Transcriptional Regulation of Endothelial Cell and Vascular Development

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Abstract: The establishment and maintenance of the vascular system is critical for embryonic development and postnatal life. Defects in endothelial cell development and vessel formation and function lead to embryonic lethality and are important in the pathogenesis of vascular diseases. Here, we review the underlying molecular mechanisms of endothelial cell differentiation, plasticity, and the development of the vasculature. This review focuses on the interplay among transcription factors and signaling molecules that specify the differentiation of vascular endothelial cells. We also discuss recent progress on reprogramming of somatic cells toward distinct endothelial cell lineages and its promise in regenerative vascular medicine. (Circ Res. 2013:112:1380-1400.)

Key Words: angiogenesis ■ endothelial cells ■ reprogramming ■ transcription factors ■ vasculogenesis

The vasculature consisting of arterial, venous, and lymphatic vessels forms through 2 distinct processes during embryogenesis: vasculogenesis, defined as de novo vessel formation induced by differentiation of angioblasts and angiogenesis defined as new vessel formation secondary to proliferation of endothelial cells (ECs) from preexisting vessels.1-3 Although vasculogenesis is the major mechanism of formation of blood island vessels, dorsal aorta, endocardium, and vitelline vessels in the embryo, angiogenesis is the predominant means of vascularization of all organs. Vasculogenesis was thought to occur only in developing embryos; recent studies show that vasculogenesis persists during vascular repair in the adult through differentiation of endothelial progenitor cells.4

Although there is no established distinction between angioblasts and endothelial progenitor cells based on specific markers, we will use the term angioblast to represent the precursor EC responsible for vasculogenesis in the developing embryo, whereas endothelial progenitor cell denotes the progenitor cell that differentiates to ECs during vessel formation in adult. We will not deal with the debate and controversy about bone marrow–derived cells that have been referred to as endothelial progenitor cells. For the definition of these controversial cells, their origins, and presumed functions, the reader is referred to the review.5

The first identifiable structures of developing mammalian embryos are blood vessels and the heart, which provide perfusion and nutrient delivery necessary for organogenesis. Early embryonic lethality is invariably the consequence of impaired cardiovascular development. The first sign of blood vessel formation occurs at the gastrulation stage as early as mouse embryonic day (E) 7.5 in the extraembryonic yolk sac blood island (Figure 1).6-8 Blood vessels in the blood island are lined by ECs and are perfused by primitive erythrocytes. The blood island subsequently fuses to form the primary plexus, the immature vascular network, which is followed by the phase of vascular remodeling in the yolk sac leading to formation of the complex yolk sac vasculature (Figure 1).

Vessel formation in the embryo proper is preceded by the appearance of angioblasts at E7.5,2 crucial cells which establish the vasculature of intraembryonic regions, including the dorsal aorta and vitelline vessels, and primary plexuses of lungs, spleen, and heart.3 The more complex phase of formation of the embryonic vascular networks occurs by angiogenesis during which newly formed vessels are stabilized through interactions of ECs with each other via endothelial junction proteins and with recruited mural cells, the pericytes, and an ordered extracellular matrix.2,3,9

The newly formed vessels of the developing embryo thereafter further specialize into arteries, veins, and capillaries, which have distinct functions based on the presence and amount of smooth muscle cells and specific extracellular matrix characteristics of the vessel wall.10 Although capillaries are not invested with smooth muscle cells, arteries develop a thick tunica medium consisting of elastic fibers and smooth muscle cells required for their vasomotor tone and conduit function. Veins by contrast contain fewer elastic fibers and smooth muscle cells (and hence are compliant) and have valves to prevent blood back-flow.10 Between E10.5 and E11.5, lymphatic ECs are generated from a subpopulation of cardinal vein ECs and the intersomitic vessels, and they migrate dorsolaterally to form lymphatic sacs and the lymphatic vasculature (the so-called third circulation), which functions to regulate tissue fluid balance and provide immune surveillance through lymphocyte trafficking (Figure 1).11,12 In this
review, we focus on transcriptional regulation and essential signaling components of vascular development and cell reprogramming by transcription factors required for differentiation of ECs and for vascular development.

### Development of Vascular Structures

#### Hemangioblasts and the Establishment of Distinct Vascular Structures

The close relationship between hematopoiesis and vessel formation has led to the canonical view that both hematopoietic cells and ECs develop from a common progenitor cell, termed the hemangioblast. However, the hemangioblast as a cell remains poorly defined and elusive. During *Drosophila* embryogenesis, the lymph gland, the major site for hematopoiesis, develops in close proximity of the aorta. Analysis of bryogenesis, the lymph gland, the major site for hematopoietic and endothelial differentiation. Further, studies in zebrafish described a *flk1* mutant displaying defects in both hematopoietic and endothelial differentiation. The cells may have a same precursor, their generation follows different routes.

The hemangioblast concept has been further challenged by the recognition of a specialized EC population capable of generating hematopoietic cells, known as the hemogenic endothelium. Zovein et al showed by lineage tracing ECs with VE-cadherin Cre recombinase-estrogen receptor T2 (tamoxifen inducible Cre) and RosaR26LacZ reporter that VE-cadherin lineage cells located within the aorta-gonad-mesonephros generated hematopoietic stem cells. In subsequent studies, the genesis of hematopoietic stem cells from ECs within the aorta-gonad-mesonephros was seen in dramatic images of these cells being shed from the endothelium of mice and *zebrafish*. In addition, it was shown that ES cell–derived blast colony–forming cells generated hematopoietic cells through a presumptive hemogenic endothelium defined by TIE2+/c-KIT+/CD41+ cells.

### First Emerging FLK1/VEGFR2+ Cells Are Multipotent Progenitors of the Cardiovascular System

Despite the controversy surrounding the hemangioblast origin of ECs and blood cells, it is clear that VEGF-FLK1 signaling is essential for EC generation and vessel development.
discuss below the developmental kinetics of EC lineage with a focus on the key role of FLK1+ cells in giving rise to ECs.

ECs are derived developmentally from the mesoderm, which can be marked definitively by the expression of Brachyury/T box gene (Figure 2). Brachyury is expressed throughout the primitive streak during gastrulation. Mice deficient in Brachyury were embryonically lethal with defects in primitive streak, notochord, and allantois. A chimeric aggregation study between Brachyury-null ES cells and wild-type embryos demonstrated that Brachyury was essential for the proper movement of mesoderm from the primitive streak. Using ES cells in which the expression of GFP is controlled by the Brachyury promoter, it was shown that Brachyury+ mesodermal cells were the first to appear and subsequently expressed FLK1, thus becoming Brachyury+FLK1+ cells during differentiation in vitro. In developing mouse embryos, the expression of Flk1 was first detected in the posterior portion of the primitive streak, followed by preferential expression in vascular ECs of the yolk sac and embryonic vasculature, including the endocardial tube. Deficiency of Flk1 induced embryonic lethality because of lack of yolk sac blood island and blood vessel and endocardium formation. A Chimeric aggregation study using wild-type embryos showed that Flk1-null ES cells failed to induce vessel development and hematopoiesis. Therefore, the first emerging FLK1+ cells represent hemangioblasts, and FLK1 is indispensable for the development of both blood and EC lineages.

Cell lineage tracing experiments have provided further insights into blood and EC potential of FLK1+ cells. FLK1+ cells present in differentiating ES cells were fractionated by the expression of SCL, a basic helix-loop-helix (bHLH) transcription factor, which plays an important role of hematopoiesis and vessel remodeling (see below), and thereby tracked for their developmental potential. To track and isolate the cells expressing SCL, a truncated human CD4 gene, a coreceptor for T-cell receptor signaling, consisting of only extracellular and transmembrane domain was knocked into the endogenous Scl locus and thus SCL+ cells could be identified with hCD4 (human cluster of differentiation 4) expression without affecting cell viability. A subset of FLK1+ cells was shown to progress to either FLK1+SCL+/hCD4+ cells or FLK1+SCL−/hCD4− cells. However, the hemangioblast activity was enriched only in the FLK1+SCL+/hCD4+ cells. Interestingly, ECs could also develop independently of SCL expression in that both FLK1+hCD4+ and FLK1+hCD4− cells generated ECs on further culture. These findings suggest that angioblasts and hemangioblasts represent 2 distinct origins of ECs. The first arising FLK1+SCL+ cells represented the in vitro equivalent of hemangioblasts, and that later ECs can also develop independently of hematopoiesis directly through angioblasts.

Other studies using the genetic cell tracing method have pointed to a broader potential of FLK1+ cells because the FLK1+ cells obtained from differentiating ES cells were also shown to generate smooth muscle cells. In addition, FLK1+ cells
cells marked by Flk1Cre;Rosa26 reporter (R26R) were detected in skeletal muscles and cardiomyocyte of E10.5 embryos, besides the expected endothelial and blood cells. Another study showed the presence of FLK1+/LacZ+ cells in cardiac and skeletal muscles; however, on reanalysis of FLK1+/LacZ mice after deleting the Neo cassette (Flk1/LacZ-neo-out), a much stronger and broader LacZ-stained cell population was found compared with a previous study with FLK1+/LacZ-neo-in mice. Thus, the FLK1+ mesoderm likely represents a multipotent progenitor cell population in addition to blood and EC progenitors of the cardiovascular system.

Transcriptional Regulation of Vessel Development

The details of transcriptional control of vessel development are summarized in Figure 3 and Table and discussed under the headings below that describe the function of individual transcription factors.

ER71/ETV2

E-26–specific (ETS) transcription factors are highly homologous in a short stretch of 85 amino acids (ETS DNA-binding domain) located at the carboxyl terminus. A winged helix-turn-helix motif formed by the ETS domain binds the consensus sequence (5′-GGAA/TCT-3′) to regulate expression of target genes. In addition to the ETS domain, all ETS factors contain a transactivation domain, and some members of the family, ETS1, FLI1 (Friend leukemia integration 1), and ERG (Ets-related gene), contain a Pointed domain that mediates protein–protein interactions and oligomerization. Up to now 27 ETS factors have been reported in humans and mice. The presence of ETS binding consensus sequence is seen in >200 genes involved in tumorigenesis, apoptosis, angiogenesis, and hematopoiesis, suggesting that ETS factors are critical in many biological and pathological processes.

Among the ETS factors regulating vascular development, recent attention has focused on ER71 (ETS-related 71; also known as ETV2). ER71 is a 38-kDa protein comprising an N-terminal transactivation domain and C-terminal ETS DNA binding domain (Figure 4A). ER71 was initially thought to be testis specific; however, more thorough analysis showed it to be present in the posterior mesoderm, extraembryonic mesoderm, lateral plate mesoderm, and cardiac crescent in embryos between E7.0 and E7.75. Most ER71+ cells were also positive for FLK1, consistent with its importance in vascular development. At later embryonic stages (E8.25–E9.5), Er71 expression was mainly seen in major vessels, specifically in the dorsal aorta, cardinal vein, brachial arch, intersomitic vessels as well as endocardium, followed by a sharp decrease beyond E11.5. This on-off expression pattern was also conserved in differentiating ES cells in vitro. Er71 expression preceded the Flk1 message and showed a transient pattern with a peak occurring at D3 of ES cell differentiation and was undetectable at D4 and thereafter. When sorted at D2.75, the Er71 message was highly enriched only in FLK1+ cells. With regard to Er71 expression relative to Flk1, a study showed an unusual result that FLK1+ cells appeared before ER71+ cells. This observation cannot be reconciled with what seems to be an important function in ER71 in giving rise FLK1+ cells. The discrepancy might be explained by different culture conditions [ie, embryoid body (EB) formation under serum versus ES differentiation on OP9 under cytokines] and the genetic manipulations performed (wild type versus Er71-Venus). In addition, another general concern is that both EB formation and OP9 coculture methods have been used in different studies for differentiating mouse ES cells in vitro, which could account for discrepancies in this and other findings. In the EB formation method, ES cells differentiate in suspension and form cell aggregates, the EBs which contain 3 embryonic germ layer lineage cells. In this protocol,
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VCAM1 indicates vascular cell adhesion molecule 1; and ENU, N-ethyl-N-nitrosourea.

*Morpholino knockdown.
EB thus mimics a 3-dimensional structure of an embryo that could be critical for cell–cell interactions needed for embryo genesis. In contrast, the ES cells differentiating in a feeder cell line OP9 do not form EBs. In this coculture system, one can efficiently evaluate the effects of added cytokines or growth factors, compared with the EB protocol. ES cells in the latter system may be directed to differentiate into certain cell lineages by factors including soluble cytokines and growth factors, as well as signals from cell surface molecules on OP9 cells. Thus, in analyzing the results of differentiation, it pays heed to note and be cognizant of the differences such as these in differentiation protocols.

Gene knockout studies showed an indispensable function of ER71 in both vessel and blood development. Mice deficient in Er71 died at E10.5 and displayed complete lack of vessel structures and hematopoietic cells. Overexpression of Er71 in ES cells induced the generation of FLK1+ cells, as well as endothelial and hematopoietic cells. ER71 was shown to function by interacting with Fox (forkhead transcription factor)-C2, which induced the expression of key endothelial genes that are critical for endothelial and hematopoietic lineages, Flk1, VE-cadherin, Tie2, Scl, Notch4, and Nfatc1 (nuclear factor of activated T cells, cytoplasmic 1). These findings have implicated a fundamental role of ER71 in vasculogenesis and hematopoiesis (Figure 4B).

A vasculogenic role of ER71 has also been reported in the zebrafish and Xenopus, indicating that the gene is evolutionarily well conserved. In zebrafish, etsrp, an Ets transcription factor in zebrafish, was expressed preferentially in the vasculature, similar to mouse ER71, and identified to be downregulated in the clo mutant. Knockdown of etsrp in zebrafish embryos led to complete absence of vascular structures, whereas overexpression of etsrp induced genes required for differentiation of endothelial and hematopoietic lineages expressing flk1 and scl. etsrp also rescued vascular defects seen in the clo mutant described above. The Xenopus ortholog er71 similarly exhibited a conserved function to the mouse and zebrafish homologs.

Although the above studies describe the important role of ER71 in differentiation of ECs and vessel development, the upstream mechanisms regulating ER71 expression itself are less well understood. Using mouse ES cell differentiation system, Yamamizu et al reported that protein kinase A signaling activated the expression of Er71 through the cAMP response
element-binding protein (Figure 4B). In another study,\textsuperscript{56} Er71 was found as one of the significantly downregulated transcripts in the cardiac progenitors deficient in Nkx2-5 (nk2 homeobox 5), a homeobox transcription factor required for heart morphogenesis, including endocardial cushion formation.\textsuperscript{60–71} Nkx2-5 was expressed in both myocardium and endocardium,\textsuperscript{60–71} but Er71 message was only detected in the endothelium/endocardium in E8.5 hearts,\textsuperscript{60} suggesting a genetic interaction of Nkx2-5 and Er71. Indeed, Nkx2-5 directly bound to the Er71 promoter through Nkx2-5 responsive element, and activated Er71 expression (Figure 4B).\textsuperscript{54} In zebrafish, foxc1a/b also bound the promoter to induce expression of etsrp (Figure 4B),\textsuperscript{72} indicating that Er71 function requires entrainment of multiple transcription factors for its full effect.

\textbf{Early Activation of ER71/ETV2 Is Critical for FLK1+VEGFR2+ Mesoderm Specification and Vessel Development}

Attempts have been made to identify the transcription factors responsible for differentiation of FLK1+ mesoderm into endothelial and hematopoietic cell versus those required for cardiac lineage specification. Studies showed that FLK1+PDGFRα (platelet-derived growth factor receptor α) cells had a distinct cardiogenic potential, whereas FLK1+PDGFRα cells were enriched in endothelial and hematopoietic progenitors.\textsuperscript{73,74} Because ER71 is an important cell fate transcriptional determinant of the FLK1+ mesoderm,\textsuperscript{55} studies were made in which ER71 was deleted in ES cells or mouse embryos.\textsuperscript{57,75} Deletion of ER71 markedly reduced the generation of FLK1+ cells.\textsuperscript{75} Interestingly, most of the FLK1+ cells derived from Er71−/− ES cells also expressed PDGFRα and differentiated into cardiac lineages.\textsuperscript{57,75} Also, overexpression of ER71 in wild-type ES cells significantly enhanced the generation of FLK1+PDGFRα cells that seen in FLK1+PDGFRα cells,\textsuperscript{75} suggesting a dominant role of ER71 for FLK1+ mesoderm specification toward endothelial and blood lineages. In addition, morpholino knockdown of etsrp in zebrafish expanded the myocardium with reduced generation of vascular ECs,\textsuperscript{64} etsrp-GFP+ cells in the absence of etsrp also expressed hand2 (heart- and neural crest derivatives-expressed protein 2),\textsuperscript{64} a cardiomyocyte marker.\textsuperscript{76,77} Ectopic expression of etsrp inhibited expression of cardiac markers but augmented endothelial markers\textsuperscript{64} further supporting the role of ER71 in mediating the differentiation of FLK1+ cells specifically toward the endothelial lineage.

The wingless-int (WNT) signaling pathway might play an important role in inducing the ability of ER71 to differentiate FLK1+ cells toward ECs.\textsuperscript{75} Overexpression of Er71 impaired cardiac mesoderm formation (as evident by decreased generation of FLK1+PDGFRα cells) and repressed expression of the WNT-β-catenin downstream genes, but this was rescued by WNT agonists. Inversely, deficiency in Er71 exhibited enhanced WNT-β-catenin activity.\textsuperscript{75} Elevated level of hemangiogenic mesoderm formation on Er71 overexpression was rescued by overexpressing β-catenin. Although these findings suggest a critical role of β-catenin in ER71-mediated FLK1+ mesoderm specification toward endothelial and hematopoietic cells, gene knockout studies in mice have not been consistent.\textsuperscript{78} Inactivation of β-catenin in FLK1+ cells (ie, Flk1Cre;flxed β-catenin mice) induced defective angiogenesis in the central nervous system with phenotypically normal development of the cardiac and hemovascular systems.\textsuperscript{78}

The findings that Nkx2-5 is expressed in both myocardium and endocardium and regulates ER71 expression\textsuperscript{56} and that ER71 specifies the endothelial and hematopoietic lineages by suppressing the cardiac lineage are seemingly contradictory.\textsuperscript{57,64,75} This apparent contradiction might be because of the definition of cardiac lineage. Endothelial lineages, including the endocardium, cardiac lineages (ie, myocardium/cardiomycocyte), and hematopoietic cells, originate from FLK1+ cells.\textsuperscript{44,45,79,80} Thus, it is plausible that ER71 can specify FLK1+ cells capable of generating hematopoietic cells and endocardium/endocardium at the expense of myocardium generation. Endocardium-specific activation of ER71 by Nkx2-5 remains unknown.

A time course study of ER71 illustrates the importance of the transient nature of its expression in the developing embryos in mediating the formation of blood vessels. The expression of ER71 became undetectable beyond E11.5 of mouse gestation and was very low in mature ECs,\textsuperscript{75–77,81} suggesting that ER71 function is tightly regulated to ensure proper development of the vascular system. In accord with this observation, a study\textsuperscript{82} has reported that persistent expression of Er71 in developing embryos induced developmental abnormalities in vessels and hematopoietic cells. The sustained expression in TIE2+ cells (Tie2Cre;RosaR26R-Er71) caused abnormal vascular development evidenced by dilated yolk sac vessels accompanied with hemorrhaging.\textsuperscript{82} This might be a function of continuous expression of proangiogenic genes, including Flik1 with reduced expression of EC maturation or stability genes Klf2 (Kruppel-like factor 2), Klf4 (Kruppel-like factor 4), Timp1 (tissue inhibitor of metalloproteinase 1), Timp2 (tissue inhibitor of metalloproteinase 2), and Cyr61 (cysteine rich protein 61). Transient expression of ER71 with continuous expression of ERG1 and FLI1 can directly convert amniotic cells to mature ECs,\textsuperscript{83} consistent with the fundamental role of the transient and early-onset of expression of ER71 in mediating EC specification in the developing embryo. In the same context, the time-dependent downregulation of ER71 expression may be needed to ensure EC maturation and formation of interendothelial junction integrity required for functionally normal vessels.

\textbf{SCL/T-Cell Acute Lymphoblastic Leukemia-1}

SCL/T-cell acute lymphoblastic leukemia transcription factor belongs to the bHLH transcription factor family.\textsuperscript{10,34,85} SCL expression is seen in yolk sac blood progenitors and ECs.\textsuperscript{86–88} Ectopic expression of scl in zebrafish induced both hematopoietic and endothelial genes.\textsuperscript{16,90} Defects in blood and vessel formation in both clo and etsrp knockout zebrafish were partially rescued by overexpressing scl.\textsuperscript{16,49,90} Although Scl+/– mice embryos died because of complete lack of hematopoiesis,\textsuperscript{86–88} chimeric analysis of wild type and Scl+/– ES cells showed that SCL was also required for vascular remodeling but apparently not for vasculogenesis.\textsuperscript{89} This was evident by the finding that the mutants with high degree of chimeraism exhibited disorganized yolk sac vessels lacking vitelline vessels.\textsuperscript{91} Further, Scl+/– ES cells failed to contribute to the formation of large vessels.\textsuperscript{91}
SCL has been postulated to be critical for the specification of the hemangioblast rather than angioblasts. Hemangioblast activity was enriched in FLK1+SCL+ cells, whereas ES cells deficient in Scl failed to generate blast colonies. Ectopic expression of Scl in the locus of Flk1 increased blast colony formation compared with Flk1+ cells. Thus, SCL seems to be dispensable for vasculogenesis but it is required for hemangioblast/vascular remodeling in the developing embryo.

Although SCL acted downstream of FLK1, the presence of a putative SCL binding site in the Flk1 promoter suggests additional regulatory roles of SCL in Flk1 expression. Studies showed that SCL working with ID1 (inhibitor of differentiation and DNA binding 1) can modulate the promoter activity of Flk1 by binding E2-2, a suppressor of Flk1 promoter activation and angiogenesis. The functional significance of SCL interaction with ID1 in regulating angiogenesis in vivo needs further investigation.

Overexpression of Er71 in ES cells and zebrafish enhanced the expression of Scl, but knockdown of Er71 induced a reduction of Scl. Genome-wide sequence analysis showed that Scl contains the binding site for ETS. Luciferase and chromatin immunoprecipitation experiments positioned Scl downstream of ER71. The mechanism of ER71 regulation of SCL and how it regulates EC and vessel development remains unexplored, and it is potentially a fruitful area because it will help to connect the function of these 2 key transcription factors.

GATA2

The GATA (GATA binding protein) transcription factors (GATA1 through GATA6) belong to C2H2 zinc-finger transcription factors and bind (T/A)GATA(G/A) in the genomic DNA. Among the known GATA members, initial analysis has described GATA1, GATA2, and GATA3 as the hematopoietic GATAs because of their preferential expression in hematopoietic cells. Gata1−/− mouse embryos displayed defects in erythropoiesis. Mouse embryos deficient in Gata2 died by E11.5 and exhibited anemia. Gata2−/− ES cells and yolk sac cells generated significantly reduced number of multipotent progenitors. However, evidence of endothelial expression of GATA2 in developing embryos suggests that GATA2 might also play an important role in vessel development. Indeed, Lugus et al. reported that expression of Gata2 was enriched in blast colony–forming cells and that overexpression of Gata2 in ES cells enhanced the generation of FLK1+ cells and ECs in vitro. The findings that several key endothelial genes contain GATA binding sites further suggest that GATA2 regulates vessel development through transcriptional activation of endothelial genes, including Flk1 and Vcadcaderin. In this regard, a recent report showed that GATA2 regulates endothelial-specific gene expression and thus endothelial specificity through epigenetic modification. In this study, the authors found that ENDOMUCIN, an endothelial-specific gene, contained preferential GATA2 binding sites in transcription start site (TSS) and −139 kb region of human dermal microvascular ECs. An epigenetic experimental approach also demonstrated that both regions are in active chromatin state in human dermal microvascular ECs.

In contrast, the erythroid cells, K562, which also highly express GATA2, contained preferential GATA2 binding sites in SCL/T-cell acute lymphoblastic leukemia-1 (ENDOMUCIN) transcription start site and −139 kb region in K562 showed enrichment of H3K9me3. Interestingly, knockdown of GATA2 in human dermal microvascular ECs significantly reduced the expression of endothelial genes, including ENDOMUCIN and KDR/VEGFR2, but also increased expression of non-EC genes, such as SM-ACTIN and SNAIL. Together, these results suggest that GATA2 plays an important role in mediating endothelial gene expression and the maintenance of EC fate. The mechanisms of GATA2-mediated EC specification in developing embryos remain to be elucidated. A fundamental question is whether ER71 can form a transcriptional complex containing GATA2 to regulate endothelial gene expression.

A recent study has also described the previously unknown function of GATA2 in lymphatic vessel development. The authors generated mice in which Gata2 is conditionally inactivated by Cre recombinase-estrogen receptor T2 under Gata2 VE enhancer and found that the conditional knockout embryos died at =E16.5 and importantly showed abnormal lymphatic vessel formation manifested by subcutaneous edema and a presence of mixed lymphatic-blood vessel structures. Because mice with germ line knockout and conditional deletion of Gata2 develop normal vascular structure during the early embryo stage (E10–E11), it seems that GATA2 plays an important role in lymphangiogenesis, but it is not required for EC specification and the initial establishment of primary vascular structure. But it is still not possible to exclude functional redundancy in studies in which Gata2 is deleted; that is, loss of GATA2 in developing mouse embryos (before E16.5) could be compensated by other GATA members, hence resulting in normal embryonic vasculature development. The finding that GATA3 directly binds the TIE2 promoter in adult ECs suggests such a redundancy concept if GATA3 is upregulated after deletion of GATA2.

Inhibitor of Differentiation and DNA Binding

ID proteins have been implicated in embryonic and postnatal angiogenesis. IDs, consisting of 4 members (ID1, ID2, ID3, and ID4), belong to the bHLH transcription factors, but lack DNA binding domains; thus, they function by sequestering transcription factors that target genes. Expression of Id1 and Id3 was detected in embryonic vessels and also mice deficient in Id1 and Id3 showed abnormal vascular development. In cultured ECs, overexpression of Id1 enhanced EC proliferation and migration, whereas Id1 knockdown inhibited these responses, suggesting a proangiogenic role of ID1. IDs together with SCL derepressed Flk1 promoter activity by interacting with E2-2, a bHLH transcription factor, functioning as a negative regulator of Flk1 expression. In addition, studies showed that ID1 and ID3 are direct downstream targets of the bone morphogenetic protein (BMP)-SMAD (smad) pathway, suggesting that BMP signaling may be important in activating IDs and hence in generating FLK1+ ECs.
Role of Other ETS Transcription Factors

The ETS transcription factors display a broad range of expression patterns and activities in developing mouse embryos and in adults. However, some of these factors show a preferential expression in ECs, and it is important to consider their role in generation of ECs and vessels during development. Expression of the ETS transcription factors, Ets1 and Fli1, can be detected in the yolk sac blood islands in the early stage embryos and their expression is maintained in developing vessels.118–120 Whereas the ETS transcription factor, Erg1, is highly expressed in mesodermal lineages, including ECs,121 another ETS transcription factor, Erv6 shows ubiquitous expression pattern throughout embryogenesis and in adults.122,123 The high degree of variability in the expression patterns of the ETS transcription factors makes it difficult to ascribe specific functions in mediating differentiation of ECs and vascular development.

Gene knockout mouse studies, however, have demonstrated in a general sense the importance of the ETS transcription factors in mediating vessel remodeling and structural integrity.124 Germ line deletion of Ets1 did not cause vascular defects.125 However, Ets1−/−;Ets2−/− mouse embryos died in utero and showed vessel branching defects,126 indicating the redundant role of ETS1/2 (c26 avian leukemia oncogene 1/2) in embryonic vessel formation. In addition, a recent study suggests a novel function of ETS1/2 in lymphatic vessel formation (see prospero homeobox transcription factor 1 [PROX1] below).127

The role of Fli1 in embryonic vessel development is intriguing. Studies from Xenopus showed that morpholino knockdown of fli1 led to a significant reduction of expression of genes critical for early hematopoietic and EC development scl, lmo2, and flik1.128 The expression of fli1 was not affected in clo mutant or in zebrafish treated with scl or lmo2 morphants. These results suggest that fli1 is one of the earliest transcription factors involved in endothelial and hematopoietic cell specification. However, knockdown of zebrafish fli1 did not recapitulate the defects seen in Xenopus.128 Similarly, Fli1−/− mouse embryos did not display severe vascular defects; Fli1−/− embryos died around E11.5 with extensive hemorrhaging,129 indicating its dispensable role in EC specification unlike the findings in Xenopus and clo mutant.128 This discrepancy might be explained by redundant role of other ETS transcription factors, such as ERG1 in zebrafish and mice knockout studies.

Homozygous mouse embryos for ERG<sup>Mold/Mold</sup> where serine at residue 329 on ERG1 is substituted with proline, resulting in nonfunctional ERG because of missense mutation, died because of hematopoietic defects and exhibited dilated vessels in the brain.130 In a subsequent study, it was demonstrated that ERG1 directly induced the expression of CDH5 (<i>vascular endothelial cadherin; cadherin 5, type 2</i>) in human vascular ECs,131 suggesting that ERG1 regulates EC–cell interaction possibly through formation of adherens junctions.

Forkhead Transcription Factors

The forkhead transcription factor family (also known as forkhead/winged helix domain) is characterized by the conserved DNA-binding domain, which recognizes the consensus sequence 5′-TTGTTTAC-3′.132,133 Among the forkhead transcription factors, FOXO and FOXC are implicated in vascular development and EC generation.133 There are 4 members of FOXO family: FOXO1/FKHR, FOXO3/FKHRL1, AFX/FOXO4, and FOXO6. FOXOs functioning mainly through phosphatase and tensin homologue deleted on chromosome 10 are involved in cell survival/apoptosis, cell cycle, DNA repair, and reactive oxygen species regulation.133,134 In mouse embryos, FoxO1 is highly expressed in the developing vessels and FoxO1−/− mice embryos failed to survive beyond E10.5 because of defective vascular development in the yolk sac and embryo.135,136 A detailed gene expression analysis uncovered decreased expression of Gja4 (gap junction-c4) and Gja5, both of which are components of gap junction.135 As gap junction genes are preferentially expressed in developing embryos,137,138 the functional interaction between FOXO1 and GJA4/GJA5 may be important in the development of the embryonic vasculature. Mice deficient in FoxO3 or FoxO4 in contrast to FoxO1 did not show vascular defects.136 However, overexpression of FoxO1 and FoxO3 inhibited endothelial tube formation and migration partly through direct inhibition of endothelial nitric oxide synthase,139 believed to be a proangiogenic factor.140 Overexpression of FOXO3 induced angiogenesis as evidenced by enhanced recovery of blow flow from ischemic injury with a concomitant increase in capillarity.139 Conditional deletion of FoxO1, FoxO3, and FoxO4 using Mx1Cre mice led to the development of hemangiomas,141 indicating suppressive function of FOXOs in EC proliferation.

FOXC1 and FOXC2, members of the C subgroup of forkhead transcription factor family, have also been found to regulate arteriovenous specification and lymphatic vessel differentiation.133 Promoters of multiple endothelial genes, Flik1, VEcadherin, Pecam1, Tie2, and Scl, contain evolutionarily conserved FOX:ETS binding motifs.63 As discussed above, ER71 and FOXC2 were shown to bind the FOX:ETS motifs present in promoters and enhancer elements of these genes and to function cooperatively to activate transcription (Figure 4B). foxc1a/foxc1b in zebrafish can also function as an upstream regulator of <i>etv6</i>, indicating a critical role of cooperation of FOXC members with ER71 in mediating EC development. FoxC1-deficient mice died pre- and postnatally with multiple vascular defects, including coarctation of the aortic arch.142 FoxC2 deficient mice exhibited similar defects but with abnormal development of lymphatic vessels manifested by lack of valves and increased vascular smooth muscle cells.143 Studies on double FoxC1/FoxC2 deleted mice demonstrated a role of FOXC in determining artery and venous EC fate as demonstrated by the presence of arteriovenous malformations in the mutant embryos and failure to express the arterial markers Dll4 (δ-like ligand) and Notch with unaltered expression of the venous markers, <i>Coup-Tfii</i> (chicken ovalbumin upstream transcription factor I) and <i>EphB4</i> (ephrin type-B receptor 4).144,145 FOXC1 and FOXC2 may function by activating the Dll4 promoter through a direct binding on the FOX binding sites of this promoter.145 FOXC2 together with [Su(H)] (suppressor of hairless)-NICD (Notch intracellular domain) may also function by directly activating the Hey2 (Hairy/enhancer-of-split related with YRPW motif protein 2) promoter to mediate arterial EC specification.146
In addition, FOXC2 functions in proper lymphatic vessel development. In the absence of FoxC2, the mutant embryos developed lymphatic vessels with defective lymphatic valves and abnormally enhanced pericytes recruitment.143 FOXC2 was shown to bind and activate the Nfatc1 promoter.147 Cyclosporine A–mediated inactivation of NFAT signaling mimicked the lymphatic phenotypes seen in Fox2−/− mouse embryos.148 Taken together with the finding that inactivating mutations in FOXC2 are responsible for lymphedema-distichiasis in human,149 it seems that FOXC2 is a versatile transcription factor that has a role in FLK1+ cell generation, arterial specification, and lymphatic vessel development (Figure 4B).

Other subclasses of forkhead transcription factors FOXF1, FOXH1, and FOXM1 have also been implicated in vessel and EC development. FoxF1 is expressed in the posterior primitive streak, allantois, amnion, and yolk sac vasculature.149,150 Germ line deletion of FoxF1 led to avascular yolk sacs and allantois.150 FOXF1 may act downstream of hedgehog signaling to induce BMP expression which is an important inducer for vasculogenesis.151 FOXF1 was also shown to function as an upstream activator of Flk1 gene expression.152 A potential binding site for FOXF1 was identified in the specific locus (distal-multitopotent-mesodermal-enhancer) of the Flk1 gene.153 In the zebrafish, foxb1 bound to the fkb1 promoter and repressed gene expression and induced defective vessel formation.154

In contrast, FOXM1 is dispensable for vessel development in developing embryos since mice lacking FoxM1 in endothelial cells (Tie2Cre;floxed FoxM1) were born alive.154,155 However FOXM1 was demonstrated to have an entirely novel function in mediating EC proliferation and vascular repair.154,155 In response to vascular injury elicited by lipopolysaccharide (LPS), vessels of mice exhibited se­


tirely impaired ability to repair compared to controls. This defective vessel repair program was ascribed to reduced expression of β-catenin in adherens junctions of ECs deficient in FoxM1 and reduced EC proliferation.154,155 Thus, FOXM1 functioned by transcriptionally activating the expression of β-catenin through FOXM1 binding sites on the promoter.155

**HEY1/HEY2**

The NOTCH pathway controls EC specification (arterial versus venous ECs) and plays an important role in EC sprouting.156,157HEY1 and HEY2, members of hairy and enhancer of split-related family of bHLH transcription factors, are direct transcriptional targets of the NOTCH pathway.158 Hey1−/− and Hey2−/− mouse embryos developed defective vessels and died at E9.5.159,160 In these mice, vasculogenesis occurred normally, but vessel remodeling in the yolk sac and placenta was impaired and some mice showed poorly developed dorsal aorta and cardinal veins. Expression of arterial markers was also significantly reduced in these mice. A similar finding was seen in gridlock (grl) zebrafish (grl is the zebrafish ortholog of mammalian Hey2) where dorsal aorta development was defective.161 These studies collectively suggest the potentially important role of HEY1/HEY2 in arteriovenous specification.

In line with the above findings, deficiency of Rbpj (recombination signal binding protein for immunoglobulin kappa J region) in mice, an obligatory transcriptional factor of the NOTCH pathway,162 also induced defective arterial vessel formation. These studies showed that the NOTCH pathway in ECs controls the development of arteries through RBPJ-HEY1/2 signaling.

**SOX7, SOX17, and SOX18, Members of the SOX Transcription Factor Family**

The SOX (Sry-Related High Mobility Group Box) transcription factor family shares a conserved DNA binding domain, known as high mobility group, that recognizes the consensus sequence 5’-(A/T)A(A/T)CCA(A/T)G-3’ present in various genes.163 Among 20 different members of the SOX factors, the SOX F group (Sox7, 17, and 18) has been found to play an important role in vascular development (Figure 4C). Expression of Sox18 and Sox7, but not Sox17, was detected between E7.5 and E8.5 in ECs of the yolk sac vasculature and the dorsal aorta, and Sox17 was expressed in ECs of the dorsal aorta at E9.5.165–167 Sox18 was also evident in ECs of the developing lymphatic vessels.168

Linkage analysis combined with DNA sequencing showed that SOX18 was responsible for the phenotypic defects observed in 4 spontaneous mouse mutants, Ragged (Ra), Ragged-Opossum (RaOp), Ragged-Jackson, and Ragged-like, all of which carried a mutation in Sox18 gene, making a dominant negative form of SOX18.169,170 RaOp homozygous mice initially developed a normal vasculature but died at E14.5 with defective lymphatic vessel development accompanied by hemorrhage and edema.170 Also, Sox18−/− mouse embryos in C57BL/6 background phenocopied the defects seen in RaOp homozygous mice;166 the mutant embryos showed lethality at E14.5 and developed no PROX1+ lymphatic ECs, indicating a critical role of Sox18/RaOp in lymphatic EC specification. Sox18 binding to sites present in Prox1 promoter may be responsible for Prox1 expression.166 Overexpression of Sox18 in differentiating ES cells induced expression of genes critical for lymphatic ECs Prox1 and Podoplanin (Figure 4D), but Sox18/RaOp suppressed these genes.168 Deficiency of Sox18 in 129-CD1 mixed background, however, did not show these defects, and the mice were born alive and apparently normal.171 This discrepancy in these mixed background mice may because of the compensatory expression of SOX7 and SOX17 in lymphatic ECs because these genetic changes did not occur in pure C57/B6 background.168,171,172 These findings collectively suggest that SOX7 and SOX17 function as genetic modifiers of lymphatic vessel development (Figure 4C).

SOX18 can also function redundantly with SOX7 or SOX17 in arterial-venous specification of ECs. Zebrafish injected with double morphants of sox7 and sox18 showed augmented expression of the venous markers dab2 (disabled homolog 2) and fibr (fms-related tyrosine kinase 4) in arteries coupled with decreased expression of the arterial markers notch3, ephrinB2 (ephrin type-B receptor 2), and dll4.173 In a similar study, double knockdown of sox7 and sox18 in zebrafish resulted in ectopic expression of fibr with concomitant decrease of ephrinB2 in the dorsal aorta.173 When both Sox17 and Sox18 were deleted in mixed background mice, the mutants displayed defects in anterior dorsal aorta and endocardial tube development.167 In contrast, Matsui et al.166 reported that mixed background Sox17−/−;Sox18−/− mice were perina­tally lethal and developed abnormal vascular structures. ECs
of Sox17−/−;Sox18− mice also showed significantly impaired tube forming ability in vitro.109 Taken together, these studies suggest that the SOX F group transcription factors function as an important regulator for EC specification, arteriovenous specification, and venous-lymphatic specification.

PROX1

Prox1 expression at E9.5 was detected in a subpopulation of cells dorsolateral to the cardinal vein.175 Thereafter, Prox1 expression became negative in the cardinal vein and it was confined to the cells migrating out of the cardinal vein which formed the lymphatic sac and eventually the lymphatic vasculature itself.176,177 Prox1 continued to be expressed in the adult lymphatic ECs. A number of studies have established a critical role of PROX1 as a supreme regulator of lymphatic EC specification and maintenance. Prox1+/− mice died shortly after birth with defects of enteric lymphatic vessels.175 Prox1+/−;Prox1LacZ/LacZ mouse embryos examined at E14.5 showed no sign of lymphatic vessel formation with normal development of blood vessels.175,177 In these mice, Prox1+/−;Prox1LacZ/LacZ cells began to bud from the cardinal vein but failed to migrate, resulting in the lack of lymphatic sac and lymphatic vessel development. Also, the cells deficient in Prox1 did not express lymphatic markers VEGFR3 and LYVE1 (lymphatic vessel endothelial hyaluronan receptor 1), while they continued to maintain their vascular endothelial identity.177 These results together show the requisite role of PROX1 activity in mediating lymphatic EC specification but they also show that PROX1 is dispensable for lymphatic EC budding from the cardinal vein.

Conditional deletion of Prox1 in embryos and in adult led to reprogramming of lymphatic ECs into blood ECs.179 Prox1 knockdown in adult lymphatic ECs showed the similar results seen in in vivo experiments.178 Overexpression of Prox1 in blood ECs induced the expression of lymphatic makers concomitant with decreased expression of vascular endothelial genes.179,180 In addition, it has been reported that PROX1 can bind with ETS1/2 and that its interaction in a synergistic manner promotes the expression of VEGFR3.177 These findings together show the essential role of PROX1 as a cell fate determinant of lymphatic ECs (Figure 4D).

A recent study on revisiting the issue of lymph-angiogenic function of PROX1 identified another source and mechanism of lymphatic endothelial progenitors.12 Using serial thickings together show the essential role of PROX1 as a cell fate determinant of lymphatic ECs.178

Chicken Ovalbumin Upstream Transcription Factor II

COUP-TFII, a member of the nuclear receptor 2F subfamily, is a nuclear orphan receptor that functions in a wide range of biological processes, including angiogenesis and neural development.182,183 Expression of COUP-TFII starting from E8.5 is limited to ECs of veins and lymphatics, not the endothelium of arteries.182,184 Targeted deletion of COUP-TFII caused abnormal development of the heart and impaired angiogenesis with missing or collapsed cardinal veins, leading to embryonic lethality.182 Inactivation of Coup-TFII in ECs of Tie2Cre mice resulted in death of mutant embryos and loss of venous identity as evident by expression of arterial markers Jag1, Notch1, ephrinB2, and Np1 (neuropilin 1) in the veins.184 Endothelial expression of COUP-TFII induced aberrant vessel development and fusion of artery–vein structures, the same defects seen in Np1 and Notch1 knockout mice.185,186 These findings suggest a regulatory loop between COUP-TFII and the NOTCH pathway needed for artery–vein specification (Figure 4D).

In support of this concept, double knockout of Coup-TFII and NOTCH signaling component, Rbpj, in ECs partially rescued the loss of venous identity seen with endothelial deletion of COUP-TFII.187

Despite the strong evidence of the critical role of COUP-TFII in venous EC specification, the mechanisms regulating the expression of COUP-TFII in this process are poorly understood. A recent study showed that Coup-TFII expression was regulated by BRG1 (brahma-related gene-1) in veins.188 BRG1 is a chromatin-remodeling enzyme and a component of SWITCH/sucrose nonfermentable (SWI/SNF)-like complex.189 Deletion of Brg1 in Tie2+ cells led to embryonic lethality and defective primitive erythropoiesis and yolk sac vessel formation.190 Venous ECs deficient in Brg1 showed decreased level of Coup-TFII expression with aberrantly elevated expression of arterial markers ephrinB2, Nrp1, and Dll4.188 BRG1 binding to the −1.2-kb promoter region of Coup-TFII was also identified, and knockdown of Brg1 caused chromosome condensation of the promoter regions. These results together suggest that BRG1 lying upstream can upregulate Coup-TFII expression through chromosome remodeling and thus induce venous endothelial cell specification.

COUP-TFII is also an important regulator of lymphatic EC identity. Coexpression of COUP-TFII and PROX1 and their interaction is involved in mediating lymphatic EC specification throughout embryogenesis and in postnatal life.191,192 The interaction of COUP-TFII and PROX1 regulated the expression of lymphatic markers Fgfr3 (fibroblast growth factor receptor 3) and Vegfr3.191,192 Further, it has been shown that COUP-TFII binds the promoter of Prox1 through COUP-TFII binding sequences. Taken together, these observations support the concept that COUP-TFII regulates lymphatic cell development through an interaction with PROX1.

Role of Crucial Signaling Pathways in Regulating Endothelial Transcription Factors and Vascular Development

VEGF Signaling

VEGF-activated signaling is the major pathway regulating multiple aspects of EC function, including survival, proliferation, and vessel permeability.193,194 On binding of VEGF, its receptor, FLK1/KDR, transmits signals through several downstream molecules MAPK-ERK, p38-MAPK, phosphatase C, and phosphatidyl inositol 3-kinase/Akt/protein kinase B to regulate endothelial function.195–198 Also (important for this review), VEGF signaling plays a critical role in vessel development during embryogenesis. VEGF+/− mouse embryos died because of defects in endothelial and hematopoietic cell development.32,199 Further, VEGF signaling has a
role in arteriovenous specification. Morpholino knockdown of \textit{vegf} in zebrafish prevented the expression of the arterial marker \textit{ephb2} accompanied by sustained expression of venous marker in dorsal aorta.\textsuperscript{200} Ectopic expression of \textit{vegf} rescued defective arterial differentiation mediated by cyclopamine, a sonic hedgehog inhibitor.\textsuperscript{200,201} VEGF also induced the expression of arterial markers \textit{EphrinB2}, \textit{Np1}, and \textit{Gja5} in primary \textit{EphrinB2−/− ECs} isolated from E10.5 mouse embryos.\textsuperscript{202} In addition, overexpression of VEGF in cardiomyocytes (α myosin heavy chain-VEGF mice) led to significant increase in number of cells expressing the arterial marker \textit{EphrinB2} with decreased number of \textit{EphB4} venous ECs.\textsuperscript{203} The molecular mechanisms of VEGF-mediated arterial specification are still unclear. Recent studies suggest that transcription factors \textit{FOXC1}/\textit{FOXC2} interact with the VEGF pathway components to promote arterial specification of ECs through the NOTCH signaling pathway (see below).

**NOTCH Signaling**

The NOTCH pathway is critical for arterial specification of embryonic vasculature.\textsuperscript{204,205} Four NOTCH receptors (1–4) and 5 ligands (Jagged1, 2, Dll1, 3, and 4) have been identified in mammals.\textsuperscript{204} Binding between the ligand and the receptor induces proteolytic cleavage of NOTCH receptor, resulting in generation of intracellular form of the receptor (notch intracellular domain, NOTCH intracellular signaling) that translocates into the nucleus to induce its downstream targets \textit{HEY1} and \textit{HEY2}.\textsuperscript{198,206} Mice lacking a single copy of \textit{Dll4} exhibited severe remodeling defects in yolk sac vessels and smaller dorsal aorta\textsuperscript{182} consistent with the expression of components of NOTCH signaling, NOTCH1, NOTCH4 and Jagged1, Jagged2, and Dll4 selectively in arterial ECs. Also, these embryos developed abnormal arteriovenous vessels because of fusion between the dorsal aorta and common cardinal vein. In a related study, \textit{Dll4}−/− mouse embryos completely lost arterial identity.\textsuperscript{207} Overexpression of \textit{Dll4} caused abnormal vessel development with enhanced arterialization.\textsuperscript{208} Mice deficient in both \textit{Notch1} and \textit{Notch4} died in utero with a severe vessel remodeling defects in both yolk sac and embryo.\textsuperscript{209} Thus, NOTCH, which can be activated by VEGF (please see below),\textsuperscript{200} has an essential role in mediating arterial specification.

The role of VEGF and NOTCH pathways in promoting arterial EC specification points to a cross-talk of both pathways. The evidence for this comes from the zebrafish in which activation of \textit{notch} was able to rescue arterial defects caused by morpholino knockdown of \textit{vegf}.\textsuperscript{202} Likewise it was shown that VEGF induced the expression of \textit{Notch1} and \textit{Dll4} through phosphatidylinositol 3-kinase/akt pathway in cultured ECs.\textsuperscript{210} The VEGF-mediated NOTCH activation was specifically seen in arterial ECs (as opposed to venous ECs) in vitro. Another study has shown that the cross-talk between both pathways could be mediated by endothelial transcription factors, \textit{FOXC1}/\textit{FOXC2}. As discussed above, deletion of \textit{FoxC1}/\textit{FoxC2} led to arterial defects in developing mouse embryo.\textsuperscript{145} In vitro analysis revealed that \textit{FOXC1}/\textit{FOXC2} upregulated the expression of the arterial endothelial markers \textit{Notch1}, \textit{Notch4}, \textit{Dll4}, \textit{Hey2}, and \textit{EphrinB2} through direct transcriptional activation.\textsuperscript{145} Interestingly, \textit{FOXC1}/\textit{FOXC2}-mediated promoter activation of \textit{Dll4} and \textit{Hey2} was augmented by VEGF treatment.\textsuperscript{146} Such augmentation was impaired by inhibiting phosphatidylinositol 3-kinase. These intriguing results suggest that the VEGF and NOTCH pathway promote arterial EC specification through FOX transcription factors. The expression of \textit{FOXC1} and \textit{FOXC2} is not activated by VEGF treatment alone,\textsuperscript{211,212} indicating a complex mechanism requiring further study. The reciprocal interaction between VEGF and NOTCH signaling has also been extensively studied during sprouting angiogenesis, where tip cells direct the growth of sprouts toward a gradient of tissue-derived VEGF. Although the review’s intent is not to cover tip cell function, information in this area can be found in other literature.\textsuperscript{213,214}

**Signaling via Bone Morphogenetic Proteins**

BMPs belong to transforming growth factor-β superfamily that regulates a multitude of biological processes, including embryonic vessel development.\textsuperscript{215,216} Approximately 20 mammalian BMPs have been identified and they function through serine/threonine kinase receptors composed of type I and type II forms.\textsuperscript{215} On binding to BMPs, the type II receptor, which is a constitutively active kinase, activates the type I receptor by phosphorylating-specific serine and threonine residues. The activated type I receptor, in turn, phosphorylates SMADs (SMAD1, 5, and 8) to transmit BMP signaling. Subsequently, the phosphorylated SMADs interact with SMAD4, a common SMAD, and translocate into the nucleus to induce the expression of genes, such as ID.\textsuperscript{114} Mouse gene knockout studies showed a key role of the BMP pathway in vascular development.\textsuperscript{216,217} Mice deficient in both \textit{Id1} and \textit{Id3}, the downstream BMP/SMAD targets, showed vascular defects in the developing brain (see also ID above).\textsuperscript{113} Although \textit{Bmp4}−/− deficient mice died without posterior mesodermal differentiation,\textsuperscript{218} germ line deletion of Alk-3, a type IA BMP receptor, failed to survive up to E9.5 stage with defects in mesoderm formation.\textsuperscript{219}

Park et al\textsuperscript{220} demonstrated that BMP4 via SMAD1/5 signaling induced the generation of FLK1+ cells from mouse ES cells in serum-free differentiation condition. Blockade of BMP4 by its antagonist, Noggin,\textsuperscript{221} reduced FLK1+ cell generation.\textsuperscript{220} GATA2 together with BMP4 was shown to also promote mouse ES cell differentiation to FLK1+ cells.\textsuperscript{105} BMP4-mediated FLK1+ cell development was mediated by the transcription factor, ER71/ETV2.\textsuperscript{55} The expression of ER71 was decreased by inhibiting BMP signaling.\textsuperscript{55} In addition, overexpression of ER71 rescued the impairment of FLK1+ cell generation by Noggin and DKK1 (WNT inhibitor).\textsuperscript{222}

**WNT Signaling**

WNT signaling is critical for embryogenesis and disease development.\textsuperscript{223,224} WNT proteins are ligands that bind their receptors to convey signals through 3 distinct signaling routes; the canonical WNT/β-catenin pathway; WNT/Ca\textsuperscript{2+} pathway; and planar cell polarity pathway.\textsuperscript{225–227} The canonical WNT/β-catenin pathway has been the best studied and has a crucial role in vascular development. In the off-state (ie, without engagement of WNT proteins and receptors), β-catenin, a downstream molecule of the pathway, is phosphorylated by GSK3β, and the marked β-catenin is ubiquitinated for degradation by proteasomes. Binding of WNT proteins

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to their receptor, Frizzled/Lrp, activates Dishevelled which inhibits GSK3β and thus releases β-catenin from the degradation pathway, allowing the translocation of β-catenin to the nucleus. Lengerke et al.228 showed that inhibition of WNT led to decreased generation of FLK1+ cells. Another report229 showed that the expression of genes associated with development of primitive streak, endoderm, and mesoderm was downregulated by inhibiting the WNT/β-catenin pathway during ES cell differentiation. Unexpectedly, however, the forced expression of the stabilized form of β-catenin failed to stimulate these marker genes. These studies suggest that the WNT/β-catenin signaling is required but it is not essential for formation of FLK1+ mesoderm. Thus, WNT/β-catenin signaling may have a supportive role in vascular development on BMP signaling. Together these studies point to a key role of WNT signaling in vascular EC specification through ID1, GATA2, and ER71/ETV2. How these factors interact with each other to activate Flk1 gene expression remains an open question.

Other studies in developing mouse embryos showed an important role of β-catenin, which can be stabilized downstream of WNT signaling, in vessel development. Inactivation of β-catenin in TIE2+ cells resulted in embryonic lethality with vascular remodeling defects and hemorrhages.230 The mutant embryos also displayed defects in endocardial cushion and cardiac valve formation.231 Further, the canonical WNTs and β-catenin were shown to be required for the development of central nervous system vessels.72,232 Embryos with sustained expression of β-catenin in TIE2+ cells lost their arteriovenous identity.233 In this study, β-catenin was shown to directly bind Dll4 promoter233 linking the Wnt and NOTCH signaling pathways. Together these findings suggest a role for WNT-β-catenin signaling in vessel development, although the role of this pathway in EC specification is less clear.

### EC Plasticity and Cell Reprogramming

It was generally accepted that terminally differentiated somatic cells are in the ground state where the fates of the cells remain unchanged throughout life. Epigenetic landscape model of Waddington234 has been supported this deterministic view. In this model, a hypothetical ball will roll down from the top of a hill and complete its journey at the lowest point; the pluripotent cell (analogous to Waddington’s ball) loses its potential, undergoes differentiation, and becomes a terminally differentiated somatic cell. However, emerging evidence has challenged this prevailing concept. Building on experiments by Gurdon235 showing that enucleated eggs receiving nuclei isolated from fully differentiated frog cells generated adult frogs, it is now clear that lineage-specific transcription factors can change identity of certain types of cells, including fully matured somatic cells. Weintraub et al.236 demonstrated that ectopically expressed MyoD, a transcription factor critical for muscle differentiation, converted fibroblasts and differentiated cell lines, including melanoma and neuroblastoma, into skeletal muscle cells. Overexpression of C/EBP (CCAAT/enhancer-binding protein) transcription factor led to cell fate change of B lymphocytes to macrophage-like cells.237 As shown by Yamanaka, the pluripotency transcription factors, OCT4 (octamer-binding transcription factor 4), NANOG, SOX2, KLF4, and MYC, are sufficient to generate ES-like cells (induced pluripotent cells) through a dedifferentiation mechanism (Figure 5A).238–241

With regard to ECs, recent studies have shown that ECs exhibit a certain degree of plasticity which is controlled by transcription factors. Overexpression of Proxl conferred the identity of lymphatic EC to blood vessel ECs.179,180 Lymphatic ECs deficient in Proxl lost their lymphatic identity and became blood vessel ECs.178 Similarly, specification of arterial and venous ECs is determined by a reciprocal function of NOTCH signaling and Coup-TfII as discussed above.184 These observations suggest that EC identity is interchangeable through transcription factor-mediated reprogramming.

Given such an important role of EC transcription factors in regulating cell fate, one can envision that appropriate combinations of endothelial transcription factors can directly reprogram terminally differentiated somatic cells into 3 different types of ECs. A study reported that a combination of ETS factors with concomitant repression of transforming growth factor-β signaling converted amniotic cells to ECs (Figure 5B).83 The key finding was that transient overexpression of ER71 converted the amniotic cells into immature ECs which subsequently became mature ECs with sustained expression of ERGI and FLI1. The authors chose midgestation c-KIT+ amniotic cells for reprogramming, but failed to reprogram postnatal fibroblasts with the same approach. Although the generated ECs showed vessel-forming capacity in vivo, it is not clear whether the amniotic cells were reprogrammed at the level of their epigenetic status in this study. Two groups have recently reported that somatic cells can be converted to cells having features of ECs. In one study, cells partially reprogrammed using the Yamanaka factors (OCT4, SOX2, KLF4, and c-MYC) were isolated (referred to as partially induced pluripotent stem) and thereafter further differentiated into ECs under defined culture conditions.242 Although partially induced pluripotent stem–derived ECs had potential for neovascularization in vivo, the identity of partially induced pluripotent stem and also partially induced pluripotent stem–derived ECs was not clear. In another study,243 human fibroblasts were induced to become CD34+ angioblast-like cells in 2 successive phases; plastic induction phase driven by ectopic expression of Yamanka factors, followed by mesodermal induction phase driven by a chemically defined medium. The CD34+ cells were able to differentiate into ECs and smooth muscle cells; however, it was not demonstrated whether ECs could be generated concomitantly with hematopoietic cells as would be expected of CD34+ cells. These studies open a new possibility of generating ECs from terminally differentiated somatic cells with certain codes of endothelial/pluripotent stem transcription factors. Considering specific functions of each endothelial transcription factors described above, it is reasonable to speculate that immature ECs derived from somatic cells could be specified into arterial, venous, or lymphatic ECs in combination of HEY1/2, Coup-TfII, and PROX1 as suggested in Figure 5B. If so, it remains to be seen whether these cells will have the ability to regenerate different portions of the vasculature.
Concluding Remarks

Cardiovascular diseases, such as high blood pressure, diabetes mellitus, coronary heart disease, and vascular diseases associated with acute and chronic inflammation, are the leading causes of morbidity and mortality in the United States and other advanced countries. Approximately 37% of the population experiences cardiovascular diseases in the United States alone. Development of new cell therapeutics based on detailed understanding of generation of ECs holds great promise. However, more knowledge and deeper understanding of EC and vascular development is urgently needed. Questions, such as how the vascular system comprising ECs develops prenatally and how it can be repaired by activating specific signaling pathways, remain to be fully addressed. In addition, there is the fundamental unexplored area of the importance of hemodynamic forces in mediating EC gene expression and functionality in arteriovenous EC specification during embryonic vessel development. Although hemodynamic forces play a key role in development of vascular diseases, such as atherosclerosis, their involvement in vascular development is unexplored territory. In this regard, an interesting study (which presages future work that is needed in this field) reported that both Gja4 and calcineurin/NFAT played a critical role in the formation of lymphatic valve. In the lymphatic valve formation, flow-dependent expression of PROX1 and FOXC2 induced expression of Gja4 and activation of calcineurin/NFAT signaling.

In the future, defining the mechanisms regulating vascular development would be the fundamental for treating diseases related to aberrant vessel growth and dysfunction of ECs. This knowledge would be a new research platform for generating functional and specific types of ECs and organ-specific ECs through directed differentiation from pluripotent stem cells and directed conversion of patient-specific somatic cells, including blood cells. This is the hope for the future but as discussed in this review some important foundations have been already laid.

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Disclosures

None.

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