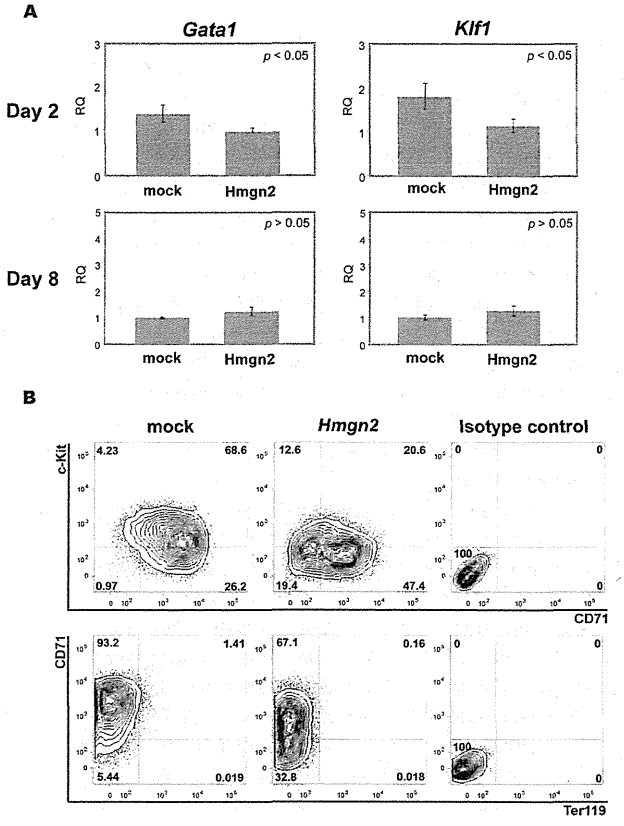

Figure 2 Ectopic expression of *Hmgn2* alters phenotypes of Friend erythroleukaemia cells

(**A**) Expression of transcription factor genes *Gata1* and *Klf1* that regulate erythroid cell differentiation. Gene expression levels in GFP$^+$ cells were quantified by real-time PCR. The *y*-axis represents the RQ of gene expression after normalization with β-actin. Two days after electroporation, *Gata1* and *Klf1* levels in *Hmgn2*-transfected cells were down-regulated ($P<0.05$) (see Supplementary Figure S2C for experimental design). We observed no significant difference in level of *Gata1* and *Klf1* at day 8 of culture ($P>0.05$). (**B**) Flow cytometric analysis for surface markers of Friend erythroleukaemia cells. Surface markers of *Hmgn2*-transfected cells were analysed at day 8 of culture. The percentage of the c-Kit$^+$/CD71$^+$ population in *Hmgn2*-transfected cells (20.6%) was decreased compared with that seen in mock controls (68.6%), whereas the percentage of the c-Kit$^+$/CD71$^-$ population in *Hmgn2*-transfected cells (12.6%) was higher than that seen in mock-transfected cells (4.23%).

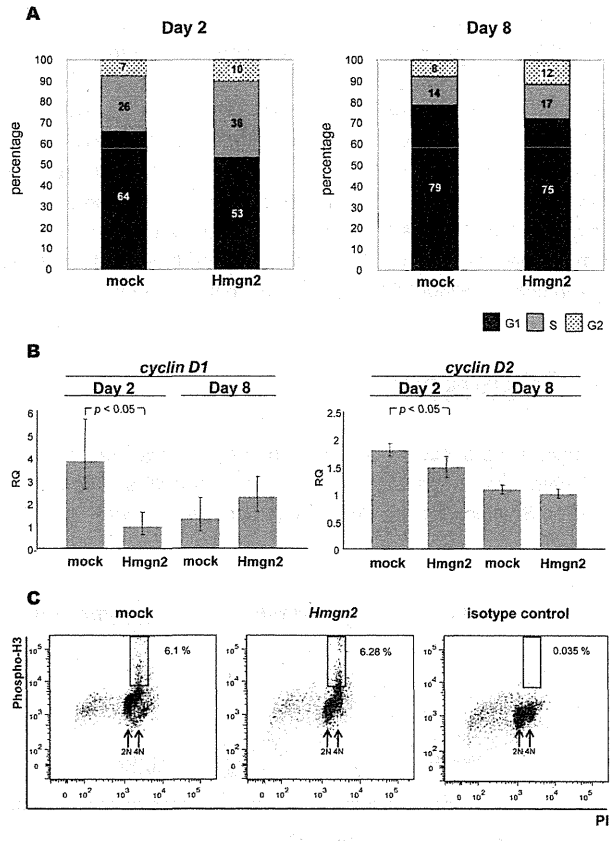

Figure 3 *Hmgn2* misexpression increases in the number of S-phase cells and down-regulates G_1-specific genes

(**A**) The percentage of cells in G_1, S and G_2 phases. Sorted GFP$^+$ cells at day 2 of culture and all cells at day 8 of culture were fixed and stained with PI. DNA content was quantified by flow cytometry. The Watson Pragmatic method was used to calculate cell percentages. (**B**) Expression of the G_1 phase-specific genes *cyclin D1* and *cyclin D2*. Gene expression was measured at days 2 and 8 of culture. The *y*-axis represents the RQ of gene expression after normalization with β-actin. (**C**) The percentage of mitotic status of *Hmgn2*-transfected cells. Sorted GFP$^+$ cells at day 2 of culture were fixed and stained with anti-phosphorylated histone H3 at Ser^{10} (phospho-H3) and PI (DNA content). Mitotic cells were defined as the cells that have 4N content of DNA and high level of histone H3 phosphorylation.

cells by flow cytometry was analysed, with evaluation of expression levels of the G_1 phase-specific genes, *cyclin D1* and *cyclin D2*. Two days after transfection with Friend erythroleukaemia cells with *Hmgn2*, 36% of GFP$^+$ cells were in S-phase, whereas 26% of mock controls were in S-phase ($P<0.05$; Figure 3A and Supplementary Figure S3 available at http://www.cellbiolint.org/cbi/036/cbi0360195add.htm). There was no significant difference in the percentage of cells in S-phase at day 8 of culture between *Hmgn2*-transfected and mock control cells (14 and 17%, respectively, $P>0.05$; Figure 3A). Two days after transfection, expression levels of both *cyclin D1* and *cyclin D2* assessed by real-time PCR were down-regulated in *Hmgn2*-transfected cells ($P<0.05$; Figure 3B). At day 8 of culture, there was no difference in expression levels of *cyclin D1* and *cyclin D2* between *Hmgn2*-transfected and mock control cells (Figure 3B).

To investigate mitosis in transfected cells, flow cytometric analysis was carried out of *Hmgn2*-transfected cells using anti-phosphorylated histone H3 at Ser^{10} (phospho-H3), an indicator of late G_2 to anaphase in the cells with 4N content of DNA. There was no significant difference at day 2 of culture in the percentage of mitotic cells between *Hmgn2*-transfected cells and mock control (6.3 and 6.1% respectively $P>0.05$; Figure 3C).

3.4. Hmgn2 expression antagonizes erythroid cell differentiation in FL

Hmgn2 expression in FL was down-regulated during erythroid differentiation (Figure 1A). To investigate whether Hmgn2 functions in erythroid differentiation in FL as well as erythroleukaemia cells, *Hmgn2* was ectopically expressed in MNCs isolated from FL at 12.5 dpc. Two days after transfection by electroporation, CD71$^+$/Ter119$^-$ cells expressing Hmgn2, as indicated by GFP

positivity, were sorted and cultured in the presence of SCF, IL-3 and EPO for 7 days. Flow cytometry showed that the percentage of c-Kit$^+$/CD71$^+$ cells in *Hmgn2*-transfected cells (26%) was higher than that seen in mock controls (15.2%), while the percentage of CD71$^+$/Ter119$^+$ cells in *Hmgn2*-transfected cells (68.4%) was lower than in mock controls (73.6%; Figure 4A). *Gata1* expression levels were 4.2-fold lower in *Hmgn2*-transfected cells than in mock controls, and *Klf1* expression was undetectable in *Hmgn2*-transfected cells (Figure 4B). These results indicate that ectopic expression of *Hmgn2* inhibits erythroid differentiation in mouse FL.

4. Discussion

Hmgn2 is ubiquitously expressed and down-regulated during the differentiation of several cell types in vertebrates (Crippa et al., 1991; Shakoori et al., 1993; Lehtonen and Lehtonen, 2001).

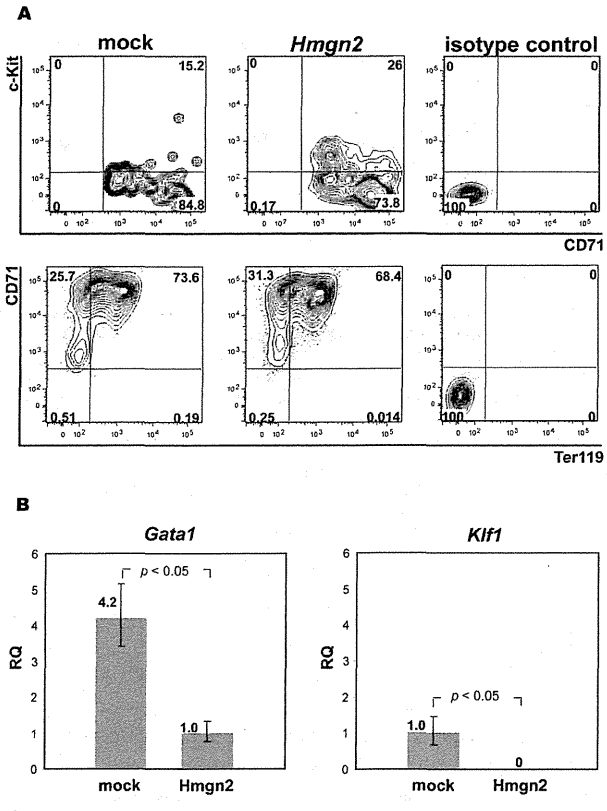

Figure 4 Ectopic expression of *Hmgn2* suppresses differentiation of erythroid progenitor cells by down-regulating *Gata1*

(**A**) Flow cytometric analysis of erythroid cells of FL MNCs transfected with *Hmgn2*/GFP expression vector. Two days after electroporation, GFP$^+$ CD71$^+$/Ter119$^-$ cells were sorted and cultured in the presence of SCF, IL-3 and EPO for 7 days. The percentage of c-Kit$^+$/CD71$^+$ cells in *Hmgn2*-transfected cells (26%) was higher than that seen in mock-transfected cells (15.2%), whereas the percentage of CD71$^+$/Ter119$^+$ cells in *Hmgn2*-transfected cells (68.4%) was lower than that seen in mock-transfected cells (73.6%). (**B**) Expression levels of *Gata1* and *Klf1* in erythroid cells were determined by RT–PCR. Two days after electroporation, *Gata1* was down-regulated in *Hmgn2*-transfected CD71$^+$/Ter119$^-$ cells compared with mock controls. *Klf1* expression was not detectable in these cells. Levels of gene expression were normalized to β-actin.

Relevant to HC (haemopoietic cell) development, injection of Hmgn2 protein into *Xenopus* embryos delayed the expression of mesoderm-specific genes (Korner et al., 2003). *Hmgn2* is also reportedly down-regulated during differentiation of erythroid cells in chicken embryos (Crippa et al., 1991) and in osteoblasts of mouse embryos (Shakoori et al., 1993). However, the role of Hmgn2 in mammalian HC development has remained unclear. The pattern of Hmgn2 gene and protein expression in mice suggests a function in erythroid differentiation (Figure 1). In mouse FL, *Hmgn2* was down-regulated during differentiation of BFU-E into reticulocytes and mature erythrocytes, when globin synthesis and enucleation occurs. Hmgn2 protein was expressed in HSCs and all stages of differentiated erythroid cells, while its expression level was down-regulated as BFU-E differentiates into mature erythrocyte. Hmgn2 therefore may regulate erythroid differentiation regardless of differentiation stage.

Friend erythroleukaemia cells are characterized as cells expressing c-Kit and CD71 (Friend 1957; Moreau-Gachelin 2008). Decrease in the number of c-Kit$^+$/CD71$^+$ cells (equivalent to CFU-E) and increase in c-Kit$^+$/CD71$^-$ cells (equivalent to BFU-E), in addition to down-regulation of *Gata1* and *Klf1* after transfection with *Hmgn2,* strongly suggest that Hmgn2 suppresses erythroid differentiation of Friend erythroleukaemia cells. During erythroid differentiation, Gata1 induces expression of CD71 and controls *Klf1* expression (Welch et al., 2004). Decrease in the number of c-Kit$^+$/CD71$^+$ cells and down-regulation of *Klf1* could be due to *Gata1* down-regulation (Figure 2A). We also showed that ectopic expression of *Hmgn2* in CD71$^+$/Ter119$^-$ FL MNCs suppresses their differentiation, indicated by the low percentage of CD71$^+$/Ter119$^+$ cells (CFU-E) and the high percentage of c-Kit$^+$/CD71$^-$ cells (BFU-E), accompanied by *Gata1* and *Klf1* down-regulation. These results are compatible with those seen in Friend erythroleukaemia cells and suggest that Hmgn2 suppresses erythroid differentiation in mice. Friend erythroleukaemia cells are comprised of c-Kit$^+$/CD71$^+$ and c-Kit$^-$/CD71$^+$ cells, equivalent to CFU-E and relatively mature cells (proerythroblasts and erythrocytes) respectively (Friend 1957; Moreau-Gachelin 2008; Supplementary Figure S4 available at http://www.cellbiolint.org/cbi/036/cbi0360195add.htm). Decrease of c-Kit$^+$/CD71$^+$ cells and increases of c-Kit$^+$/CD71$^-$, c-Kit$^-$/CD71$^+$ and c-Kit$^-$/CD71$^-$ cells were observed after transfection with *Hmgn2,* implying that differentiation arrest occurred at CFU-E. In mouse FL, expression level of *Hmgn2* declined from CFU-E to proerythroblast ($P<0.05$; Figure 1A). Hmgn2 may therefore function particularly at CFU-E during erythroid differentiation.

To investigate how Hmgn2 down-regulates erythroid differentiation, we analysed cell cycle status and related gene expression in *Hmgn2*-misexpressing cells. Following *Hmgn2* transfection at 2 and 8 days of culture, the percentage of Friend erythroleukaemia cells in S-phase increased, whereas the percentage of cells in G_1 decreased (Figure 3A). Cyclins D1 and D2 are G_1 phase-specific cyclins predominantly expressed in human, chicken and mouse erythroid cells (Dolznig et al., 1995; Dai et al., 2000). Observation of down-regulation of *cyclin D1* and *cyclin D2* genes 2 days after *Hmgn2* transfection is compatible with a low percentage of cells in G_1 and a high percentage in S-phase (Yang et al., 2006). Hmgn2 reportedly enhances DNA

replication of M13 DNA containing SV40 (simian virus 40) origin *in vitro* (Vestner et al., 1998). The data suggests that Hmgn2 does not function in mitosis of Friend erythroleukaemia cells (Figure 3C). It will be necessary to investigate whether Hmgn2 functions in mitosis during erythroid differentiation.

During erythroid differentiation, erythroblasts lose their proliferation capability and exit the cell cycle (Buttitta and Edgar, 2007) and condense chromatin. However, we do not know what is the key regulator underlying these molecular mechanisms. Our novel finding is that Hmgn2 is involved in erythroid differentiation. Therefore, further study of Hmgn2 should enable us to investigate the role of DNA replication and cell mitosis in erythroid differentiation. In conclusion, Hmgn2 appears to reduce compaction of chromatin fibres and facilitates accessibility of DNA polymerase to nucleosomes, enhancing DNA synthesis (Vestner et al., 1998; Bustin, 2001). An increase in the number of Friend erythroleukaemia cells in S-phase and a decrease of those in G_1 after *Hmgn2* misexpression might be due to enhanced DNA replication and/or blocking entry of mitosis in S-phase that promotes down-regulation of erythroid differentiation.

5. Conclusion

We have shown that ectopic expression of *Hmgn2* altered differentiation of mouse erythroid cells *in vitro*. In Friend erythroleukaemia cells, a decrease in relatively mature c-Kit$^+$/CD71$^+$ erythroid cells and an increase in immature c-Kit$^+$ CD71$^-$ erythroid cells occurred, whereas in FL HCs there was also a decrease in relatively mature CD71$^+$/Ter119$^+$ erythroid cells and an increase in relatively immature c-Kit$^+$/CD71$^+$ erythroid cells. An increase in the number of S-phase cells and a decrease in the number of G_1-phase cells in erythroleukaemia suggests that Hmgn2 antagonizes mouse erythroid differentiation may be due to enhancement of DNA replication and/or blocking entry of mitosis in S-phase.

Author Contribution

Kasem Kulkeaw performed the research and wrote the manuscript. Tomoko Inoue, Chiyo Mizuochi, Yuka Horio and Yasushi Ishihama performed the research. Daisuke Sugiyama designed and performed the research and wrote the manuscript.

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Molecular Pathways Governing Development of Vascular Endothelial Cells from ES/iPS Cells

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Abstract Assembly of complex vascular networks occurs in numerous biological systems through morphogenetic processes such as vasculogenesis, angiogenesis and vascular remodeling. Pluripotent stem cells such as embryonic stem (ES) and induced pluripotent stem (iPS) cells can differentiate into any cell type, including endothelial cells (ECs), and have been extensively used as in vitro models to analyze molecular mechanisms underlying EC generation and differentiation. The emergence of these promising new approaches suggests that ECs could be used in clinical therapy. Much evidence suggests that ES/iPS cell differentiation into ECs in vitro mimics the in vivo vascular morphogenic process. Through sequential steps of maturation, ECs derived from ES/iPS cells can be further differentiated into arterial, venous, capillary and lymphatic ECs, as well as smooth muscle cells. Here, we review EC development from ES/iPS cells with special attention to molecular pathways functioning in EC specification.

Keywords Endothelial cells · Differentiation · Molecular pathways · Embryonic stem cells · Induced pluripotent stem cells

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Introduction

Diverse pathways give rise to the vertebrate circulatory system, which forms early in embryogenesis. Vascular ECs initially differentiate from mesoderm-derived angioblasts, which form a primitive vascular plexus at or near their sites of origin during the onset of vascularization (vasculogenesis) [1]. Arterially-fated angioblasts adjacent to the endoderm form the first artery (the dorsal aorta) ventrally, whereas venous-fated angioblasts migrate dorsally and form the first vein (the cardinal vein) adjacent to the neural tube [2]. The angioblast develops to ultimately form arterial and venous ECs, depending on activation of specific molecular pathways and expression of other factors. Through a sequential maturation process, arterial and venous ECs become functionally specified, giving rise to functional blood vessels such as arteries, veins and capillaries [3]. Arteries transport oxygen-rich blood to all tissues, and veins bring oxygen-depleted blood back to the heart. Arteries are thicker-walled vessels surrounded by multiple layers of vascular smooth muscle cells. Veins are thinner-walled vessels with little smooth muscle and exhibit valves to prevent backflow of blood (Fig. 1).

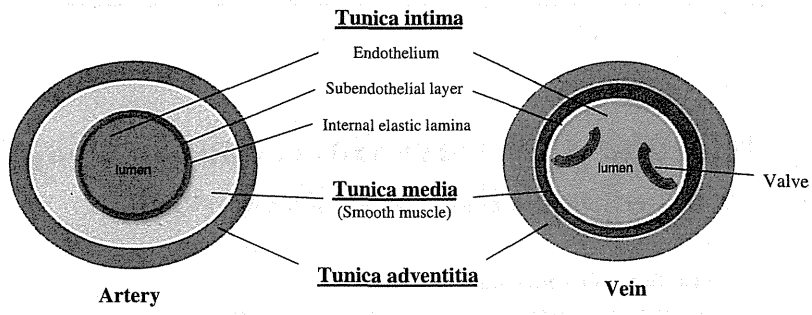

Fig. 1 Morphology of arteries and veins in the vascular network. Depicted are schematics representing cross-sections of arteries and veins. Arteries exhibit thicker smooth muscle walls compare to veins, while by comparison veins have a thicker tunica adventitia

Key Regulators of the Vascular Network

Two mechanisms are central to formation of the vascular network: vasculogenesis and angiogenesis [1]. Vasculogenesis gives rise to anatomically and physiologically distinct vessels such as arteries and veins, whereas angiogenesis is a process in which new capillaries form from the parent vessel [1, 4]. Arterial and venous ECs have been molecularly defined and are specified embryonically prior to establishment of the circulation or tubulogenesis (the formation of a branching structure from specific ducts) [3]. Arterial ECs are characterized by expression of Ephrin-B2 ligand, which binds to the EphB4 receptor expressed on venous ECs to regulate arterial-venous EC specification before the circulatory system is established [5, 6]. In addition, as they form primitive blood vessels exhibiting lumens, venous ECs further differentiate into lymphatic ECs in the presence of specific stimuli mentioned in this review.

Molecular pathways underlying EC lineage commitment and differentiation into mature cell types have been widely studied using animal models and ES/iPS cells [7–10]; however, how these complex signaling pathways and downstream molecules act together in arterial-venous EC specification remains to be fully understood. Multistep regulatory systems implicated in arterial, venous and lymphatic EC specification are summarized in Fig. 2 and reviewed below.

Arterial EC Specification

The Sonic Hedgehog (SHH) Signaling Pathway

SHH is the mammalian homologue of the *Drosophila* hedgehog protein. In flies, hedgehog is the ligand activating a pathway composed of the Patched receptor, the signal transducer Smoothened and a cytoplasmic component, Cubitus interruptus (also known as GLI3 protein in mammals), which is tethered to the Smoothened carboxyl tail and plays an indispensable role in governing cell fate and embryonic patterning [11, 12]. Patched forms a complex with the seven-pass membrane protein, Smoothened, maintaining it in an inactive form. In flies, Hedgehog-bound Patched receptor dissociates from Smoothened, allowing activated Smoothened to disengage from Cubitus interruptus, which then enters the nucleus to activate downstream targets [13]. Wild-type zebrafish embryos exposed to cyclopamine, a hedgehog inhibitor, or treated with *vegf* anti-sense morpholinos show down-regulation of artery-specific markers such as Ephrin-B2, while microinjection of *vegf* mRNA into zebrafish embryos lacking Shh activity rescues arterial EC specification [8]. These observations suggest that the SHH pathway acts upstream of VEGF in arterial EC specification in zebrafish [8]. However, in zebrafish embryos in which Notch signaling is blocked, injection of *vegf* mRNA fails to induce expression of arterial markers, such as Ephrin-B2 and *notch5* (also known as *notch3*), suggesting that VEGF acts downstream of SHH but upstream of Notch signaling [14].

Vascular Endothelial Growth Factor (VEGF)

VEGF family proteins stimulate vasculogenesis and angiogenesis and through EC proliferation, survival and migration. In mammals, the family is composed of VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PIGF) [15]. VEGF-A and its receptor VEGFR-2 and co-receptor neuropilin-1 (NP-1) are involved in arterial EC specification [16]. VEGF-A binding to VEGFR-2 stimulates receptor auto-phosphorylation and rapid recruitment of SH2-containing intracellular signaling molecules such as phosphoinositide 3-kinase (PI3Ks), mitogen-activated protein kinase (MAPK)/ERK, Src family tyrosine kinases, Ras, phospholipase Cγ1, Shc, Grb2 and Nck adaptor molecules [17]. In humans and mice, VEGF-A exists in at least three isoforms: $VEGF_{120}$, $VEGF_{164}$ and $VEGF_{188}$ in mice, and $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ in humans [18, 19]. Each reportedly functions uniquely in vascular development, as they differ in receptor binding, matrix association and angiogenic activity [20]. Transgenic mice that selectively express single isoforms of $VEGF_{120}$ and $VEGF_{164}$ show normal arterial development in retina, but those expressing an isoform of $VEGF_{188}$ exhibit normal venular outgrowth with impaired arterial development [21], suggesting that $VEGF_{120}$ and $VEGF_{164}$ drive differentiation toward arterial EC specification, whereas $VEGF_{188}$ governs venous EC specification [21]. Loss of $VEGF_{164}$ in mice down-regulates Ephrin-B2 expression, suggesting that $VEGF_{164}$ accelerates arterial EC specification [21].

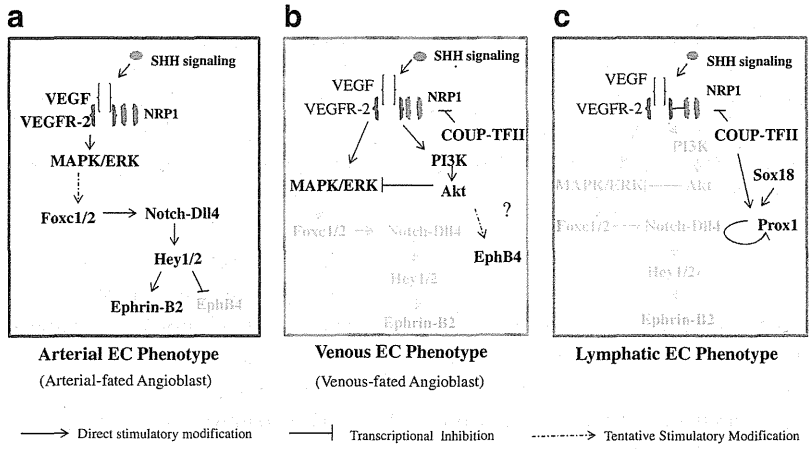

Fig. 2 Model of arterial, venous and lymphatic EC specification. **a** Arterial-fated angioblast forms arterial ECs in the presence of Shh molecules that induce the signaling protein vascular endothelial growth factor (VEGF). VEGF interacts with VEGFR-2 and *neuropilin1 (NRP1)* activating Notch-Dll4 and downstream Notch signaling to induce arterial phenotypes. **b** The venous-fated angioblast forms venous ECs in the presence of COUP-TFII by suppressing expression of Notch-Dll4 and their effectors and NRP1. **c** The transcription factor Sox18 cooperates with COUP-TFII to activate expression of the homeobox transcription factor Prox1. Prox1 expression becomes independent of external stimuli and regulates its own expression to maintain lymphatic EC identity

Notch Signaling

Notch signaling is highly conserved among species, and it regulates the fate of numerous embryogenic tissues [22]. There are 4 types of Notch receptors (Notch1, 2, 3 and 4) and several ligands of the Delta-like (Dll) and Jagged families. A Notch effector, HERP (HES-related repressor protein; also called Hey/Hesr/HRT/CHF/gridlock) plays critical roles in promoting arterial EC specification [23–25]. Notch receptors 1 and 4 and their ligand delta-like 4 (Dll4) are specifically detected in Ephrin-B2-positive ECs of the dorsal aorta and umbilical artery [26]. Dll4 was originally identified as a ligand for Notch1 and Notch4 during vascular development [27]. In fact, although Notch1 and Dll4 reportedly function in arterial EC specification, Notch4 does not [27, 28]. In addition, *Dll4*-ablated mouse embryos show a reduction in the diameter of the dorsal aorta, and arterial ECs exhibit an irregular morphology, suggesting that *Dll4* functions in arterial EC specification [2].

In vitro analysis indicates that VEGF-A induces *Notch1* and *Dll4* expression in human arterial ECs through the PI3Ks/Akt pathway [28]. By contrast, in zebrafish embryos, chemical inhibition of the PI3Ks/Akt pathway by GS4898 induces arterial EC specification by activating MAPK pathways [29]. These opposing outcomes are likely due to the difference of species. Furthermore, in mouse pulmonary ECs, Foxc1 and Foxc2 up-regulate expression of *Dll4* and *Hey2* (a HERP homologue) through binding to their promoter regions, suggesting that Foxc induces arterial EC specification through Notch signaling [30]. *Hey2*-ablated mice die by postnatal day 10 due to severe cardiac defects but show no obvious abnormality in ECs, while *Hey1*-ablated mice develop normally [23, 31–33]. However, *Hey1/2* double knockout mutants die by embryonic day (E) 9.5 due to failure of vascular remodeling and exhibit low expression of arterial EC markers such as Ephrin-B2, NP-1 and CD44 [23]. Over-expression of *Hif1a* (hypoxia-inducible factor 1-alpha) in T17b EC progenitor lines up-regulates *Dll4* and *Hey2* and down-regulates *COUP-TFII* (a regulator of venous EC specification), implying that hypoxia determines arterial EC specification [34].

Venous EC Specification

COUP-TFII

The orphan nuclear receptor, COUP-TFII (chicken ovalbumin upstream-transcription factor II; official gene symbol; *Nr2f2*) is detected in the cardinal vein but not in the dorsal aorta in the mouse embryo by immunohistochemistry and genetic approach using *COUP-TFII/lacZ* 'knock-in' mouse model [35, 36]. COUP-TFII governs venous EC specification by suppressing expression of arterial specific genes, such as *NP-1* and *Notch1* [35]. Endothelial-specific *COUP-TFII* knockout mice (*Tie2*-Cre/+; *COUP-TFII*$^{flox/flox}$ mice) exhibit thin and well-dilated vessels and die of hemorrhage by E12 [35]. In the veins of these mutant mice, expression of arterial EC markers such as NP-1, Jagged1, Notch 1, HESR-1 (*Hey1*) and Ephrin-B2 appear in venous ECs [35]. Immunohistochemical staining of expression of the venous EC marker EphB4 indicates reduced expression, suggesting that *COUP-TFII* acts at upstream of NP-1 to suppress Notch signaling in venous EC specification [35]. In addition, endothelial cell-specific overexpression of *COUP-TFII* in transgenic mouse embryos impairs angiogenesis in the yolk sac and down-regulates

expression of Jagged1 as detected by immunohistochemical staining in aortic ECs, promoting embryonic lethality by E10-E11.5 [35]. These phenotypes resemble those displayed by *NP-1* and *Notch1* knockout embryos, suggesting that COUP-TFII functions upstream of NP-1 and Notch1 to regulate arterial-venous EC specification.

Capillary EC Formation

Capillaries, which are the smallest blood vessels, connect arteries and veins. Although EC studies relevant to capillaries have emerged in the past decade [37], how capillary ECs are specified remains elusive. Capillaries form a large network of capillary beds (collections of capillaries) supplying oxygen and nutrients to tissue [38]. The three structurally different types of capillaries include continuous capillaries, fenestrated capillaries and sinusoidal capillaries (sinusoids) (Table 1) [39]. Among them, sinusoids exhibit a discontinuous basal lamina enabling formation of open pores, but lack a diaphragm [40]. Such open pores increase capillary permeability, allowing transport of small molecules [40]. Liver sinusoidal endothelial cells line liver sinusoids and allow medium-sized proteins such as albumin to be transported into the circulation [41]. Liver sinusoidal endothelial cells also exclude large circulating particles such as blood cells, chylomicrons and platelets [41]. In addition, liver sinusoidal endothelial cells reportedly clear the macromolecule hyaluronan from the circulatory system and critically regulate liver regeneration by secreting angiogenic factors such as wingless-related MMTV integration site 2 (Wnt2) and hepatocyte growth factor (HGF), which promote hepatocyte proliferation [42]. The lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1), a member of the scavenger receptor family, is expressed on embryonic blood vessels [43] and liver sinusoidal endothelial cells surface (Fig. 3). LYVE-1 is not expressed on other hepatic cells or on the conventional endothelium [44].

Cysteine-rich transmembrane BMP regulator 1 (CRIM1) mRNA and protein are expressed in human capillary ECs that also express platelet endothelial cell-adhesion molecule (PECAM)-1 (also known as CD31) [45]. *CRIM1* knockdown with antisense oligonucleotides in cultured human umbilical vein ECs impairs capillary formation in Matrigel assay (a method for quantifying angiogenesis), implying that *CRIM1* functions in capillary formation in vivo [45]. In addition, capillary formation, particularly sprouting, is induced in human umbilical vein EC spheroids in collagen gels following bone morphogenetic protein (BMP)-4 treatment, via both Smad and Extracellular-Signal Regulated Kinase (ERK) signaling [46]. Blocking ERK signaling with a *MEK* inhibitor impairs capillary formation in vitro, suggesting that the ERK pathway is essential for capillary formation through BMP signaling.

Lymphatic EC Specification

The specification of lymphatic ECs occurs after arterial-venous EC specification [47]. The lymphatic network forms a second vascular system, which complements the vascular network by modulating tissue fluid balance, allowing interstitial protein transport, and initiating the immune response [48]. The network is composed of a highly branched network of lymphatic capillaries and vessels [48]. Lymphatic capillaries are blind-ended vessels comprised of a single, non-fenestrated EC layer, whereas lymphatic vessels are lined with ECs and exhibit a thin wall with valves to transport fluid to the lymph node [48, 49]. After venous EC specification, a subpopulation of venous ECs is able to acquire a lymphatic EC fate by expressing the transcription factors *Sox18* (SRY (sex determining region Y)-box 18) and *Prox1* (Prospero-related homeobox 1, a homeobox transcription factor) [50].

SOX-18 is first expressed in ECs lining the dorsolateral sector of the cardinal vein at E9 in mice, approximately 1.5 day prior to the onset of PROX-1 expression [51]. Lymphatic ECs expressing *Prox1* reportedly sprout from intersomitic vessels as well as the cardinal vein to form lymph sacs [52]. In *Vegfc*-deficient embryos, lymphatic ECs failed to

Table 1 The vascular-capillary network: summary of characteristics of continuous, fenestrated and sinusoidal capillaries

Characteristics	Capillaries		
	Continuous	Fenestrated	Sinusoidal
Morphology	Uninterrupted lining	Have pores (60–80 nm in diameter)	Larger pores (30–40 μm in diameter)
Diaphragm	Yes	Yes (slit pores)	No
Basal lamina	Continuous	Continuous	Discontinuous
Molecules and cells	Water, ions	Small molecules, limited amount of proteins	Red blood cells, white blood cells, serum proteins
Transport	Diffusion	Diffusion	Gap Junctions
Location	Skeletal muscle, fingers, gonads, skin, blood–brain-barrier	Endocrine glands, intestine, pancreas, kidney glomeruli	Bone marrow, liver, lymph nodes, adrenal gland, spleen

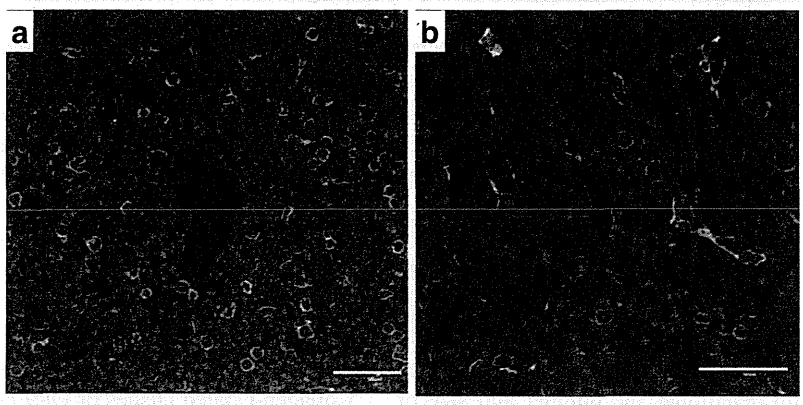

Fig. 3 Immunohistochemistry of fetal liver. Section of fetal liver were prepared from ICR mouse embryos at E14.5, stained with the indicated antibodies and observed under confocal microscopy. **a** Confocal image of hematopoietic stem cells and progenitors expressing c-Kit (*green*; hematopoietic stem cells), Lyve-1 (*red*; sinusoidal endothelial cells) and TOTO-3 (*blue*; nuclear staining). **b** Higher magnification image of fetal liver tissue expressing Lyve-1 (*green*; sinusoidal endothelial cells), c-Kit (*red*; hematopoietic stem cells) and TOTO-3 (*blue*; nuclear staining). *Scale bars* indicate 50 (**a**, **b**) micrometers

sprout, as observed by immunohistochemistry of the jugular region using lymphatic EC markers such as VEGFR-3, LYVE-1 and podoplanin [53]. In addition, VEGF-C (a VEGFR-3 ligand) rescues lymphatic EC sprouting in whole-mount explants of this mutant [53]. In addition to SOX18, COUP-TFII is required to activate *PROX1* through direct binding to the *Prox1* promoter region in a study using cultured human lymphatic ECs [54]. Proximity ligation assays indicate that endogenous PROX1 and COUP-TFII interact in the nuclei of human dermal lymphatic ECs [55]. PROX1 is co-expressed with COUP-TFII in mouse lymphatic ECs at E11.5 [55]. When *COUP-TFII* is over-expressed in human dermal lymphatic ECs, *PROX1*, *CCNE1* and *VEGFR3* mRNAs are down-regulated [55]. Taken together, *Sox18* and *COUP-TFII* are required for *Prox1* up-regulation in venous ECs, and *Vegfc* is required for lymphatic EC sprouting after lymphatic EC specification by *Prox1*.

In Vitro Differentiation of ECs from ES and iPS Cells

ES and iPS cells are of great interest for their potential use in vascular EC differentiation for purposes of regenerative medicine [56]. Pluripotent human ES cells develop into various cell types found in the adult human body, including cells that form the human vasculature [57, 58]. Like ES cells, iPS cells can be reprogrammed from adult cells and differentiated into the three germ layers. Recently, iPS cell generation has become technically simpler, enabling establishment of patient-specific pluripotent stem cells with fewer ethical concerns [59]. However, investigators have devised different methods to differentiate ECs from ES and iPS cells. For better understanding of EC differentiation from ES/iPS cells, we review these methods below.

EC Differentiation from Embryoid Bodies (EBs)

Induction of ECs Through EB Formation from Mouse ES Cells

Conventional methods to form EBs include liquid suspension culture in petri-dishes, methylcellulose semisolid culture, or hanging drop culture [60]. Alternatively, ES cells can also be plated in semisolid 1 % methylcellulose-containing medium, on mouse embryonic fibroblasts (MEFs), or in Matrigel to form EBs [61, 62]. ES cell lines established from the inner cell mass of the mouse blastocyst maintain pluripotency when cultured in the presence leukemia inhibitory factor (LIF) [63]. EBs are three-dimensional (3D) multicellular aggregates formed spontaneously from ES cells in vitro in the absence of LIF. After 3–8 days of suspension culture, EBs form all three germ layers, like cells that found in the developing embryo and yolk sac [64]. After 8–10 days, approximately 50 % of EBs begin to form a large cystic structure resembling the visceral yolk sac of the post-implantation embryo [64]. Capillary-like structures reportedly form on the surface of ES-derived cystic EBs, suggesting that during in vitro differentiation these cells undergo vasculogenesis and angiogenesis [10].

Alternatively, EBs can be formed in 3 days of ES cells cultured using the hanging drop method and then transferred to gelatin-coated wells of Chamber slides to allow attachment [65]. To promote EC differentiation from EBs, EBs are maintained in endothelial growth medium-2 (EGM-2), which contains VEGF-A and basic fibroblast growth factor (bFGF), and 5 % fetal bovine serum [65, 66]. Using this method, ES

cells differentiate into ECs after 10 days of culture, as detected by the presence of mRNA and protein of EC markers such as FLK-1, CD31 and VE-cadherin [65]. When ECs are cultured for 5 more days in Matrigel, they form capillary-like structures [65]. However, capillary-like structures are not formed when gelatin-coated dishes are used [65].

To induce EB formation, ES cells are trypsinized and plated in semisolid 1 % methylcellulose-containing medium (at day 0) [62]. EBs are cultured for up to 11 days in the presence of recombinant VEGF, erythropoietin (EPO) and bFGF to induce ECs [62]. The composition of growth factors in the culture medium determines the number and size of EBs and whether capillary-like structures appear in EBs [67]. *Flk-1* expression, an early marker of EC precursors, is up-regulated by day 3 of EB development [62, 68]. Expression of genes such as CD31 and *Tie-2,* both are the markers of ECs and hematopoietic progenitor cells is detected on day 4 [62]. Early co-expression of three genes (*Flk-1*, *Pecam-1* and *Tie-2*) reflects commitment towards an endothelial lineage [62]. On day 5, *Cdh5* and *Tie-1* expression begins in the culture system. Immunohistochemical analysis shows growth factor receptors (FLK-1 and TIE-2), adhesion molecules (CD31 and VE-cadherin) and endothelial specific antigens (MECA-32 and MEC-14) are expressed from day 5 to day 11 [62].

Induction of ECs Through EB Formation from Human ES Cells

Human ES cells also differentiate into ECs through EB formation. Human ES cells (H9 clone) are maintained in an undifferentiated state in the presence of LIF and bFGF [69]. To form EBs, ES cells are dissociated and then grown in the absence of LIF and bFGF on petri dishes for a maximum of 13–15 days to allow cell aggregation [70, 71]. Levels of EC-specific mRNAs such as CD31, VE-cadherin and CD34 increase during the EB differentiation period [71, 72]. To evaluate whether EC surface markers are expressed in this process, CD31-expressing cells were isolated by flow cytometry and re-cultured on a 1 % gelatin-coated plates in EGM-2 medium for several passages [73]. Several adhesion molecules were detected at adherens junctions, including N-cadherin and VE-cadherin, and cells spontaneously formed capillary-like structures when maintained in Matrigel for several days [73]. On the other hand, capillaries can also be formed by seeding 8 to 9 day-old EBs in Matrigel supplemented with TGF-β1, activin-A, insulin-like growth factor and retinoic acid [70].

In another study, human ES cells were cultured in vitro for 2 weeks and then transplanted into severe combined immunodeficient (SCID) mice, where they formed capillaries along with the mouse vasculature [73]. Alternatively, lentiviral transduction of human ES cells with a fragment containing the genomic locus of the human EC-specific gene VE-cadherin (*CDH5*) from a bacterial artificial chromosome favors EC formation [74]. To induce EB formation using these cells, cells transduced with this sequence upstream of a GFP marker (hVPr-GFP) were grown to confluence on Matrigel in the absence of FGF-2 [74]. On day 4, EBs were replated on Matrigel-coated plates and supplemented with VEGF-A. By day 8, hVPr-GFP+ cells not only expressed EC-specific markers such as VE-cadherin, CD31, D34 and FLK-1 but also formed capillary-like structures. These cells were negative for α-SMA (a smooth muscle marker) and CD45 (a hematopoietic cell marker) [74].

EC Differentiation from ES Cells Cultured on Type IV Collagen-Coated Dishes or OP9 Feeder Layers

Induction of ECs from Mouse ES Cells

In addition to the methods cited above, EC lineages can be differentiated from mouse ES cells by seeding the latter on feeder cell layers or on collagen type IV- or gelatin-coated culture plates. In these culture systems, Flk-1-expressing cells derived from differentiating ES cells reportedly represent common vascular progenitor cells, as they can give rise to vascular cell types, including arterial, venous and capillary, which eventually form a functional vascular network supported by mural cells (pericytes or vascular smooth muscle cells) and basement membrane [75]. At day 4 of ES cell culture, Flk-1-expressing cells are re-plated with medium supplemented with VEGFs to promote EC differentiation [76]. After 4 more days, cells express VE-cadherin+ and can be isolated using flow cytometry [76]. Cells are maintained in EGM-2 medium on fibronectin-coated plates for 1 to 2 passages [76]. When ECs are placed in a 3D-type 1 collagen matrix in the presence of phorbol myristate acetate (PMA), VEGF165 or bFGF, they differentiate into capillary-like tubular networks [77].

Foxo1, a member of the Foxo subfamily of forkhead box transcription factors, regulates EC morphology in mouse in response to $VEGF_{165}$ [78]. To assess *Foxo1* function in ECs, *Foxo1*(+/+) and (−/−) ES cells were cultured on OP9 feeder cell layers [78]. After 4 days Flk-1+/E-cadherin+ cells were isolated and allowed to form aggregates in the presence of VEGF-A. When grown on type I collagen gel, *Foxo1*(+/+) vascular progenitors formed long capillary-like structures, whereas *Foxo1*(−/−) cells formed short sprouts with irregular shape (Fig. 4), suggesting that *Foxo1* deficiency perturbs the EC microtubular system [78].

Induction of ECs from Non-Human Primate ES Cells

Monkey ES cells are morphologically more similar to human ES cells than are mouse cells. Monkey ES (R278.5) cells are derived from rhesus monkey blastocysts and exhibit cell surface markers that differ from mouse ES cells [79]. For example, undifferentiated monkey ES cells express VEGFR-2 [80]. Monkey ES cells can be maintained in an undifferentiated

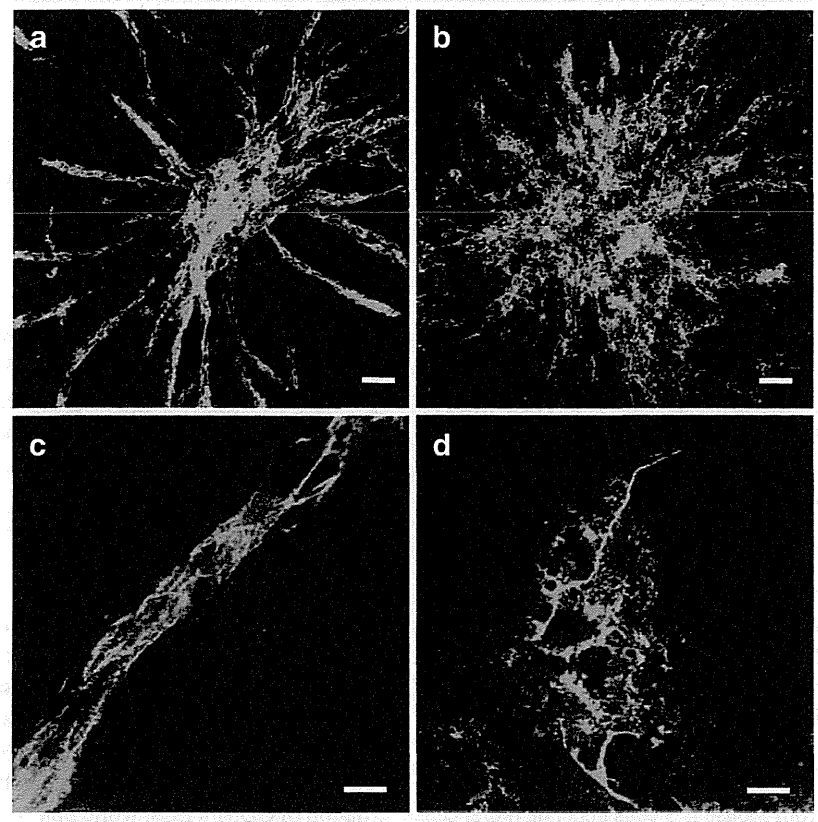

Fig. 4 Immunofluorescence staining of ES cell-derived ECs. Shown is immunofluorescence staining with VE-cadherin antibody (*green*) of ES cell-derived ECs. Flk-1+ mesodermal cells are induced from *Foxo1*(+/+) (*left*, *top* and *bottom panels*) and *Foxo1*(−/−) (*right*, *top* and *bottom panels*) ES cells. FACS-purified Flk-1+ cells were cultured in Type I collagen gels in the presence of VEGF for 4 days. Capillary-like structures are revealed by immunofluorescence. *Foxo1*(+/+) ECs show spindle-shaped elongation, as revealed by VE-cadherin staining along the long axis of the cells, and cells are organized into long cord-like structures (**b**, **c**). *Foxo1*(−/−) ECs fail to elongate and produce only short bundles with irregularly kinked adherens junctions (**b**, **d**). Images were derived from an Olympus FV1000D confocal microscope, using a 20x (**a**, **b**) or 60x (**c**, **d**) objective lens. *Scale bars* indicate 50 (**a**, **b**) or 10 (**c**, **d**) micrometers

state in the absence of LIF and have a longer doubling time than do mouse ES cells [79]. When cultured on OP9 feeder layers, they lose VEGFR-2 expression by day 4 and then re-acquire it at day 8 [80]. Culture of VEGFR-2+/VE-cadherin- cells sorted by flow cytometry on the OP9 feeder layers results in their differentiation into ECs expressing CD31, VE-cadherin and eNOS (endothelial nitric oxide synthase) after 5 days [80]. VEGFR-2+/VE-cadherin- cells differentiate into SMA+ calponin+ mural cells when cultured on a collagen type IV-coated dish [80]. In the presence of VEGFs, VEGFR-2+/VE-cadherin- cells form CD31+ ECs surrounded by SMA+ mural cells . When these differentiated VEGFR-2+ cells are further cultured in a 3D culture system, capillary-like structures form, indicating that VEGFR-2 is expressed on both undifferentiated monkey ES cells and on EC progenitors differentiated from those cells [80].

Induction of ECs from Human ES Cells

Like monkey ES cells, undifferentiated human ES cells express VEGFR-2 [81]. They also express AC133, c-Kit and tumor rejection antigen 1–60 (TRA1-60, a marker of human ES cells), but not CD34 [81]. Human HES3 ES cells give rise to colonies of EC precursor and further differentiate into ECs on OP9 feeder layers [81]. Differentiated HES3 cells express VEGFR-2 but not TRA1-60 on day 8 of culture [81]. Isolated VEGFR-2+/TRA1-60- cells do not differentiate efficiently into CD34+ /VE-cadherin+/CD31+/eNOS+ ECs on collagen type IV-coated dishes in the presence of VEGFs and fetal bovine serum without feeder cell layers after 8 days [81]. Following continuous EC culture, CD31+ cells formed capillary-like structures with morphology similar to that of vessel endothelium [81].

Culture of human ES cells on collagen type IV-coated dishes for 6 days promoted CD31, CD34, AC133, TIE-2 and GATA3 expression, indicative of ECs [82]. After seeding ECs in 3D collagen gels or Matrigel in the presence of VEGF, a capillary-composed of elongated ECs formed within the matrix [71]. Several studies show that cultured ECs derived from human ES cells form capillary tubes when ECs are cultured in Matrigel [71, 81].

ECs derived from ES cells in vitro reportedly exhibit properties similar to those of arterial-venous-lymphatic ECs

[83]. These cells trans-differentiate into smooth muscle cells in vitro and that process is inducible by culture on laminin-1 coated-dishes or in the presence of TGF-β1 [84]. Their smooth muscle phenotype is further supported by expression of both CD31 and α-SMA markers [85].

EC Differentiation from iPS Cells

Induction of ECs from Mouse iPS Cells

Mouse iPS cells established from fibroblasts form EC precursors expressing Flk-1, when cultured for 5 days on OP9 feeder layers [86]. Those EC precursors also express eNOS, CD31, VE-cadherin and *Tie1* [86]. Mouse iPS cells also differentiate into Flk-1+ cells when cultured for 4 to 4.5 days on gelatin-coated dishes without LIF [87]. Flk-1+ cells form and are re-plated on confluent mitomycin-C-treated OP9 feeder layers, eventually giving rise to VE-cadherin+ EC colonies [87]. Colony size and number are enhanced by angiopoietin-1 treatment, which promotes vasculogenesis [87]. Implantation of Flk-1+ cells from mouse iPS cells enhances ischemia-induced angiogenesis in a mouse hind limb model of ischemia: injected Flk-1+ cells derived cells from iPS cells incorporated into the capillary network and promoted angiogenesis in vivo [88]. Flk-1+ cells derived from mouse iPS cells generate mural cells in addition to ECs when cultured on OP9 feeder cell layers, like ES cells [89]. By varying cultivation methods, iPS cells form CD31+/CXCR4+ arterial ECs or lymphatic EC colonies expressing Lyve1+ or Prox1+ [89, 90].

Induction of ECs from Human iPS Cells

ECs can also be differentiated from human iPS cells [6, 90]. The morphologies and characteristics of three human ES cell lines were compared with four human iPS (201B6, 201B7, 253G1, and 253G4) lines produced from human skin fibroblasts by standard reprogramming techniques [90]. Those human iPS cell lines were differentiated into ECs using a 2D culture system [90]. The EC markers VE-cadherin was detected after 10 days of differentiation, and VE-cadherin+ cells expressed the EC markers FLK-1, CD31 and CD34 [90]. Further culture of CD31+/CD34+ cells in Matrigel in the presence of VEGFs or platelet-derived growth factor (PDGF)-BB formed capillary-like structures [90]. This study shows that human iPS-derived and ES-derived ECs possess similar properties in vivo, as they expressed VE-cadherin, CD31, vWF (von Willebrand factor) and CD34 [90]. In addition, VE-cadherin+ ECs and SMA+ mural cells derived from iPS cells exhibited characteristics identical to human ES cell-derived cells, among them, Flk-1 expression [90].

Human fetal, neonatal and adult fibroblasts are reprogrammed into human iPS cell lines with *POU5F1*, *SOX2*, *NANOG* and *LIN28* and can be maintained in an undifferentiated state by culture with MEFs [6, 91]. To induce human iPS cell lines towards EC development, human iPS cell lines can be cultured on OP9 feeder cell layers for 8 days without cytokines. ECs (CD31+/CD34+/CD43-) are detected and further cultured on fibronectin-coated plastic dishes supplemented with endothelium serum-free medium in the presence of bFGF, acidic FGF and heparin [6]. Cultured ECs form a single layer and express VE-cadherin, vWF, VEGFR-2, CD31, CD49d and CD105 [6]. Capillary-like structures form when ECs are seeded on growth factor-reduced Matrigel in the presence of endothelial serum-free medium supplemented with VEGFs [6]. These analyses indicate no morphological differences between ECs derived from human ES and iPS cells, and both cell types show identical gene expression patterns [6, 91].

Indian Hedgehog (IHH) and BMP-4 Signaling During EC Differentiation

In vitro differentiation of nullizygous ES cells is used as an alternative to the analysis of knock-out mouse embryos. A mouse ES cell line nullizygous for the Indian hedgehog gene, *Ihh*(−/−) was employed to examine the role of *Ihh* during angiogenesis in vitro. *Ihh*(−/−) ES cell-derived EBs failed to form blood islands and exhibited reduced differentiation of CD31+ ECs, while *Ihh*(−/−) embryos formed blood vessels and revealed fewer numbers of ECs in yolk sac [92]. EBs that were derived from ES cells deficient for Smoothend also resembled the phenotype of *Ihh*(−/−) EBs, suggesting that IHH signaling is required during early angiogenesis [92].

Addition of recombinant Ihh in the co-culture of human ES cells on OP9 feeder layers promoted differentiation of CD31+ VE-cadherin+ ECs [93]. Inhibition of BMP-4 signaling by neutralizing antibody suppressed the Ihh-induced EC differentiation, while recombinant BMP-4 rescued EC differentiation by cyclopamine (hedgehog inhibitor) [93]. Therefore, hedgehog facilitates EC differentiation via BMP-4 signaling. BMP-4 is known to promote vasculogenesis in EB formation [93]. Hence, addition of BMP-4 in an EB formation culture of human ES cells increased formation and outgrowth of KDR/VEGFR2+ capillary-like network structures [94]. Recently, soluble recombinant endoglin (*Eng*), an auxiliary receptor of TGF-β family, was shown to enhance BMP-4-induced EC differentiation in a culture of mouse ES cells on gelatin-coated plates [95]. This in vitro result is consistent with the defects of angiogenesis and cardiovascular development found in the *Eng*-deficient mouse embryos [96, 97].

Arterialization of ES Cell-Derived ECs

As shown in the case of vasculogenesis and angiogenesis in the mouse embryos, VEGF is a key regulator of EC differentiation from ES cells. When Flk1/VEGFR2+ mesodermal cells derived from mouse ES cells were subcultured to induce

EC differentiation on collagen-type IV coated dishes in the presence of high concentration of VEGF (50 ng/mL), expression of arterial marker genes such as *Dll4*, *Notch4* and *Ephrin-B2* were elevated in differentiating ECs [98]. In contrast, the venous marker gene *COUP-TFII* was upregulated in ECs differentiating in the presence of lower concentration of VEGF. The VEGF-dependent arterialization was blocked by γ-secretase, a multi-subunit protease complex involved in the processing of Notch, ErbB4, E-cadherin, Ephrin-B2 and CD44, implying involvement of Notch signaling in this process [98]. Addition of 8bromo-cAMP or adrenomedullin with VEGF further enhanced the differentiation of arterial ECs from Flk-1/VEGFR2+ mesodermal cells derived from mouse ES cells [99]. The arterialization induced by VEGF and cAMP was abolished in RPBJκ-deficient ES cells which lack Notch signaling, although the constitutive active Notch1 alone was not enough to induce arterial ECs [99]. Therefore, coordinated signaling of VEGF, cAMP and Notch1 is likely to be necessary for arterial EC differentiation.

ECs reportedly respond to shear stress generated by blood flow by undergoing changes in cell morphology and function as well as gene expression [100]. Exposure of mouse ES cell-derived Flk-1/VEGFR2+ mesodermal cells to laminar shear stress in a subculture on collagen-type IV coated coverslips resulted in an increase expression of Ephrin-B2, an arterial EC marker in the differentiating ECs [100]. Addition of either VEGF receptor kinase inhibitor or γ-secretase inhibitor suppressed the shear stress-induced increase in the expression of Ephrin-B2 [100], also suggesting that VEGF and Notch signaling pathways play an important role in arterial EC specification.

Growth Factors Sufficient for EC Differentiation

One of the advantages of in vitro differentiation of ES cells is that cell differentiation can be induced in serum-free defined conditions, thereby enabling combination of growth factors sufficient for differentiation of a certain cell lineage to be determined. For instance, low concentration of BMP-4 (1 ng/mL) in a serum-free medium was reportedly sufficient for inducing Brachyury+ VEGFR2+ mesodermal cells from mouse ES cells on collagen-type IV coated dishes [101]. The Brachyury+ VEGFR2+ mesodermal cell subset has a high potential to differentiate into ECs, and high dose of activin was shown to antagonize the BMP-4-induced generation of this endothelial-competent mesoderm subset [101]. On the other hand, recent study showed that combination of canonical Wnt agonist GSK inhibitor (glycogen synthase kinase inhibitor), activin and FGF2 induced VEGFR2+ mesodermal cell differentiation from Fgf5+ epiblast-stage cells that were initially induced from mouse ES cells [102]. The VEGFR2+ mesodermal cells were further induced to CD31+ AcLDL+ ECs in the presence of VEGF, cAMP, BMP-4, FGF2 and ALK inhibitor [102]. The reason for the discrepancy in the combination of factors which induced mesodermal cell differentiation is not fully understood. Differences of serum-free media and extracellular matrices as well as the timing of growth factor addition may influence the fate of differentiating ES cells. Furthermore, above studies did not even describe the minimum requirement of growth factors for EC differentiation from ES cells. Indeed, BMP-4 and VEGF in a serum-free medium are sufficient to induce CD31+VE-cadherin+ ECs from mouse ES cells on collagen-type IV coated dishes (M.O., unpublished observation), although involvement of endogenous factor(s) secreted from differentiating ES cells (including ECs themselves) cannot be excluded.

Lymphatic Specification of ES Cell-Derived ECs

Another advantage of in vitro differentiation of ES cells is that cell fate can be manipulated by regulating expression of transcription factors. Regulation of the lymphatic marker *Prox1* expression by Sox18 was tested by using forced expression of wild-type Sox18 or dominant-negative Sox18RaOp in the EB formation of mouse ES cells [51]. Expression of *Prox1* and *podoplanin* was increased through over-expression of wild-type Sox18, while Sox18RaOp inhibited induction of the lymphatic markers [51]. Neither of the treatments affected vascular EC differentiation as monitored by the expression of *Vegfr2*, *Tie2* and *VE-cadherin* [51], suggesting that Sox18 regulates *Prox1* expression in an endothelial-specific manner.

In order to identify the target genes of *Prox1*, a mouse ES cell line in which forced expression of *Prox1* is induced by a tetracycline-regulatable gene expression system was employed [103]. Gene expression profiles of *Prox1*-induced ECs differentiated from ES cells revealed that FoxC2, angiopoietin-2 and HoxD8 are the candidate targets of *Prox1*. Further investigations showed that HoxD8 up-regulated *Prox1* expression in ECs, suggesting a positive-feedback-loop mechanism in which *Prox1* expression is maintained by HoxD8 after maturation of lymphatic vessels where Sox18 expression ceases [103]. Taken together with these in vitro data, Sox18-Prox1-HoxD8 axis is likely to play a pivotal role in the regulation of lymphangiogenesis during embryogenesis.

Future Perspectives

Vasculogenesis and angiogenesis are important processes in formation of a functional vascular network. Formation of that network is driven by diverse biological activities, including EC differentiation, migration, and cell-cell adhesion. Studies indicate that ES/iPS-derived ECs mimic characteristics of ECs in vivo. Therefore, in vitro differentiation of the ES/iPS cells could provide opportunities to further explore the roles of the ECs that can be targeted towards vascular development. In vitro differentiation of ES cell systems provides an in vitro

model for current research. Recently, we reported that ECs function as hematopoietic niche cells through stem cell factor production in the aorta and placenta in mid-gestational mouse embryos [104]. In fetal liver, liver sinusoidal endothelial cells express TGF-β1, which up-regulates extra-cellular matrix production in mouse hepatoblasts, and Wnt2 and HGF as mentioned in this review. [42, 105]. This finding suggests that some types of ECs regulate others through cytokine production. Thus, in vitro EC differentiation of ES/iPS cells could allow understanding of crosstalk between ECs and other cells, as well as serve as the basis for future regenerative medicine.

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Conflict of Interest Statement The authors indicate no potential conflicts of interest.

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REVIEW

Hematopoietic cell differentiation from embryonic and induced pluripotent stem cells

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Abstract

Pluripotent stem cells, both embryonic stem cells and induced pluripotent stem cells, are undifferentiated cells that can self-renew and potentially differentiate into all hematopoietic lineages, such as hematopoietic stem cells (HSCs), hematopoietic progenitor cells and mature hematopoietic cells in the presence of a suitable culture system. Establishment of pluripotent stem cells provides a comprehensive model to study early hematopoietic development and has emerged as a powerful research tool to explore regenerative medicine. Nowadays, HSC transplantation and hematopoietic cell transfusion have successfully cured some patients, especially in malignant hematological diseases. Owing to a shortage of donors and a limited number of the cells, hematopoietic cell induction from pluripotent stem cells has been regarded as an alternative source of HSCs and mature hematopoietic cells for intended therapeutic purposes. Pluripotent stem cells are therefore extensively utilized to facilitate better understanding in hematopoietic development by recapitulating embryonic development *in vivo*, in which efficient strategies can be easily designed and deployed for the generation of hematopoietic lineages *in vitro*. We hereby review the current progress of hematopoietic cell induction from embryonic stem/induced pluripotent stem cells.

Introduction

Hematopoietic stem cells (HSCs) lay the foundation of hematopoiesis to generate all functional hematopoietic lineages, including erythrocytes, leukocytes (neutrophils, basophils, eosinophils, lymphocytes, monocytes and macrophages) and platelets [1]. Perturbations in the hematopoietic system have been reported to cause numerous diseases such as anemia, leukemia, lymphomas and thrombocytopenia. Currently, HSC transplantation and hematopoietic cell transfusion are useful treatments for some hematological diseases, such as thalassemia and leukemia. However, some conventional sources of HSCs – such as cord blood, bone marrow and peripheral blood – are limited in usage, owing to the limited number of transplantable cells as well as inefficient strategies for expanding those cells. Additionally, problems such as shortage of human leukocyte antigen-matched donors, adverse effects from graft-versus-host disease, viral contamination and immunoreactions impede the utility of readily available HSCs. The use of alternative sources for these cells will therefore be a great advantage for regenerative medicine.

Pluripotent stem cells are one of the potential sources for HSCs and the *in vitro* model for further elucidating the regulatory mechanisms underlying embryonic hematopoietic development. Embryonic stem (ES) cells are pluripotent cells established from the inner cell mass of blastocyst-stage embryos, in both mouse and human [2,3], and are capable of giving rise to three germ layers after directed differentiation in culture [3,4]. However, manipulation of human ES cells raises some ethical issues and immunoreactions. Induced pluripotent stem (iPS) cell technology has made a groundbreaking discovery to circumvent the problems of ethical and practical issues in using ES cells [5]. It is of great importance to develop efficient and controllable induction strategies to drive hematopoietic differentiation from ES/iPS cells in culture prior to the realization of pluripotent cell-derived therapies. To review current progress of

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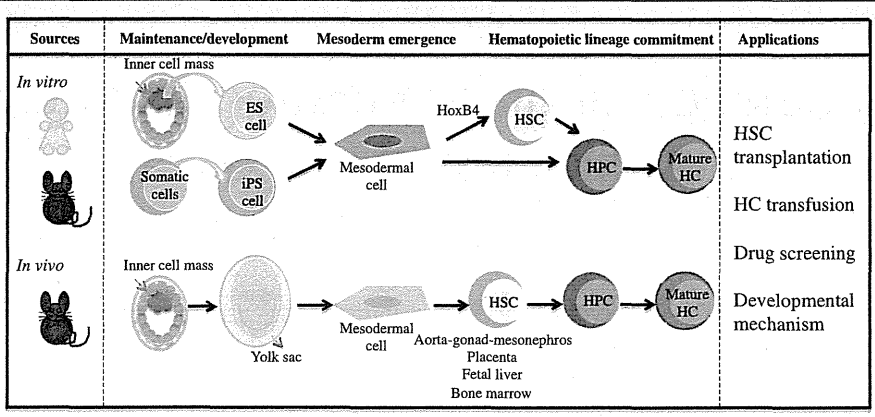

Figure 1 Schematic representations of hematopoietic development from *in vivo* and *in vitro* models. Both human and mouse *in vitro* models have been established for hematopoietic differentiation in a defined culture system from embryonic stem (ES) and adult cell-derived induced pluripotent stem (iPS) cells. For the *in vivo* model, the mouse inner cell mass undergoes differentiation, later forming the yolk sac, which generates mesodermal cells and induces hematopoietic stem cells (HSCs), hematopoietic progenitor cells (HPCs) and mature hematopoietic cells (HCs). Successfully generated HSCs from both *in vitro* and *in vivo* models might be applied to HSC transplantation for hematopoietic disorders. Further differentiation of HSC in a cytokine-defined culture system produces hematopoietic cells for hematopoietic cell transfusion. Thorough understanding of molecular mechanism on these models will be beneficial for both drug screening as well as the mechanism of hematopoiesis development.

differentiation protocol from ES/iPS cells, we first summarize the knowledge of hematopoietic development during early mouse hematopoiesis followed by the manipulation of ES/iPS cells in hematopoietic cell induction (Figure 1).

Embryonic hematopoiesis

Studies of hematopoietic development during embryogenesis *in vivo* are important to gain insight into its underlying mechanisms, whereby accumulated knowledge will facilitate the induction of HSCs, hematopoietic progenitor cells (HPCs) and mature hematopoietic cells from pluripotent stem cells in culture. In mouse blastocyst, the inner cell mass at 3.5 days post coitum (dpc) comprises a population of cells – which can give rise to a derivative of three germ layers (endoderm, mesoderm and ectoderm) – that eventually develop into both intraembryonic and extraembryonic tissues as embryo develops [6]. The hematopoietic system that derives from the mesodermal germ layer can be classified into two waves. The first hematopoiesis (primitive hematopoiesis) begins to develop primitive erythroid and macrophage progenitors in the yolk sac (YS) blood islands at 7.0 dpc [7]. Para-aortic splanchnopleural regions that will develop into aorta–gonad–mesonephros (AGM) already possess hematopoietic precursors beginning at 8.5 dpc [8]. Before the establishment of circulation (8.0 dpc), both YS and para-aortic splanchnopleural-derived mesodermal cells acquire HSC activity after co-culturing with AGM-derived stromal cells [9]. After circulation commences, $CD34^{+}c\text{-}Kit^{+}$ cells derived from both YS and para-aortic splanchnopleura at 9.0 dpc were able to reconstitute the hematopoietic system in newborn recipient pups, but not in adult recipient mice [10]. These findings demonstrate that both YS and para-aortic splanchnopleura possess HSC potential that can contribute to definitive hematopoiesis under a favorable microenvironment.

The first definitive HSCs that can reconstitute the adult hematopoietic system appear in the AGM region at 10.5 dpc followed by the YS, placenta and liver, spanning from 11.0 to 11.5 dpc [11-13]. YS cells expressing *Runx1* at 7.5 dpc progressed into fetal lymphoid progenitors at 16.5 dpc in both fetal liver and thymus as well as adult HSCs in 9-month-old to 12-month-old mouse bone marrow [14]. In view of these results, both the YS and the AGM region contribute to HSC generation. However the extent of their contribution still remains unclear. To address this issue, YS–YS chimeric embryos were generated before blood circulation at 8.25 dpc, where no B-cell activity was detected, which is relevant to HSC activity in the early mouse embryo. As the chimeric embryos develop into 11.0 dpc equivalent in whole embryo culture, the grafted YS cells contributed to B-cell activity in the AGM region, but with low frequency [15]. This observation implies that the main source of HSCs is derived from the AGM region.

In addition to the YS and the AGM region, the placenta is another site for HSC generation. The placenta exchanges oxygen and nutrient between mother and fetus, and is formed around 9.0 dpc after fusion of chorion and allantois. A fluorescent-labeled allantois region at 8.25 dpc could be detected in the hematopoietic cell cluster after 42 hours of whole embryo culture, suggesting that allantois alone possesses HSC potential

and also proposing that the placenta is likely to be an ixndependent site of HSC generation, regardless of cells circulating from other hematopoietic organs [16]. Taken together, the YS, AGM region and placenta contribute to HSC generation prior to the HSC expansion in the fetal liver .

Gene and protein markers for mesodermal and hematopoietic lineages

During early embryonic development, *Brachyury* that is indispensable for mesodermal formation expresses transiently [17]. Subsequently tyrosine kinase *Flk1* for blood-island formation and vasculogenesis expresses in hemangioblasts, which is the common embryonic endothelial and hematopoietic precursor [18].

Transcription factors characterized to be involved in the hematopoietic lineages are shown below. In the transition of mesodermal to hematopoietic lineages, *Scl* is indispensable for the development of all hematopoietic lineages, in which a homozygous mutant $Scl^{-/-}$ model showed undetectable hematopoietic lineages in both *in vivo* and *in vitro* studies [19]. *Runx1* plays an important role in governing definitive hematopoiesis but not primitive hematopoiesis through the observation of blast colony-forming cells, which are both hematopoietic and endothelial cell precursors from ES cells and equivalent of hemangioblast, using a $Runx1^{-/-}$ ES cell culture model [20]. The GATA family of transcription factors, especially GATA-1 and GATA-2, are differentially regulated at multiple steps from the early development of hemangioblast to hematopoietic commitment [21].

Surface markers of hematopoietic lineages are briefly described. Cells expressing endothelial markers, such as CD34, PECAM-1 (CD31) and VE-cadherin, are closely associated with both hematopoietic and endothelial cell commitment [22]. Expression of CD41 initiates the primitive and definitive hematopoiesis, whereas HPCs are highly dependent on c-Kit expression [23]. Additionally, CD34 as a marker for HSCs and CD45 as a marker for pan-leukocyte and/or maturation of HSCs are utilized respectively [24,25].

Differentiation of embryonic stem/induced pluripotent stem cells and hematopoietic cell induction

Mouse ES/iPS cells can be maintained and propagated indefinitely on a layer of mitotically inactivated mouse embryonic fibroblasts (MEFs) and/or leukemia inhibitory factor in culture. On the contrary, human ES cells achieve an undifferentiated state on a layer of MEFs and/or basic fibroblast growth factor. After removal of leukemia inhibitory factor and basic fibroblast growth factor, these ES cells can differentiate into any kind of cell depending on appropriate culture conditions. One of the ultimate goals of *in vitro* differentiation of ES/iPS cells is to generate HSCs with the acquisition of long-term reconstitution capacity.

By recapitulating mouse embryogenesis, *in vitro* differentiation of ES/iPS cells toward hematopoietic development has been technically approached: through formation of a cystic-like structure that exhibits three germ layers that are able to generate mesodermal cells (embryoid body (EB) formation); through feeder cell co-culture that can provide ES/iPS cells an intimate cell contact with secreting factor(s) that promote cell proliferation and differentiation (feeder cell co-culture); and through an extracellular matrix-coated dish that supports differentiation of ES/iPS cells (Figure 2).

Embryoid body formation

EBs are three-dimensional cell aggregates formed in suspension culture and they mimic the spatial organization of the embryo through enhancing cell–cell interactions to form three germ layers [3,4]. There are a number of approaches to EB formation, such as methylcellulose or suspension culture medium either using a bacterial-grade dish, a methylcellulose-coated plate, a low-adherent micro-well plate or the hanging drops method. To specifically induce a hematopoietic lineage, single-cell suspension of EBs are directed into methylcellulose culture medium that functions to support hematopoietic development in the presence of hematopoietic cytokines, such as stem cell factor (SCF), granulocyte colony-stimulating factor (CSF), macrophage CSF, granulocyte–macrophage CSF, erythropoietin (Epo) and interleukins (ILs) such as IL-1, IL-3, IL-4 and IL-6 [26]. In the absence of cytokines in culture, EB cells themselves reportedly possess the capability to secrete proteins having macrophage CSF, IL-3 and Epo activity in order to support the early development of erythroid and macrophage precursors [27].

Using mouse ES cells, 75% of blast colonies were generated from EBs of methylcellulose culture consisting of bipotential precursors (hematopoietic and endothelial cells) as blast colony-forming cells at day 2.5, and then decreased at day 4 (<15%) [28]. In mouse ES cells with GFP targeted to the *Brachyury*, EB formation at day 2.5 in suspension culture gave rise to $GFP^{-}Flk1^{-}$, $GFP^{+}Flk1^{-}$ and $GFP^{+}Flk1^{+}$ distinct populations, which are comparable with the developmental progression ranging from pre-mesoderm to pre-hemangioblast mesoderm to the hemangioblast, respectively [29].

EBs derived from human ES cells formed three embryonic germ layers that can be identified using the following markers: ζ-globin, neurofilament 68Kd and α-fetoprotein [30]. By differentiating human ES cells through EBs at day 3.0 to 4.0, blast colony-forming cells were generated in liquid culture and expressed *FLK1*, *BRACHYURY* and *SCL* genes [31]. Differentiation of