22.杉山大介、疋田隼人、Kulkeaw Kasem、水落ちよ、堀尾有可、Jones Rhodri、竹原徹郎
胎生期肝臓における造血・血管新生制御
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APOA-1はマウス、ヒトにおいて造血幹細胞から赤血球への新しい成熟マーカーである
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24.杉山大介
造血発生システムの動作原理に基づく新規再生医療法の開発
第 23 回セルセラピーセミナー，前橋，2010 年 6 月 25 日
口頭発表（特別講演）

H．知的財産権の出願・登録状況

（予定を含む。）

1．特許取得

・ 発明の名称:　アセチル化酵素競合阻害因子Gm16515の新規用途
・ 代表発明者　杉山　大介　准教授　（　医学研究院）
・ 出願番号:　特願2012-080211
・ 出願日:2012/3/30
・ 特許事務所:　三枝国際特許事務所
・ 担当者　三角　可恵(　知的財産本部　)

2．実用新案登録

特になし

3.その他

特になし

別紙４

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
Sugiyama D, Inoue T and Kulkeaw K.	Stem cell maintenance in embryos and adults.	Roger Kasunic	Year Book of SCIENCE & TECHNOLOGY 2011	McGRAW-HILL	New York	2011	258-261

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Tan KS, Tamura K, Lai MI,Veerakumarasivam A, Nakanishi Y, Ogawa M, Sugiyama D.	Molecular pathways governing development of vascular endothelial cells from ES/ iPS cells	Stem Cell Reviews and Reports	DOI 10.1007/s12015-013-9450-7		Manuscript accepted in May 2013
Lim WF, Inoue T,Tan KS, Lai MI, Sugiyama D.	Hematopoietic cell differentiation from embryonic and induced pluripotent stem cells	Stem Cell Research & Therapy			Manuscript accepted in May 2013
Sugiyama D, Kulkeaw K, Mizuochi C.	TGF-beta-1 up-regulates extra-cellular matrix production in mouse hepatoblasts.	Mechanisms of Development	130(2-3)	195-206	2013
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Mizuochi C, Fraser ST, Biasch K, Horio Y, Kikushige Y, Tani K, Akashi K, Tavian M, Sugiyama D.	Intra-aortic clusters undergo endothelial to hematopoietic phenotypic transition during early embryogenesis.	PLoS One	7(4)	e35763	2012

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Inoue T, Sugiyama D, Kurita R, Oikawa T, Kulkeaw K, Kawano H, Miura Y, Okada M, Suehiro Y, Takahashi A, Marumoto T, Inoue H, Komatsu N, and Tani K.	APOA-1 is a novel marker of erythroid cell maturation from hematopoietic stem cells in mice and humans.	Stem Cell Reviews and Reports	7(1)	43-52	2011
Sasaki T, Mizuochi C, Horio Y, Nakao K, Akashi K and Sugiyama D.	Regulation of hematopoietic cell clusters in the placental niche through SCF/c-Kit signaling in embryonic mouse.	Development	137(23)	3941-3952	2010

次ページより研究成果の刊行物（13 点）の別刷を添付する。

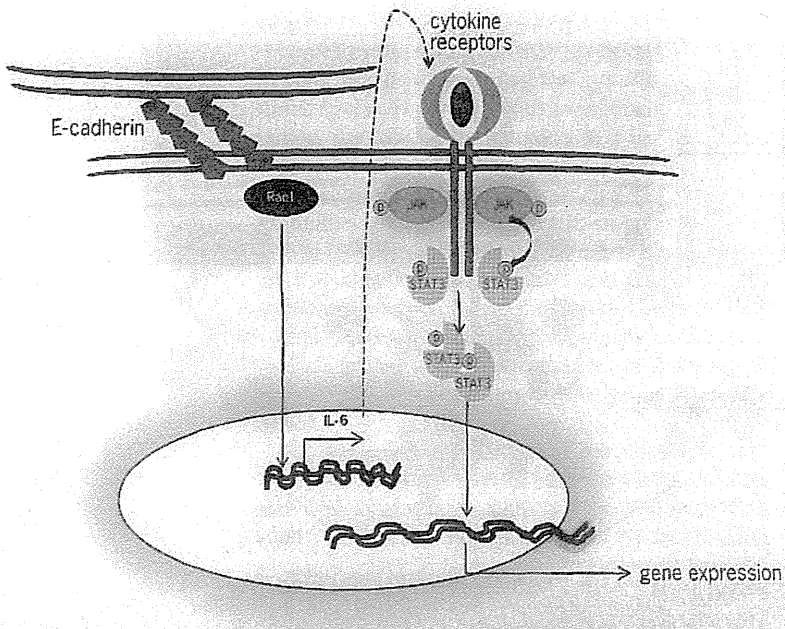

Fig. 2. Model of STAT3 activation by cadherin and Rac1/Cdc42. In an alternative mode of activation, cell–cell contact through cadherin engagement increases Rac1 and Cdc42 proteins, which induce IL-6 transcription. An increase in IL-6 production stimulates cytokine receptors, which then activate STAT3 through receptor-mediated activation.

of a Crohn's disease–like colitis, whereas immunohistochemical studies showed activated STAT3 expression in the intestines of patients with inflammatory bowel disease. Genome-wide association studies (GWAS) uncovered the single-nucleotide polymorphism, rs12948909, within the *STAT3* gene in patients with Crohn's disease and ulcerative colitis.

Role of STAT3 in cancer. STAT3 is constitutively activated in cancers of the breast, prostate, lung, head, and neck, as well as in multiple myeloma and leukemia. A constitutively active form of STAT3 alone is sufficient to transform cultured fibroblasts to anchorage independence (the ability of cells to proliferate in the absence of adhesion to extracellular matrix proteins) and points to a causal role for STAT3 in cancer development. Most of the described oncogenic functions of STAT3 depend on its Tyr^{705} phosphorylation status; however, an additional oncogenic role for STAT3 has been recently described that is dependent on serine phosphorylation and takes place in mitochondria. Recent evidence also suggests a crucial role for STAT3 in selectively inducing and maintaining a procarcinogenic inflammatory microenvironment, both at the initiation of malignant transformation and during cancer progression. STAT3 is linked to inflammation-associated tumorigenesis that is initiated by genetic alterations in malignant cells, as well as by many environmental factors, including chemical carcinogens, sunlight, infection, cigarette smoking, and stress.

Drug targets for STAT3 inhibition. The large body of data validating STAT3 as a target for cancer therapy, and the tolerance of normal cells for the loss of STAT3 function, has driven the effort to identify molecules that inhibit STAT3. In cell lines, inhibition of STAT3 activity using genetic or pharmacologic approaches reduces cancer cell growth and induces apoptosis. A few of the strategies to inhibit STAT3 that are currently under exploration are based on targeting the different structural domains of the protein and on direct targeting of the upstream components of the pathway, including the inhibition of tyrosine kinases that block aberrant STAT3 signaling. As STAT3 is negatively regulated through numerous mechanisms [for example, suppressors of cytokine signaling (SOCS), protein inhibitor of activated STAT (PIAS), protein phosphatases, and ubiquitin-dependent proteasomal degradation], additional strategies targeting STAT3 are also being actively investigated. Although few clinical trials targeting STAT3 are in place in 2011, it is likely to become an important targeting tool in the future as more is understood about the significance of this molecule in cancer and other diseases.

For background information *see* CANCER (MEDICINE); CELL (BIOLOGY); CELL BIOLOGY; CELLULAR IMMUNOLOGY; CYTOKINE; GENE; ONCOGENE; ONCOLOGY; PROTEIN; PROTEIN KINASE; SIGNAL TRANSDUCTION; TRANSCRIPTION; TUMOR SUPPRESSOR GENES in the McGraw-Hill Encyclopedia of Science & Technology. Rachel A. Altura

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Stem cell maintenance in embryos and adults

Stem cells are capable of self-renewal. Provided they have the ability to differentiate into various types of mature cells, they are classified as pluripotent stem cells. Examples of well-studied stem cell types include embryonic stem cells, induced pluripotent stem cells, and tissue-specific stem cells, which enable the corresponding tissues to sustain cell-based homeostasis and which contribute to regenerative mechanisms. These tissue-specific stem cells have been found in blood, nervous tissue, mesenchyme, and skin, and they are regulated both intrinsically and extrinsically. Intrinsic regulation is programmed by genes and transcription factors that are active in the stem cells themselves, whereas extrinsic regulation is accomplished by cytokines and matrices secreted by niche cells surrounding stem cells. Among tissue-specific stem cells, hematopoietic stem cells (HSCs) are the stem cells in the blood lineage and

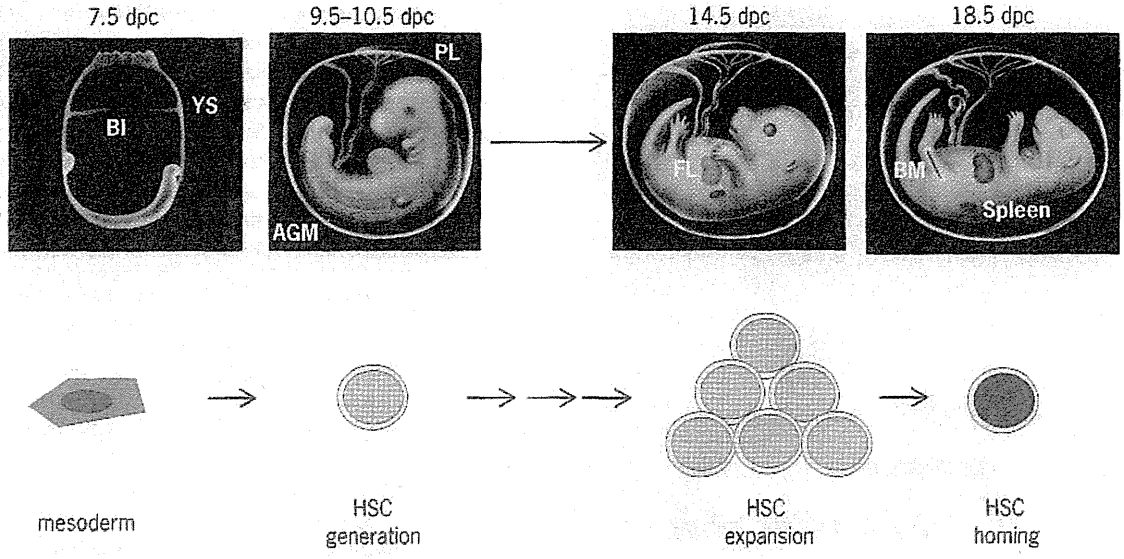

Fig. 1. Schema of hematopoietic development during embryogenesis in mice. Hematopoietic stem cells (HSCs) are generated in the yolk sac (YS), aorta-gonad-mesonephros (AGM) region, and placenta (PL) from early- to mid-gestation periods [7.0–11.0 days postcoitum (dpc)]. They expand to the fetal liver (FL) at midgestation and later migrate (home) temporarily to the fetal spleen and finally to fetal bone marrow (BM). The blood island (BI) is the first site of hematopoiesis.

they have been used in patients for transplantation therapy in the fields of hematology and regenerative medicine. Most sources of HSCs rely on healthy donors and umbilical cord blood. However, problems remain in HSC transplantation, including shortages of donors and risks for donors [for example, transplant rejection and graft-versus-host disease (GVHD)]. Understanding of HSC regulation, especially with regard to niche cells, will enable us to improve HSC therapy through novel stem cell engineering.

HSC maintenance in embryos. In the mouse embryo, the site for blood production changes during the 20-day gestation period. HSCs are initially generated in the yolk sac, aorta-gonad-mesonephros region, and placenta. This initial stage is followed by expansion of HSCs into the fetal liver. Then, later in gestation, after residing temporarily in the fetal spleen, HSCs finally reside in the fetal bone marrow (**Fig. 1**).

The yolk sac is a membranous sac attaching to an embryo and contains the blood islands, which are the first site of hematopoiesis (the process by which the cellular elements of the blood are formed), particularly erythropoiesis (the process by which erythrocytes are formed), and development of the circulatory system after 7.0 days postcoitum (dpc, which is a designation of embryonic age). Among the three germ layers (endoderm, mesoderm, and ectoderm), mesodermal cells differentiate into both hematopoietic cells inside and endothelial cells (ECs) outside of the blood islands. Endodermal cells differentiate into unclassified mesenchymal cells (MCs) that fill out the other spaces of the yolk sac. Therefore, both ECs and MCs likely comprise a niche for yolk sac hematopoiesis. Members of the Hedgehog family of proteins, which are important regulators of many developmental processes, reportedly function in yolk sac hematopoiesis, but the niche regulation of yolk sac hematopoiesis remains unclear.

After yolk sac hematopoiesis, HSCs are generated in the aorta-gonad-mesonephros region, where aggregates of the cells expressing HSC surface protein markers (for example, c-Kit, CD31, and CD34) are observed and display HSC activity. These aggregates are named hematopoietic clusters or aortic clusters. At 10.5 dpc, these hematopoietic clusters are frequently observed attaching to the walls of large arteries (as if they are generated from EC layers). Because the aorta-gonad-mesonephros region consists of ECs, gonad cells, mesonephros cells, and unclassified MCs, the cells likely comprise a niche for HSC generation. MCs of the aorta-gonad-mesonephros region reportedly increase the number of HSCs at 10.5 dpc through the Hedgehog signaling pathway. In addition, ECs of the aorta-gonad-mesonephros region at 10.5 dpc likely have a role in HSC regulation through secretion of a cytokine known as stem cell factor (SCF). Taken together, several cell components comprise a niche for HSC generation.

The placenta connects the fetus to the mother and is highly vascularized in structure. It functions not only in gas exchange and fetal nutrition but also in hematopoiesis at 8.5–13.5 dpc. In particular, HSCs are generated in the placenta at 11.5 dpc, with their numbers dramatically increasing at 11.5–12.5 dpc. Similar to the aorta-gonad-mesonephros region, hematopoietic clusters are observed in the vasculature of the placenta (**Fig. 2**). To examine the roles of niche cells surrounding the clusters, they were isolated by both laser-capture microdissection and flow cytometry methods. Among several cytokine genes, only the *SCF* gene was expressed particularly in the niche cells. Administration of blocking antibody to c-Kit, the receptor for SCF, clearly demonstrated that SCF/c-Kit signaling is pivotal in HSC regulation in the placenta.

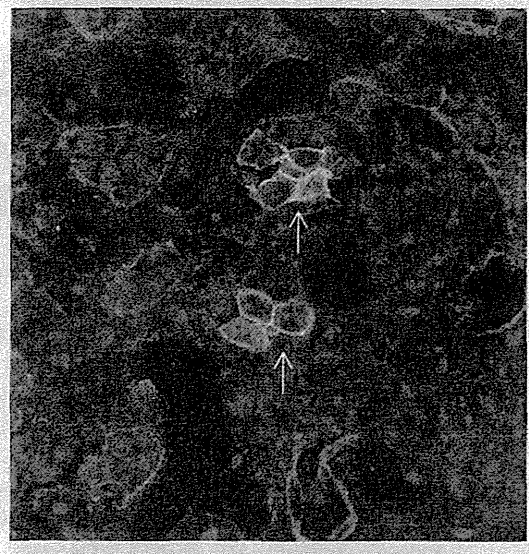

Fig. 2. Confocal image of hematopoietic clusters in the aorta of the placenta (PL) at 11.5 days postcoitum (dpc). Sections of the placenta were made from ICR mouse strain embryos at 11.5 dpc, stained with antibodies (CD34 and c-Kit). CD34/c-Kit double-positive cells indicate hematopoietic clusters (arrows), equivalent to hematopoietic stem cells (HSCs). TOTO-3 was used as a stain for nuclei.

After HSCs are generated in the yolk sac, aorta-gonad-mesonephros region, and placenta, they migrate to the fetal liver, where the numbers of HSCs dramatically increase at 12–16 dpc and they differentiate particularly into erythrocytes. The fetal liver consists of hepatoblasts (hepatocyte precursors), ECs, and hematopoietic cells (as well as other cells). Among them, hepatoblasts have a role in HSC differentiation, particularly erythropoiesis through secretion of mainly erythropoietin (EPO), a cytokine that regulates erythropoiesis, and SCF. In addition, hepatoblasts increase the number of HSCs through production of several other cytokines, including angiopoietin-like 3 (Angptl3), insulin-like growth factor-2 (IGF2), SCF, and thrombopoietin (TPO). Taken together, hepatoblasts comprise a niche for HSC regulation through cytokine secretion. However, it remains unclear how HSCs maintain their self-renewal capability under extensive cytokine exposures that stimulate HSC differentiation. Further investigation is necessary to resolve how niche cells regulate HSC potency in the fetal liver.

After the numbers of HSCs increase in the fetal liver, they migrate to the fetal spleen. At this location, HSCs differentiate particularly into macrophages at 13.5–14.5 dpc. Fetal spleen-derived stromal cells enable HSCs to differentiate into macrophages, but not lymphocytes. It is not known how HSC differentiation is regulated in this tissue. After transient hematopoiesis in the fetal spleen, HSCs gradually migrate to the bone marrow after 16.5 dpc, and they remain there for the duration of their lives. Here, the stromal cells and MCs secrete a chemokine, called CXC-chemokine ligand 12 (CXCL12), which attracts the HSCs expressing the CXCL12 receptor CXCR-4 and results in HSCs migrating to the bone marrow. Aside from this homing (migration) mechanism, little is known about niche regulation in fetal bone marrow.

During embryogenesis, spatiotemporal regulation of HSCs occurs extrinsically by several niche cell components. Accumulation of evidence about embryonic HSC regulation by niche cells will facilitate the development of novel therapies of in vitro generation, expansion, and differentiation of HSCs from embryonic stem (ES) cells and induced pluripotent stem (iPS) cells in the future.

HSC maintenance in adults. In adults, HSCs residing in the bone marrow self-renew slowly and are able to differentiate into leukocytes, erythrocytes, and platelets, thereby maintaining homeostasis in peripheral blood. Compared to embryonic HSCs, one important characteristic of adult HSCs is their cell cycle status. The cell cycle is the series of events for cell division and duplication. It consists of five phases: G0, G1, S, G2, and M phases. The G0 phase is the state in which cells stop cell division and is called quiescence. The cell cycle status of most adult HSCs is G0 phase, whereas that of embryonic HSCs is a non-G0 phase. This quiescent status is essential in interrupting the exhaustion of HSCs and is tightly regulated by surrounding niche cells. In response to blood cell loss, HSCs exit from quiescence, divide, and differentiate into mature blood cells.

Bone marrow consists of several kinds of cells, including osteoblasts (which generate bone), ECs, neural cells, fibroblasts (stellate connective tissue cells found in fibrous tissue), hematopoietic cells, and MCs. Several reports suggest that both osteoblasts and ECs comprise niches for HSC regulation. Osteoblastic niches are located at the endosteal region (the inner surface of bone), where HSCs bind through their expression of adhesion molecules, including neural cadherin (N-cadherin) and very late antigen 4 (VLA4) integrin, which bind to the same and/or other adhesion molecules expressed on osteoblasts. Moreover, N-cadherin interacts with the extracellular matrix, including an acidic glycoprotein (osteopoietin) and a hyaluronic acid secreted from osteoblasts. These adhesion molecules are important for retaining HSCs in the osteoblastic niches. To maintain their quiescent status in the osteoblastic niches, osteoblasts secrete several molecules: (1) vascular endothelial growth factor (angiopoietin-1), (2) TPO, (3) SCF, and (4) CXCL12. Angiopoietin-1, TPO, SCF, and CXCL12 respectively bind to their corresponding receptors of Tie2, MPL, c-Kit, and CXCR4 expressed on HSCs. The bindings of these molecules result in inhibition of HSC division to maintain the quiescent status of HSCs.

In contrast to the osteoblastic niches, HSCs residing in endothelial niches are not quiescent. The endothelial niches are located in the center of the bone marrow cavity and are composed of sinusoid ECs expressing vascular endothelial cadherin (VE-cadherin). In response to blood cell loss, sinusoid ECs secrete CXCL12 to attract HSCs from osteoblastic niches to endothelial niches, resulting in HSC proliferation and differentiation to replenish blood cells.

The endothelial niches are also important for regulating HSC migration from the osteoblastic niches to blood circulation. Administration of granulocyte colony-stimulating factor (G-CSF) causes endothelial niches to secrete neutrophil protease, which degrades adhesion molecules and keeps HSCs in the osteoblastic niches. Using this mechanism, HSCs are harvested from peripheral blood by G-CSF administration and are clinically used for HSC transplantation therapy.

Recently, it has been reported that mesenchymal stem cells (MSCs) expressing nestin, an intermediate filament protein, constitute an essential niche in the bone marrow. Loss of nestin-expressing MSCs reduces the number and homing capacity of HSCs. However, it remains unclear how MSCs cooperate with osteoblastic and endothelial niches.

The quiescent status of HSCs is regulated extrinsically by several niche cell components. With this in mind, stem cells in leukemia are considered to be an important target for therapy because leukemic stem cells may be quiescent in osteoblastic niches, causing relapse of leukemia after complete remission. Thus, understanding of adult HSC regulation by niche cells will facilitate the development of novel technologies to freely control the quiescent status of HSCs and leukemic stem cells, ultimately leading to therapies targeting leukemic stem cells as well as HSC expansion and differentiation in the future.

For background information *see* CELL (BIOLOGY); CELL CYCLE; CELL DIFFERENTIATION; CELL LINEAGE; CYTOKINE; EMBRYOLOGY; EMBRYONIC DIFFERENTIATION; GENETIC ENGINEERING; HEMATOPOIESIS; REGENERATIVE BIOLOGY; STEM CELLS; TRANSPLANTATION BIOLOGY in the McGraw-Hill Encyclopedia of Science & Technology.

Daisuke Sugiyama; Tomoko Inoue; Kasem Kulkeaw

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Strategic decision making

Strategic decisions are decisions that critically influence the performance and survival of a firm. These strategic decisions are typically made by the senior managers of a firm, and generally involve high levels of uncertainty and complexity, occur in dynamic contexts, and include many different stakeholders, often with conflicting interests. Examples of strategic decisions include deciding whether a firm should collaborate with another firm to develop an innovative new product or go it alone, and whether a company should diversify into a new industry or market as opposed to concentrating on its core businesses.

A common theme—risk—underlies many of the theories and perspectives used to examine strategic decision making. Consistent with common usage, managers use the term risk to refer to the possibility of outcomes that are worse than their expected levels, and the degree to which this poor performance could hurt managers and their firms. This usage of risk differs from the usage in many academic fields that associate risk with variability of outcomes or choices involving well-specified potential outcomes, where each outcome is associated with a probability of occurrence. Researchers of strategic decision making generally use the term risk when referring to decisions that involve uncertainty or ambiguity.

Three theoretical perspectives, namely, the behavioral theory of the firm (BTOF), behavioral decision theory (BDT), and agency theory, dominate the work on strategic decision making. These theories are described in the following text.

Behavioral theory of the firm (BTOF). According to this theory, firms consist of coalitions of stakeholders. As such (and in contrast to traditional economic conceptualizations of firms as entities with a single goal or a well-defined set of many goals), firms have aspiration levels on many dimensions of performance. That is, firms seek to achieve desired levels of performance related to areas such as sales, profits, and so on. Various factors influence aspiration, including a firm's performance relative to its peers and the firm's historical performance. The theory predicts that the firm will make few changes to its existing routines and will operate as usual when the performance of a firm exceeds its aspiration level. However, when the performance of a firm falls below its aspiration level, the firm will seek to raise its performance by making changes that typically increase firm risk (see **illustration**). In this theory (and again in contrast to traditional economic theory), firms do not seek optimal results; rather, they seek to "satisfice," or produce good-enough results; in this context, the term satisfice refers to aiming to achieve satisfactory results. Empirical work on the behavioral theory of the firm has used field or archival data on firms.

Although the above description captures the essence of the theory, it should be noted that the theory also takes into account contingent factors that influence the likelihood that firms will change their routines in response to performance below

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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).

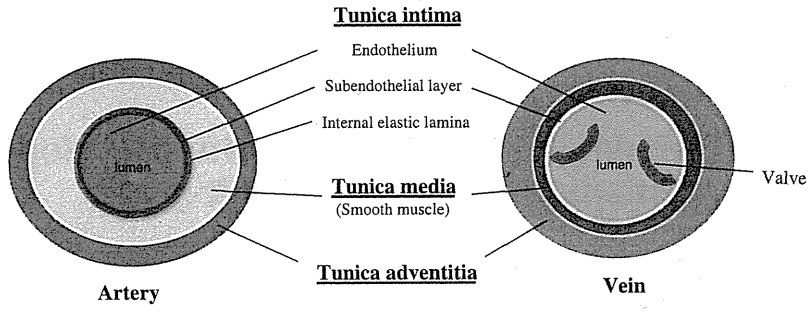

Fig. 1 Morphology of arteries and veins in the vascular network. Depicted are schematics representing cross-sections of arteries and veins. Arteries exhibit thicker smooth muscle walls compare to veins, while by comparison veins have a thicker tunica adventitia

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].

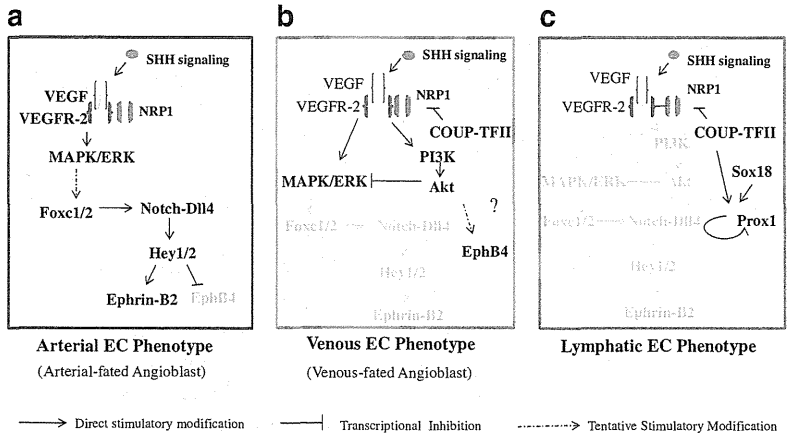

Fig. 2 Model of arterial, venous and lymphatic EC specification. **a** Arterial-fated angioblast forms arterial ECs in the presence of Shh molecules that induce the signaling protein vascular endothelial growth factor (VEGF). VEGF interacts with VEGFR-2 and *neuropilin1 (NRP1)* activating Notch-Dll4 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

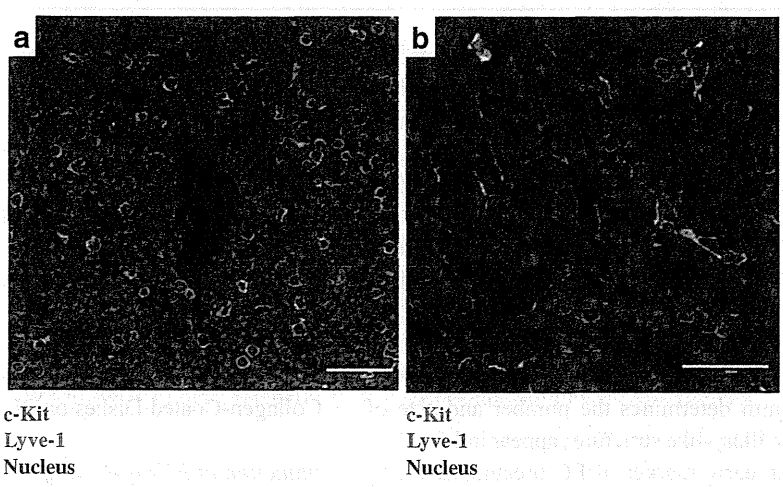

Fig. 3 Immunohistochemistry of fetal liver. Section of fetal liver were prepared from ICR mouse embryos at E14.5, stained with the indicated antibodies and observed under confocal microscopy. **a** Confocal image of hematopoietic stem cells and progenitors expressing c-Kit (*green*; hematopoietic stem 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

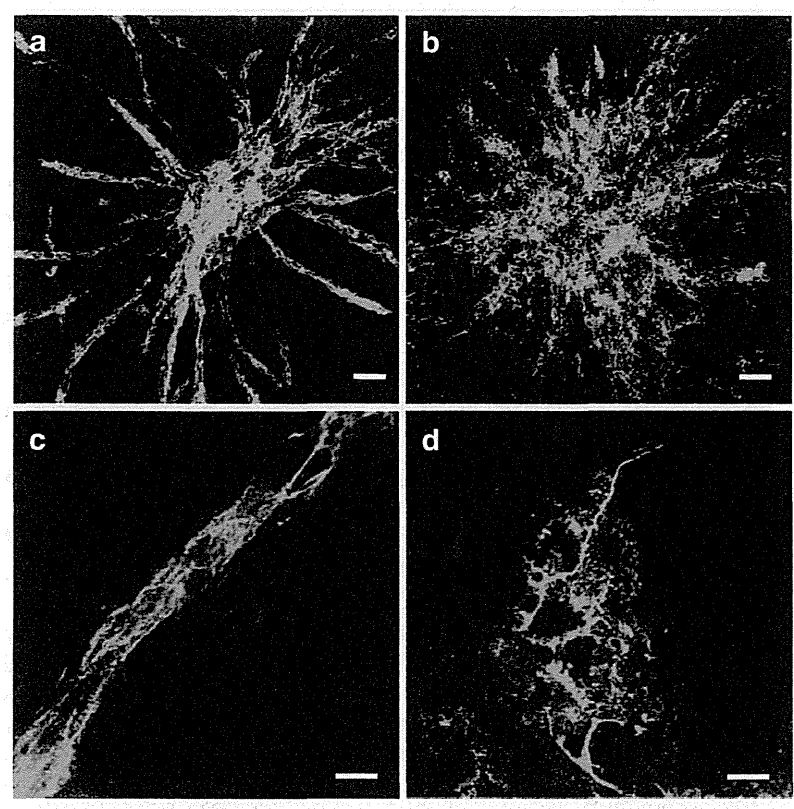

Fig. 4 Immunofluorescence staining of ES cell-derived ECs. Shown is immunofluorescence staining with VE-cadherin antibody (*green*) of ES cell-derived ECs. Flk-1+ mesodermal cells are induced from *Foxo1*(+/+) (*left*, *top* and *bottom panels*) and *Foxo1*(−/−) (*right*, *top* and *bottom panels*) ES 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 replated 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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