$ epithelioid cells (cores). Subsequently, VE-cadherin$^+$ core-forming epithelioid cells undergo endothelial-to-hematopoietic transition, giving rise to CD235a$^{+/-}$ CD41a$^{+/-}$ primitive blood cells.[31] Although our and other studies demonstrated that BL-CFCs (HBs) produce mostly primitive hematopoietic cells,[37,50] the possibility that HBs have definitive hematopoietic potential cannot be entirely excluded. It is possible that BL-CFCs have both primitive and definitive hematopoietic potential, but our culture conditions used for HB detection selectively support development of primitive hematopoietic cells and inhibit HBs maturation into definitive hematopoietic cells. In order to test this hypothesis, we would need to isolate BL-CFCs and culture them in conditions that support definitive hematopoietic cell differentiation. Unfortunately, these studies can't be performed at present time, since HB-specific markers are currently unknown. Although selection of APLNR$^+$ or KDR$^+$ cells from day 3 differentiated hESCs significantly enriches for BL-CFCs, the percentage of BL-CFCs within these populations remain a low 1.5–4%.[31,38]

Defining Endothelial Progenitor Stage in PSC Differentiation Cultures

Since VE-cadherin plays essential role in maintaining of vascular integrity and is considered as one of the most specific marker for endothelial cells,[40,41,55] we defined the beginning of endothelial lineage development in PSC differentiation cultures by the upregulation of VE-cadherin. The emerging VE-cadherin$^+$ cells generated from PSCs represent endothelial progenitors, which should not be equated entirely with endothelial cells forming the lining of already established blood vessels in the embryo. During development, the earliest VE-cadherin$^+$ cells are detected within the Flk1+ mesoderm in the extraembryonic region (the future yolk sac) of E6.75 gastrulating mouse embryo.[56] Although these cells coexpress CD31, CD34, Tie2 and endoglin, they are considered as mesoderm, since they form masses of morphologically indistinguishable mesodermal cells rather than distinct vascular structures. The similar type of VE-cadherin$^+$ cells was detected within visceral yolk sac mesoderm at later stages of development (E7.0–7.5).[56,57] VE-cadherin$^+$ cells at this location are bipotential and capable of generating primitive and definitive hematopoietic and endothelial cells.[56,57] When the rudimentary intraembryonic circulatory system is established in the mouse between E8.0 and E8.5, VE-cadherin is detected in the endocardial primordia, definitive endocardium and aortic primordia (loosely associated cords of primordial endothelial cells formed by segregation from mesoderm at initial stages of vasculogenesis).[58] These data indicate significant functional diversity of emerging VE-cadherin$^+$ cells in the embryo. Similarly, our studies revealed that VE-cadherin$^+$ cells generated from PSCs are phenotypically and functionally diverse. However, we consider all of them as angiogenic/endothelial progenitors, because all first emerging VE-cadherin$^+$ populations express typical endothelial genes and possess endothelial potential. Following acquisition of VE-cadherin expression, cells become smaller compared with HVMPs and eventually acquire typical endothelial or hematopoietic morphology, indicative of maturation and terminal differentiation. The markers that discriminate VE-cadherin$^+$ progenitors from mature endothelial cells remain largely unknown. Since vWF is not detected in emerging endothelial cells, but rather upregulated in endothelial cell cultures generated from early VE-cadherin$^+$ cells (**Fig. 2B**), its expression may be helpful to discriminate immature and mature endothelial cells.

CD235a erythroid marker is detected within the earliest VE-cadherin$^+$ cells, designated as AHPs. These cells have the capacity to form myeloid and erythroid colonies, which morphologically resemble BL-CFC (HBs) colonies. In addition, similar to HBs, AHPs possess endothelial potential and require FGF2 for colony formation. Because of these properties, they could easily be confused with HBs. However, in contrast to HBs, colony development from AHPs is hematopoietic cytokine-dependent and does not proceed through endothelial core stage. The in vivo equivalent of AHPs remains unclear. Because AHPs have broad erythroid and myeloid differentiation potential, they may represent a transient wave of definitive erythromyeloid hematopoiesis, similar to the one described in mouse yolk sac.[59]

Hemogenic Endothelium as a Platform for the Study HSC Development In Vitro

Identifying immediate precursors of blood cells from differentiating hPSCs will be critical to model blood development in vitro. This system especially provides an opportunity to identify the factors required for blood cells to acquire self-renewing potential, i.e., HSC properties, as they emerge from endothelium. Recently, other groups have demonstrated the endothelial origin of blood cells during hPSCs differentiation. Using hESCs engineered to express fluorescent reporters under VE-cadherin or CD41a promoter, the James group was able to directly observe the formation of round blood cells from VE-cadherin$^+$ epithelioid precursors.[60] The Keller group demonstrated that CD43$^-$ cells expressing endothelial marker can be differentiated into T cells, indicating that HE generated in vitro are multipotent and have lymphoid potential.[61] However, it remains to be determined whether we can recapitulate somatic HSC specification from HE in vitro as it occurs in vivo. Several studies have reported hematopoietic engraftment from hESC- and iPSC-derived hematopoietic cells.[62-67] Although human cells were detected in the recipient bone marrow 3–6 mo after transplantation of differentiated hESCs to immunecompromised mice, the engraftment rate was very low

and mostly restricted to the myeloid lineage. In the mouse system, development of HSCs with robust engraftment potential from mESCs requires the overexpression of *HoxB4* or *Cdx4* genes to specify ESC derived cells toward HSC fate.[68,69] Several groups engineered human ESCs to overexpress *HOXB4*.[70,71] However, hematopoietic cells generated from these cell lines failed to engraft. Because specification of mouse ESCs to HSCs can be achieved only within particular differentiation window (the stage at which CD41a$^+$c-kit$^+$ cells emerge),[70-72] it is possible that HOXB4-sensitive window and stage of differentiation was missed and/or not appropriately targeted in human cells. It is also possible that species-specific differences exist between human and mouse ESCs. Our molecular profiling studies demonstrated that hESC-derived hematopoietic progenitors already express a high level of *HOXB* cluster genes, while *HOXA* cluster genes are significantly reduced as compared with phenotypically similar hematopoietic cells from fetal liver.[73] Therefore, different *HOX* genes in humans may be required for respecification of primitive hematopoietic cells toward definitive HSCs. It should be noted that Hoxb4-induced HSCs from mouse ESCs are not entirely similar to somatic HSCs. ESC-HSCs lack HoxA9 expression and transcriptional response to Notch signaling.[74] Hematopoiesis from these cells is skewed toward the myeloid lineage,[68] indicating that HoxB4-induced cells are not completely transformed into somatic HSC-like cells. Further studies are needed to determine the factors necessary to complete the somatic HSC program in the developing hematopoietic cells from in vitro-generated HE.

In conclusion, identification of HEPs and distinct stages of angiohematopoietic development from hPSCs in our studies establishes a platform for further investigation of stage-specific molecular pathways required for the specification and expansion of HSCs from hPSCs.

Disclosure of Potential Conflicts of Interest

I.S. is a founder, stock owner, and consultant of Cellular Dynamics International (CDI). I.S. is also a founder, stock owner and serves on the Board of Directors of Cynata.

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