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 etiology 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 towards distinct endothelial cell lineages and its promise in regenerative vascular medicine.
A. Introduction

The vasculature consisting of arterial, venous and lymphatic vessels forms through two 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 from pre-existing vessels.\(^1\)\(^-\)\(^3\) While 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 (EPCs).\(^4\)

Although there is no established distinction between angioblasts and EPCs based on specific markers, we will use the term angioblast to represent the precursor endothelial cell responsible for vasculogenesis in the developing embryo, whereas EPC denotes the progenitor cell that differentiates to endothelial cells 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 “EPCs”. 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 extra-embryonic yolk sac blood island (Figure 1).\(^6\)\(^-\)\(^8\) Blood vessels in the blood island are lined by endothelial cells 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, crucial cells which establish the vasculature of intra-embryonic regions including the dorsal aorta and vitelline vessels, and primary plexuses of lungs, spleen, and heart. The more complex phase of formation of the embryonic vascular networks occurs by angiogenesis during which newly formed vessels are stabilized through interactions of endothelial cells with each other via endothelial junction proteins and with recruited mural cells, the pericytes, and an ordered extracellular matrix.

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 extra-cellular matrix characteristics of the vessel wall. While 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. Lymphatic endothelial cells are generated from a sub-population of cardinal vein endothelial cells as well as the intersomitic vessels, and they migrate dorso-laterally 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).

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 endothelial cells and for vascular development. Abbreviations are listed in Table 1.

B. 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 endothelial cells develop from a common progenitor cell, termed the hemangioblast.\textsuperscript{6-8} However, the hemangioblast as a cell remains poorly defined and elusive. During \textit{Drosophila} embryogenesis, the lymph gland, the major site for hematopoiesis, develops in close proximity of the aorta.\textsuperscript{13} Analysis of expression markers and lineage tracing studies using Flp - FRT (flippase - flippase recognition target) recombination indicated that the cardioblast, a type of vascular progenitor cell, and lymph gland (comprising hematopoietic cells) developed from a common progenitor population in the cardiogenic mesoderm.\textsuperscript{14} Studies in zebrafish described a \textit{cloche} (clo) mutant displaying defects in both hematopoietic and endothelial differentiation.\textsuperscript{15-17} \textit{clo} was shown to act upstream of the hematopoietic and endothelial genes \textit{flk1} (\textit{vascular endothelial growth factor receptor 2}), \textit{scl} (\textit{stem cell leukemia}), and \textit{etsrp} (\textit{ets related protein}). Another report using zebrafish showed that \textit{lysocardiolipin acyltransferase} (\textit{lycat}) was critical for both hematopoietic and endothelial cell development.\textsuperscript{18} Fate mapping in zebrafish showed the existence of bipotential cells (hemangioblast-like cells) in embryos capable of generating both hematopoietic and endothelial cells, but not other cell types.\textsuperscript{19}

Initial evidence of the existence of hemangioblasts in mammalian cells comes from studies using the \textit{in vitro} embryonic stem (ES) cell differentiation system.\textsuperscript{20-22} Vascular endothelial growth factor (VEGF) stimulation of ES cells induced the formation of a transient cell population (termed BL-CFCs, blast colony-forming cells) expressing hematopoietic and endothelial lineage markers. These cells upon re-plating differentiated clonally into distinct hematopoietic and endothelial cell populations. Studies in mice showed that Brachyury/GFP\textsuperscript{+}FLK1\textsuperscript{+} cells appearing at E7.5 embryos formed blast colonies similar to the ES cell-derived blast colonies and gave rise to both hematopoietic and endothelial cells.\textsuperscript{23}

While the above studies suggested that endothelial cells share their early developmental program with hematopoietic cells, others have challenged this fundamental concept. In
zebrafish, lineage tracing experiments showed that only a certain fraction of endothelial cells originated from hemangioblasts\textsuperscript{19} raising the possibility of another population giving rise to endothelial cells. Studies have also reported a differential time course of emergence of endothelial and hematopoietic cells in the developing mouse embryo.\textsuperscript{24} Angioblasts were found as early as E5.5 whereas hematopoietic progenitors were observed at E6.5,\textsuperscript{24} suggesting non-parallel events. While these studies showed that generation of hematopoietic and endothelial cells can follow a different time course, they do not rule out the presence of a common precursor cell. A plausible explanation for these findings is that generation of hematopoietic and endothelial cells is a stochastic process, thus, while 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 endothelial cell population capable of generating hematopoietic cells, known as the hemogenic endothelium.\textsuperscript{25,26} Zovein et al\textsuperscript{25} showed by lineage tracing endothelial cells with VE-cadherin CreERT (Cre recombinase - estrogen receptor T2; tamoxifen inducible Cre) and RosaR26LacZ reporter that VE-cadherin lineage cells located within the aorta-gonad-mesonephros (AGM) generated hematopoietic stem cells. In subsequent studies, the genesis of hematopoietic stem cells from endothelial cells within the AGM was seen in dramatic images of these cells being “shed” from the endothelium of mice and zebrafish.\textsuperscript{27-29} Additionally, it was shown that ES cell-derived BL-CFCs generated hematopoietic cells through a presumptive hemogenic endothelium defined by TIE2\textsuperscript{hi}c-KIT\textsuperscript{+}CD41\textsuperscript{−} cells.\textsuperscript{30}

**First emerging FLK1/VEGFR2\textsuperscript{+} cells are multipotent progenitors of the cardiovascular system**

Despite the incompletely controversy surrounding the hemangioblast origin of endothelial cells and blood cells, it is clear that VEGF-FLK1 signaling is essential for endothelial cell generation and vessel development.\textsuperscript{31,32} We will discuss below the developmental kinetics
of endothelial cell lineage with a focus on the key role of FLK1+ cells in giving rise to endothelial cells.

Endothelial cells 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 endothelial cells of the yolk sac and embryonic vasculature including the endocardial tube. Deficiency of Flk1 induced embryonic lethality due to 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 endothelial cell lineages.

Cell lineage tracing experiments have provided further insights into blood and endothelial cell potential of FLK1+ cells. FLK1+ cells present in differentiating ES cells were fractionated by the expression of SCL (stem cell leukemia), a basic helix-loop-helix 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 co-receptor 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 expression without affecting cell viability.\textsuperscript{22} 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.\textsuperscript{22} Interestingly, endothelial cells could also develop independently of SCL expression in that both FLK1+hCD4+ and FLK1+hCD4- cells generated endothelial cells on further culture.\textsuperscript{22} These findings suggest that angioblasts and hemangioblasts represent two distinct origins of endothelial cells. The first arising FLK1+SCL+ cells represented the \textit{in vitro} equivalent of hemangioblasts, and that later endothelial cells can also develop independently of hematopoiesis directly through angioblasts.

Other studies utilizing the genetic cell tracing method have pointed to a broader potential of FLK1+ cells as the FLK1+ cells obtained from differentiating ES cells were also shown to generate smooth muscle actin (SMA) cells.\textsuperscript{42,43} In addition, FLK1+ cells marked by Flk1Cre;Rosa26 reporter (R26R) were detected in skeletal muscles and cardiomyocyte of E10.5 embryos,\textsuperscript{44} besides the expected endothelial and blood cells.\textsuperscript{44} Another study\textsuperscript{45} showed the presence of FLK1+/LacZ+ cells in cardiac and skeletal muscles; however, upon re-analysis 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 to a previous study with FLK1+/LacZ-neo-in mice.\textsuperscript{31} Thus, the FLK1+ mesoderm likely represents a multipotent progenitor cell population in addition to blood and endothelial cell progenitors of the cardiovascular system.

C. Transcriptional regulation of vessel development

The details of transcriptional control of vessel development are summarized in Figure 3 as well as Table 2 and discussed under the headings below that describe the function of individual transcription factors.

\textit{ER71 (ETS-related 71) / ETV2}

ETS (E-twenty-six specific) transcription factors are highly homologous in a short stretch of 85 amino acids (ETS DNA binding domain) located at the carboxyl terminus.\textsuperscript{46-48} A winged
helix-turn-helix motif formed by the ETS domain binds the consensus sequence (5’-GGAA/T-3’) to regulate expression of target genes.\textsuperscript{47} 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 (PNT) domain that mediates protein-protein interactions and oligomerization.\textsuperscript{46,49} Up to now 27 ETS factors have been reported in humans and mice.\textsuperscript{48} The presence of ETS binding consensus sequence is seen in more than 200 genes involved in tumorigenesis, apoptosis, angiogenesis, and hematopoiesis,\textsuperscript{50} 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 (also known as ETV2).\textsuperscript{51} ER71 is a 38kDa protein comprising a N-terminus transactivation domain and C-terminus ETS DNA binding domain (Figure 4A).\textsuperscript{52,53} ER71 was initially thought to be testis-specific,\textsuperscript{53,54} however, more thorough analysis showed it to be present in the posterior mesoderm, extra-embryonic mesoderm, lateral plate mesoderm, and cardiac crescent in embryos between E7.0-E7.75.\textsuperscript{55-58} Most ER71\textsuperscript{+} cells were also positive for FLK1\textsuperscript{57} 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, branchial arch, intersomitic vessels as well as endocardium, followed by a sharp decrease beyond E11.5.\textsuperscript{55-58} This “on-off” expression pattern was also conserved in differentiating ES cells \textit{in vitro}.\textsuperscript{55,57} 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\textsuperscript{+} cells.\textsuperscript{55,57}

With regard to \textit{Er71} expression relative to \textit{Flk1}, a study showed an unusual result that FLK1\textsuperscript{+} cells appeared prior to ER71\textsuperscript{+} cells.\textsuperscript{57} This observation cannot be reconciled with what appears to be an important function in ER71 in giving rise FLK1\textsuperscript{+} cells. The discrepancy might be explained by different culture conditions [i.e., embryoid body (EB) formation under serum vs.
ES differentiation on OP9 under cytokines] and the genetic manipulations carried out (wild type vs. Er71-VENUS). In addition, another general concern is that both EB formation and OP9 co-culture 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 three embryonic germ layer lineage cells. In this protocol, EB thus mimics a three-dimensional structure of an embryo that could be critical for cell-cell interactions needed for embryogenesis. In contrast, the ES cells differentiating in a feeder cell line OP9 do not form EBs. In this co-culture system, one can efficiently evaluate the effects of added cytokines or growth factors, compared to 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 FoxC2, which induced the expression of key endothelial genes that are critical for endothelial and hematopoietic lineages, Flk1, VEcadherin, Tie2, Scl, Notch4, and Nfatc1. 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. 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*. 17,65,66 *etsrp* also rescued vascular defects seen in the *clo* mutant described above. 17 The *Xenopus* ortholog *er71* similarly exhibited a conserved function to the mouse and zebrafish homologs. 67

While the above studies describe the important role of ER71 in differentiation of endothelial cells and vessel development, the upstream mechanisms regulating ER71 expression itself are less well understood. Using mouse ES cell differentiation system, Yamamizu et al 68 reported that protein kinase A (PKA) signaling activated the expression of *Er71* through the cAMP response element binding protein (CREB) (Figure 4B). In another study, 56 *Er71* was found as one of the significantly downregulated transcripts in the cardiac progenitors deficient in *Nkx2-5*, a homeobox transcription factor required for heart morphogenesis including endocardial cushion formation. 69-71 *Nkx2-5* was expressed in both myocardium and endocardium, 69-71 but *Er71* message was only detected in the endothelium/endocardium in E8.5 hearts, 56 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). 56 In zebrafish, *foxc1a/b* also bound the promoter to induce expression of *etsrp* (Figure 4B), 72 indicating that ER71 functions requires entrainment of multiple transcription factors for its full effect.

*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 vs. 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} Since ER71 is an important cell fate transcriptional determinant of the FLK1\textsuperscript{+} 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\textsuperscript{+} cells.\textsuperscript{75} Interestingly, most of the FLK1\textsuperscript{+} cells derived from Er71\textsuperscript{-/-} ES cells also expressed PDGFR\textgreek{a} and differentiated into cardiac lineages.\textsuperscript{57,75} Also overexpression of ER71 in wild type ES cells significantly enhanced the generation of FLK1\textsuperscript{+}PDGFR\textgreek{a} cells over that seen in FLK1\textsuperscript{+}PDGFR\textgreek{a}\textsuperscript{-} cells,\textsuperscript{75} suggesting a determinant role of ER71 for FLK1\textsuperscript{+} mesoderm specification towards endothelial and blood lineages. In addition, morpholino knockdown of etsrp in zebrafish expanded the myocardium with reduced generation of vascular endothelial cells.\textsuperscript{64} etsrp-GFP\textsuperscript{+} cells in the absence of etsrp also expressed hand2,\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\textsuperscript{+} cells specifically towards the endothelial lineage.

The WNT signaling pathway might play an important role in inducing the ability of ER71 to differentiate FLK1\textsuperscript{+} cells towards endothelial cells.\textsuperscript{75} Overexpression of Er71 impaired cardiac mesoderm formation (as evident by decreased generation of FLK1\textsuperscript{+}PDGFR\textgreek{a}\textsuperscript{-} cells) and repressed expression of the WNT-\textbeta-\catenin downstream genes, but this was rescued by WNT agonists. Inversely, deficiency in Er71 exhibited enhanced WNT-\textbeta-\catenin activity.\textsuperscript{75} Elevated level of hemangiogenic mesoderm formation upon Er71 overexpression was rescued by overexpressing \textbeta-\catenin. Although these findings suggest a critical role of \textbeta-\catenin in ER71-mediated FLK1\textsuperscript{+} mesoderm specification towards endothelial and hematopoietic cells, gene knockout studies in mice have not been consistent.\textsuperscript{78} Inactivation of \textbeta-\catenin in FLK1\textsuperscript{+} cells (i.e., Flk1Cre;floxed \textbeta-\catenin mice) induced defective angiogenesis in the central nervous system with phenotypically normal development of the cardiac and hemo-vascular 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 due to the definition of “cardiac lineage”. Endothelial lineages including the endocardium, cardiac lineages (i.e. myocardium/cardiomyocyte) and hematopoietic cells originate from FLK1\textsuperscript{+} cells.\textsuperscript{44,45,79,80} Thus, it is plausible that ER71 can specify FLK1\textsuperscript{+} cells capable of generating hematopoietic cells and endothelium/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 endothelial cells,\textsuperscript{55-58,81} 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\textsuperscript{+} 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 pro-angiogenic genes including Flk1 with reduced expression of endothelial cell 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 endothelial cells,\textsuperscript{83} consistent with the fundamental role of the transient and early-onset of expression of ER71 in mediating endothelial cell specification in the developing embryo. In the same context, the time-dependent downregulation of ER71 expression may be needed to ensure endothelial cell maturation and formation of inter-endothelial junction integrity required for functionally normal vessels.
**SCL/TAL1 (stem cell leukemia/T-cell acute lymphoblastic leukemia)**

SCL/TAL transcription factor belongs to the basic helix loop helix (bHLH) transcription factor family.\(^4^,8^,5^,8^,5\) SCL expression is seen in yolk sac blood progenitors and endothelial cells.\(^8^,^6^,^8^,^8\) Ectopic expression of *scl* in zebrafish induced both hematopoietic and endothelial genes.\(^1^,^6^,8^,^9\) Defects in blood and vessel formation in both *clo* and *etsrp* knock-down zebrafish were partially rescued by overexpressing *scl*.\(^1^,^6^,8^,^9,^1^0\) Although *Scl*\(^-/-\) mice embryos died due to complete lack of hematopoiesis,\(^8^,^6^,^8^,^8^,^8^,^8^,^8^,^8^ chimeric analysis of wild type and *Scl*\(^-/-\) ES cells showed that SCL was also required for vascular remodeling but apparently not for vasculogenesis.\(^9^,^1^,^1^1\) This was evident by the finding that the mutants with high degree of chimerism exhibited disorganized yolk sac vessels lacking vitelline vessels.\(^9^,^1^,^1^1\) Further, *Scl*\(^-/-\) ES cells failed to contribute to the formation of large vessels.\(^9^,^1^,^1^1\)

SCL has been postulated to be critical for the specification of the hemangioblast rather than angioblasts.\(^2^,^1^,^2^,^2^,^8^,^9^,^2^,^2^) Hemangioblast activity was enriched in FLK1\(^+\)SCL\(^+\) cells, whereas ES cells deficient in *Scl* failed to generate blast colonies.\(^2^,^1^,^2^,^2^,^9^,^2^) Ectopic expression of *Scl* in the locus of Flk1 increased blast colony formation compared to Flk1\(^+/+\) cells.\(^4^,^3^) Thus, SCL appears to be dispensable for vasculogenesis but it is required for hemangioblast/vascular remodeling in the developing embryo.

Although SCL acted downstream of FLK1,\(^2^,^2^,^4^,^3^) the presence of a putative SCL binding site in the Flk1 promoter\(^9^,^3^) 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.\(^9^,^4^) 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*.\(^1^,^7^,^5^,^5^) Genome wide sequence analysis showed that *Scl* contains the binding site for ETS.\(^6^,^3^) Luciferase and ChIP experiments positioned *Scl*
downstream of ER71. The mechanism of ER71 regulation of SCL and how it regulates endothelial cell and vessel development remains unexplored, and it is potentially a fruitful area since it will help to connect the function of these two key transcription factors.

**GATA2 (GATA binding protein 2)**

The GATA 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 six GATA members, initial analysis has described GATA1, GATA2 and GATA3 as the “hematopoietic GATAs” due to their preferential expression in hematopoietic cells. \(^{97}\) Gata1\(^{-/-}\) mouse embryos displayed defects in erythropoiesis. \(^{98,99}\) Mouse embryos deficient in Gata2 died by E11.5 and exhibited anemia. \(^{100}\) Gata2\(^{-/-}\) ES cells and yolk sac cells generated significantly reduced number of multipotent progenitors. \(^{100,101}\) However, evidence of endothelial expression of GATA2 in developing embryos \(^{102-104}\) suggests that GATA2 might also play an important role in vessel development. Indeed, Lugus et al.\(^{105}\) reported that expression of Gata2 was enriched in BL-CFCs and that overexpression of Gata2 in ES cells enhanced the generation of FLK1\(^{+}\) cells as well as endothelial cells *in vitro*. The findings that several key endothelial genes contain GATA binding sites \(^{106-108}\) further suggest that GATA2 regulates vessel development through transcriptional activation of endothelial genes including Flk1 andVEcadherin. In this regard, a recent report showed that GATA2 regulates endothelial specific gene expression and thus endothelial specificity through epigenetic modification. \(^{109}\) In this study, the authors found that ENDOMUCIN, an endothelial specific gene, contained preferential GATA2 binding sites in transcription start site (TSS) as well as -139 kb region of human dermal microvascular endothelial cells (HMVECs). An epigenetic experimental approach also demonstrated that both regions are in active chromatin state in HMVECs. In contrast, the erythroid cells, K562, which also highly express GATA2, contained preferential GATA2 binding sites in SCL/TAL1. \(^{109}\) ENDOMUCIN TSS and -139 kb region in K562 showed enrichment of H3K9me3. \(^{109}\) Interestingly, knockdown of GATA2 in HMVECs significantly reduced the expression of endothelial genes...
including *ENDOMUCIN* and *KDR/VEGFR2*, but also increased expression of non-endothelial cell 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 endothelial cell fate. The mechanisms of GATA2-mediated endothelial cell 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-ERT under Gata2 vascular endothelial (VE) enhancer, and found that the CKO 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. Since mice with germ line knockout and conditional deletion of Gata2 develop normal vascular structure during the early embryo stage (E10-E11), it appears that GATA2 plays an important role in lymphangiogenesis, but it is not required for endothelial cell 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 (prior to 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 endothelial cells suggests such a redundancy concept if GATA3 is upregulated following deletion of GATA2.

**ID (Inhibitor of Differentiation and DNA binding)**

ID proteins have been implicated in embryonic and postnatal angiogenesis. IDs, consisting of 4 members (ID1, ID2, ID3, ID4), belong to the bHLH transcription factors, but lack DNA binding domains; thus, they function by “sequestering” transcription factors that target genes. Expression of *ld1* and *ld3* was detected in embryonic vessels and also mice deficient in *ld1* and *ld3* showed abnormal vascular development. In cultured endothelial cells,
overexpression of \(ld1\) enhanced endothelial cell proliferation and migration, whereas \(ld1\) knockdown inhibited these responses,\(^{114,115}\) suggesting a pro-angiogenic role of ID1. IDs together with SCL de-repressed \(Flk1\) promoter activity by interacting with E2-2, a basic helix-loop-helix transcription factor, functioning as a negative regulator of \(Flk1\) expression.\(^{94,116}\) In addition, studies showed that ID1 and ID3 are direct downstream targets of the BMP-SMAD pathway\(^{114,117}\) suggesting that BMP signaling may be important in activating IDs and hence in generating FLK1\(^+\) endothelial cells.

### Role of Other ETS Transcription Factors

The ETS transcription factors display a broad range of expression patterns and activities in developing mouse embryos as well as in adults. However, some of these factors show a preferential expression in endothelial cells, and it is important to consider their role in generation of endothelial cells 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 endothelial cells,\(^{121}\) another ETS transcription factor \(Etv6\) 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 endothelial cells 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 \textit{in utero} and showed vessel branching defects,\(^{126}\) indicating the redundant role of ETS1/2 in embryonic vessel formation. In addition, a recent study suggests a novel function of ETS1/2 in lymphatic vessel formation (see 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 endothelial cell development *scl, lmo2* and *flk1*. 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*. Similarly, *Fli1*−/− mouse embryos did not display severe vascular defects; *Fli1*−/− embryos died around E11.5 with extensive hemorrhaging, indicating its dispensable role in endothelial cell specification unlike the findings in *Xenopus* and *clo* mutant. 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>Mld2/Mld2</sup> where serine at residue 329 on ERG1 is substituted with proline, resulting in non-functional ERG due to missense mutation, died due to hematopoietic defects and exhibited dilated vessels in the brain. In a subsequent study, it was demonstrated that ERG1 directly induced the expression of *CDH5* (*vascular endothelial cadherin; cadherin 5, type 2*) in HUVECs, suggesting that ERG1 regulates endothelial cell-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 the DNA-binding domain, which recognizes the consensus sequence 5′-TTGTTTAC-3′. Among the forkhead transcription factors, FOXO and FOXC are implicated in vascular development and endothelial cell generation. There are four members of FOXO family, FOXO; FOXO1/FKHR, FOXO3/FKHRL1, AFX/FOXO4 and FOXO6. FOXOs functioning mainly through PTEN (phosphatase and tensin homologue deleted on chromosome 10) are involved in cell survival/apoptosis, cell cycle, DNA repair and reactive oxygen species regulation. In mouse embryos, *FoxO1* is highly expressed in the
developing vessels and FoxO1<sup>−/−</sup> mice embryos failed to survive beyond E10.5 because of defective vascular development in the yolk sac and embryo.<sup>135,136</sup> A detailed gene expression analysis uncovered decreased expression of Gja4 and Gja5, both of which are components of gap junction.<sup>135</sup> As gap junction genes are preferentially expressed in developing embryos,<sup>137,138</sup> 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.<sup>136</sup> However, overexpression of FoxO1 and FoxO3 inhibited endothelial tube formation and migration partly through direct inhibition of eNOS,<sup>139</sup> believed to be a proangiogenic factor.<sup>140</sup> Overexpression of FOXO3 induced angiogenesis as evidenced by enhanced recovery of blood flow from ischemic injury with a concomitant increase in capillarity.<sup>139</sup> Conditional deletion of FoxO1, FoxO3 and FoxO4 using Mx1Cre mice led to the development of hemangiomas,<sup>141</sup> indicating suppressive function of FOXOs in endothelial cell proliferation.

FOXc1 and FOXc2, members of the C subgroup of forkhead transcription factor family, have also been found to regulate arterio-venous specification and lymphatic vessel differentiation.<sup>133</sup> Promoters of multiple endothelial genes Flk1, VEcadherin, Pecam1, Tie2 and Scl, contain evolutionarily conserved FOX:ETS binding motifs.<sup>63</sup> 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/fox1b in zebrafish can also function as an upstream regulator of etsrp,<sup>72</sup> indicating a critical role of cooperation of FOXC members with ER71 in mediating endothelial cell development. FoxC1 deficient mice died pre- and post-natally with multiple vascular defects including coarctation of the aortic arch.<sup>142</sup> FoxC2 deficient mice exhibited similar defects but with abnormal development of lymphatic vessels manifested by lack of valves and increased vascular smooth muscle cells.<sup>143</sup> Studies on double FoxC1/FoxC2 deleted mice demonstrated a role of FOXC in determining artery and venous endothelial cell fate as demonstrated by the presence of arterio-
venous malformations in the mutant embryos and failure to express the arterial markers Dll4 and Notch with unaltered expression of the venous markers, Coup-TFII and EphB4.\textsuperscript{144,145} FOXC1 and FOXC2 may function by activating the Dll4 promoter through a direct binding on the FOX binding sites of this promoter.\textsuperscript{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 endothelial cell specification.\textsuperscript{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.\textsuperscript{143} FOXC2 was shown to bind and activate the Nfatc1 (nuclear factor of activated T cells, cytoplasmic 1) promoter.\textsuperscript{147} Cyclosporine A-mediated inactivation of NFAT signaling mimicked the lymphatic phenotypes seen in Fox2\textsuperscript{−/−} mouse embryos.\textsuperscript{147} Taken together with the finding that inactivating mutations in FOXC2 are responsible for lymphedema-distichiasis in human,\textsuperscript{148} it appears that FOXC2 is a versatile transcription factor that has a role in FLK1\textsuperscript{+} 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 endothelial cell development. FoxF1 is expressed in the posterior primitive streak, allantois, amnion and yolk sac vasculature.\textsuperscript{149,150} Germ line deletion of FoxF1 led to avascular yolk sacs and allantois.\textsuperscript{150} FOXF1 may act downstream of hedgehog signaling to induce BMP expression which is an important inducer for vasculogenesis.\textsuperscript{151} FOXF1 was also shown to function as an upstream activator of Flk1 gene expression.\textsuperscript{152} A potential binding site for FOXF1 was identified in the specific locus (Distal-Multipotent-Mesodermal-Enhancer, DMME) of the Flk1 gene.\textsuperscript{152} In the zebrafish, foxh1 bound to the flk1 promoter and repressed gene expression and induced defective vessel formation.\textsuperscript{153} In contrast, FOXM1 is dispensable for vessel development in developing embryos since mice lacking FoxM1 in endothelial cells
(Tie2Cre;floxed FoxM1) were born alive.\textsuperscript{154,155} However FOXM1 was demonstrated to have an entirely novel function in mediating EC proliferation and vascular repair.\textsuperscript{154,155} In response to vascular injury elicited by lipopolysaccharide (LPS), vessels of Tie2Cre;floxed FoxM1 mice exhibited severely 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.\textsuperscript{154,155} Thus, FOXM1 functioned by transcriptionally activating the expression of β-catenin through FOXM1 binding sites on the promoter.\textsuperscript{155}

**HEY1/HEY2 (Hairy/enhancer-of-split related with YRPW motif protein1/2)**

The NOTCH pathway controls endothelial cell specification (arterial vs. venous endothelial cells) and plays an important role in endothelial cell sprouting.\textsuperscript{156,157} HEY1 and HEY2, members of hairy and enhancer of split-related family of bHLH transcription factors, are direct transcriptional targets of the NOTCH pathway.\textsuperscript{158} Hey1\textsuperscript{−/−} and Hey2\textsuperscript{−/−} mouse embryos developed defective vessels and died at E9.5.\textsuperscript{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.\textsuperscript{161} These studies collectively suggest the potentially important role of HEY1/HEY2 in arterio-venous 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,\textsuperscript{162} also induced defective arterial vessel formation. These studies showed that the NOTCH pathway in endothelial cells controls the development of arteries through RBPJ-HEY1/2 signaling.
SOX7, SOX17 and SOX18, members of the SOX (Sry-related HMG box) transcription factor family

The SOX (SRY-like HMG-box) transcription factor family shares a conserved DNA binding domain, known as HMG (high mobility group), that recognizes the consensus sequence 5’-(A/T)(A/T)CCA(A/T)G-3’ present in various genes. 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 endothelial cells of the yolk sac vasculature and the dorsal aorta, and Sox17 was expressed in endothelial cells of the dorsal aorta at E9.5. Sox18 was also evident in endothelial cells of the developing lymphatic vessels.

Linkage analysis combined with DNA sequencing showed that SOX18 was responsible for the phenotypic defects observed in four spontaneous mouse mutants, Ragged (Ra), Ragged-Opposum (RaOp), Ragged-Jackson, and Ragged-like, all of which carried a mutation in Sox18 gene, making a dominant negative form of SOX18. RaOp homozygous mice initially developed a normal vasculature but died at E14.5 with defective lymphatic vessel development accompanied by hemorrhage and edema. Also Sox18−/− mouse embryos in C57BL/6 background phenocopied the defects seen in RaOp homozygous mice; the mutant embryos showed lethality at E14.5 and developed no PROX1 (Prospero homeobox transcription factor 1) lymphatic endothelial cells, indicating a critical role of Sox18/RaOp in lymphatic endothelial cell specification. SOX18 binding to sites present in Prox1 promoter may be responsible for Prox1 expression. Overexpression of Sox18 in differentiating ES cells induced expression of genes critical for lymphatic endothelial cells Prox1 and Podoplan (Figure 4D), but Sox18/RaOp suppressed these genes. Deficiency of Sox18 in 129-CD1 mixed background, however, did not show these defects, and the mice were born alive and apparently normal. This discrepancy in these mixed background mice may due to the compensatory expression of SOX7 and SOX17 in lymphatic endothelial cells since these genetic changes did not occur in
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 endothelial cells. Zebrafish injected with double morphants of sox7 and sox18 showed augmented expression of the venous markers dab2 and flt4 in arteries coupled with decreased expression of the arterial markers notch3, ephrinB2 and dll4. In a similar study, double knockdown of sox7 and sox18 in zebrafish resulted in ectopic expression of flt4 with concomitant decrease of ephrinB2 in the dorsal aorta. When both Sox17 and Sox18 were deleted in mixed background mice, the mutants displayed defects in anterior dorsal aorta and endocardial tube development. In contrast, Matsui et al. reported that mixed background Sox17<sup>-/-</sup>;Sox18<sup>-/-</sup> mice were perinatally lethal and developed abnormal vascular structures. Endothelial cells of Sox17<sup>-/-</sup>;Sox18<sup>-/-</sup> mice also showed significantly impaired tube forming ability in vitro. Taken together, these studies suggest that the SOX F group transcription factors function as an important regulator for endothelial cell specification; arterio-venous specification, and venous-lymphatic specification.

**PROX1 (Prospero homeobox transcription factor 1)**

Prox1 expression at E9.5 was detected in a subpopulation of cells dorsolateral to the cardinal vein. 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. Prox1 continued to be expressed in the adult lymphatic endothelial cells. A number of studies have established a critical role of PROX1 as a supreme regulator of lymphatic endothelial cell specification and maintenance. Prox1<sup>-/-</sup> mice died shortly after birth with defects of enteric lymphatic vessels. Prox1<sup>LacZ/LacZ</sup> mouse embryos examined at E14.5 showed no sign of lymphatic vessel formation with normal development of blood vessels. In these mice, Prox1<sup>LacZ/LacZ</sup> 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.\textsuperscript{177} These results together show the requisite role of PROX1 activity in mediating lymphatic endothelial cell specification but they also show that PROX1 is dispensable for lymphatic endothelial cell budding from the cardinal vein.

Conditional deletion of Prox1 in embryos as well as adult led to reprogramming of lymphatic endothelial cells into blood endothelial cells.\textsuperscript{178} Prox1 knockdown in adult lymphatic endothelial cells showed the similar results seen in in vivo experiments.\textsuperscript{178} Overexpression of Prox1 in blood endothelial cells induced the expression of lymphatic makers concomitant with decreased expression of vascular endothelial genes.\textsuperscript{179,180} In addition, it has been reported that PROX1 can bind with ETS1/2 and that its interaction in a synergistic manner promote the expression of VEGFR3.\textsuperscript{127} These findings together show the essential role of PROX1 as a cell fate determinant of lymphatic endothelial cells (Figure 4D).

A recent study on revisiting the issue of lymph-angiogenic function of PROX1 identified another source and mechanism of lymphatic endothelial progenitors.\textsuperscript{12} Using serial thick vibratome sections, Yang et al\textsuperscript{12} found at E10.5 distinct PROX1\textsuperscript{+} cell populations in the intersomitic vessels (ISV) as well as the cardinal vein. These PROX1\textsuperscript{+} cells at both sites acquired expression of PODOPLANIN, which was identified as another accessory protein involved in the specification of lymphatic endothelial cells.\textsuperscript{181}

\textbf{COUPTFI1 (Chicken ovalbumin upstream transcription factor II)}

COUPTFI1, a member of the NR (nuclear receptor) 2F subfamily, is a nuclear orphan receptor that functions in a wide range of biological processes including angiogenesis and neural development.\textsuperscript{182,183} Expression of Coup-TFI1 starting from E8.5 is limited to endothelial cells of veins and lymphatics, not the endothelium of arteries.\textsuperscript{182,184} Targeted deletion of Coup-TFI1 caused abnormal development of the heart and impaired angiogenesis with missing or collapsed cardinal veins, leading to embryonic lethality.\textsuperscript{182} Inactivation of Coup-TFI1 in
endothelial cells of *Tie2Cre* mice resulted in death of mutant embryos and loss venous identity as evident by expression of arterial markers *Jag1, Notch1, ephrinB2* and *Np1* in the veins.\(^\text{184}\) 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.\(^\text{185,186}\) 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 endothelial cells partially rescued the loss of venous identity seen with endothelial deletion of COUP-TFII.\(^\text{187}\)

Despite the strong evidence of the critical role of COUP-TFII in venous endothelial cell 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 gene1) in veins.\(^\text{188}\) BRG1 is a chromatin-remodeling enzyme and a component of SWITCH/sucrose non-fermentable (SWI/SNF)-like complex.\(^\text{189}\) Deletion of *Brg1* in TIE2\(^+\) cells led to embryonic lethality and defective primitive erythropoiesis and yolk sac vessel formation.\(^\text{190}\) Venous endothelial cells deficient in *Brg1* showed decreased level of *Coup-TFII* expression with aberrantly elevated expression of arterial markers *ephrinB2, Nrp1,* and *Dll4.*\(^\text{188}\) 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 *Coup-TFII* expression through chromosome remodeling and thus induce venous endothelial cell specification.

COUP-TFII is also an important regulator of lymphatic endothelial cell identity. Co-expression of COUP-TFII and PROX1 and their interaction is involved in mediating lymphatic endothelial cell specification throughout embryogenesis and in postnatal life.\(^\text{191,192}\) The interaction of COUP-TFII and PROX1 regulated the expression of lymphatic markers *Fgfr3* and *Vegfr3.*\(^\text{191,192}\) 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.

D. 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 endothelial cell function including survival, proliferation and vessel permeability.\(^\text{193,194}\) Upon binding of VEGF, its receptor, FLK1/KDR, transmit signals through a number of downstream molecules MAPK-ERK, p38-MAPK, phospholipase C, and phosphatidylinositol 3-kinase (PI3K)/Akt/protein kinase B to regulate endothelial function.\(^\text{195-198}\) Also (importantly for this review), VEGF signaling plays a critical role in vessel development during embryogenesis. \(\text{Vegf}^{+/}\) mouse embryos died due to defects in endothelial and hematopoietic cell development.\(^\text{32,199}\) Further, VEGF signaling has a role in arteriovenous specification. Morpholino knockdown of \(\text{vegf}\) in zebrafish prevented the expression of the arterial marker \(\text{ephb2}\) accompanied by sustained expression of venous marker in dorsal aorta.\(^\text{200}\) Ectopic expression of \(\text{vegf}\) rescued defective arterial differentiation mediated by cyclopamine, a sonic hedgehog inhibitor.\(^\text{200,201}\) VEGF also induced the expression of arterial markers \(\text{EphrinB2}, \text{Np1},\) and \(\text{Gja5}\) in primary \(\text{EphrinB2}\) endothelial cells isolated from E10.5 mouse embryos.\(^\text{202}\) In addition, overexpression of VEGF in cardiomyocytes (\(\alpha\text{MHC-VEGF}\) mice) led to significant increase in number of cells expressing the arterial marker \(\text{EphrinB2}\) with decreased number of \(\text{EphB4}\) venous endothelial cells.\(^\text{203}\) The molecular mechanisms of VEGF-mediated arterial specification are still unclear. Recent studies suggest that transcription factors FOXC1/FOXC2 interact with the VEGF pathway components to promote arterial specification of endothelial cells through the NOTCH signaling pathway (see below).

**NOTCH Signaling**

The NOTCH pathway is critical for arterial specification of embryonic vasculature.\(^\text{204,205}\) Four NOTCH receptors (1 through 4) and five ligands (jagged1, 2, Delta like ligand (Dll)1, 3, 4)
have been identified in mammals. Binding between the ligand and the receptor induces proteolytic cleavage of NOTCH receptor, resulting in generation of intracellular form of the receptor (NICD, NOTCH intracellular domain) that translocates into the nucleus to induce its downstream targets HEY1 and HEY2. Mice lacking a single copy of Dll4 exhibited severe remodeling defects in yolk sac vessels and smaller dorsal aorta consistent with the expression of components of NOTCH signaling, NOTCH1, NOTCH4 and Jagged1, Jagged2 and Dll4 selectively in arterial endothelial cells. Also, these embryos developed abnormal arteriovenous vessels due to fusion between the dorsal aorta and common cardinal vein. In a related study, Dll4−/− mouse embryos completely lost arterial identity. Overexpression of Dll4 caused abnormal vessel development with enhanced arterialization. Mice deficient in both Notch1 and Notch4 died in utero with a severe vessel remodeling defects in both yolk sac and embryo. Thus, NOTCH, which can be activated by VEGF (please see below), has an essential role in mediating arterial specification.

The role of VEGF and NOTCH pathways in promoting arterial endothelial cell specification points to a crosstalk of both pathways. The evidence for this comes from the zebrafish in which activation of notch was able to rescue arterial defects caused by morpholino knockdown of vegf. Likewise it was shown that VEGF induced the expression of Notch1 and Dll4 through PI3K/AKT pathway in cultured endothelial cells. The VEGF-mediated NOTCH activation was specifically seen in arterial endothelial cells (as opposed to venous endothelial cells) in vitro. Another study has shown that the cross-talk between both pathways could be mediated by endothelial transcription factors, FOXC1/FOXC2. As discussed above, deletion of FoxC1/FoxC2 led to arterial defects in developing mouse embryo. In vitro analysis revealed that FOXC1/FOXC2 upregulated the expression of the arterial endothelial markers Notch1, Notch4, Dll4, Hey2 and EphrinB2 through direct transcriptional activation. Interestingly, FOXC1/FOXC2-mediated promoter activation of Dll4 and Hey2 was augmented by VEGF treatment. Such augmentation was impaired by inhibiting PI3K. These intriguing results
suggest that the VEGF and NOTCH pathway promote arterial endothelial cell specification through FOX transcription factors. The expression of FOXC1 and FOXC2 is not activated by VEGF treatment alone, 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 towards 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 literatures.

**Signaling via Bone Morphogenetic Proteins**

Bone morphogenetic proteins (BMPs) belong to transforming growth factor (TGF) β superfamily that regulates a multitude of biological processes including embryonic vessel development. Approximately 20 mammalian BMPs have been identified and they function through serine/threonine kinase receptors composed of type I and type II forms. Upon 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.

Mouse gene knockout studies showed a key role of the BMP pathway in vascular development. Mice deficient in both Id1 and Id3, the downstream BMP/SMAD targets, showed vascular defects in the developing brain (see also “ID” above). While Bmp4 deficient mice died without posterior mesodermal differentiation, germ line deletion of Alk-3, a type IA BMP receptor, failed to survive up to E9.5 stage with defects in mesoderm formation.

Park et al 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, reduced FLK1⁺ cell generation. GATA2 together with BMP4 was shown to also promote mouse ES cell differentiation to FLK1⁺ cells. BMP4-mediated FLK1⁺
cell development was mediated by the transcription factor, ER71/ETV2.\(^{55}\) The expression of ER71 was decreased by inhibiting BMP signaling.\(^ {55}\) In addition, overexpression of ER71 rescued the impairment of FLK1\(^+\) cell generation by Noggin and DKK1 (WNT inhibitor).\(^ {222}\)

**WNT Signaling**

WNT signaling is critical for embryogenesis and disease development.\(^ {223,224}\) WNT proteins are ligands that bind their receptors to convey signals through three distinct signaling routes; the canonical WNT/β-catenin pathway; WNT/Ca\(^{2+}\) pathway; and planar cell polarity pathway (PCP).\(^ {225-227}\) The canonical WNT/β-catenin pathway has been the best studied and has a crucial role in vascular development. In the off-state (i.e., 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 to their receptor, Frizzled/Lrp (Low-density lipoprotein receptor), activates Dishevelled (Dvl) 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 report\(^ {229}\) 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 upon BMP signaling. Together these studies point to a key role of WNT signaling in vascular endothelial cell 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 \(\beta\)-catenin were shown to be required for development of CNS vessels.\(^{78,232}\) Embryos with sustained expression of \(\beta\)-catenin in TIE2\(^+\) cells lost their arteriovenous identity.\(^{233}\) In this study, \(\beta\)-catenin was shown to directly bind *Dll4* promoter\(^{233}\) linking the Wnt and NOTCH signaling pathways. Together these findings suggest a role for WNT-\(\beta\)-catenin signaling in vessel development, although the role of this pathway in endothelial cell specification is less clear.

### E. Endothelial cell plasticity and cell reprogramming

It is now generally accepted that terminally differentiated somatic cells are in the "ground state" where the fates of the cells remain unchanged throughout life. Waddington's epigenetic landscape model has been supported this deterministic view.\(^{234}\) 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 upon experiments by John Gurdon\(^{235}\) 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 as well as 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, NANOG, SOX2, KLF4 and MYC, are sufficient to generate ES-like cells (induced pluripotent cells, iPSCs) through a de-differentiation mechanism (Figure 5A).\(^{238-241}\)
With regard to endothelial cells, recent studies have shown that endothelial cells exhibit a certain degree of plasticity which is controlled by transcription factors. Overexpression of Prox1 conferred the identity of lymphatic endothelial cell to blood endothelial cells.\(^{179,180}\) Lymphatic endothelial cells deficient in Prox1 lost their lymphatic identity and became blood endothelial cells.\(^{178}\) Similarly, specification of arterial and venous endothelial cells is determined by a reciprocal function of NOTCH signaling and COUP-TFII as discussed above.\(^{184}\) These observations suggest that endothelial cell identity is interchangeable through transcription factor-mediated reprogramming.

Given such an important role of endothelial cell transcription factors in regulating cell fate, one can envision that appropriate combinations of endothelial transcription factors can directly reprogram terminally differentiated somatic cells into three different types of endothelial cells. A study reported that a combination of ETS factors with concomitant repression of TGFβ signaling converted amniotic cells to endothelial cells (Figure 5B).\(^{83}\) The key finding was that transient overexpression of ER71 converted the amniotic cells into immature endothelial cells which subsequently became mature endothelial cells with sustained expression of ERG1 and FLI1. The authors chose mid-gestation c-KIT\(^+\) amniotic cells for reprogramming, but failed to reprogram postnatal fibroblasts with the same approach. Although the generated endothelial cells 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 endothelial cells. In one study, cells partially reprogrammed using the Yamanaka factors (OCT4, SOX2, KLF4 and c-MYC) were isolated (referred to as PiPS: partially induced pluripotent stem) and thereafter further differentiated into endothelial cells under defined culture conditions.\(^{242}\) Although PiPS-derived endothelial cells had potential for neovascularization in vivo, the identity of PiPS and also PiPS-derived endothelial cells was not clear. In another study,\(^{243}\) human fibroblasts were induced to become CD34\(^+\) angioblast-like cells in two 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 endothelial cells as well as smooth muscle cells; however, it was not demonstrated whether endothelial cells could be generated concomitantly with hematopoietic cells as would be expected of CD34+ cells. These studies open a new possibility of generating endothelial cells 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 endothelial cells derived from somatic cells could be specified into arterial, venous or lymphatic endothelial cells 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.

F. Concluding remarks

Cardiovascular diseases such as high blood pressure, diabetes and 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 suffers from cardiovascular diseases in the United States alone. Development of new cell therapeutics based upon detailed understanding of generation of endothelial cells holds great promise. However, more knowledge and deeper understanding of endothelial cell and vascular development is urgently needed. Questions such as how the vascular system comprising endothelial cells develops prenatally and how it can be repaired by activating specific signaling pathways remain to be fully addressed. Additionally, there is the fundamental unexplored are of the importance of hemodynamic forces in mediating endothelial cell gene expression and functionality in arteriovenous endothelial cell specification during embryonic vessel development. Although hemodynamic forces play a key role in development of vascular diseases such as atherosclerosis,244-246 their involvement in vascular development is
unexplored territory. In this regard, an interesting study\textsuperscript{247} (which presages future work that is needed in this field) reported that both $Gja4$ and calcinerin/NFAT played a critical role for 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 endothelial cells. This knowledge would be a new research platform for generating functional and specific types of endothelial cells as well as organ-specific endothelial cells 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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**Competing interests statements**

The authors declare that they have no competing financial interests.
<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Full name</th>
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<tr>
<td>Bmp4</td>
<td>bone morphogenetic protein 4</td>
</tr>
<tr>
<td>Brg1</td>
<td>brahma-related gene 1</td>
</tr>
<tr>
<td>Coup-TFII</td>
<td>chicken ovalbumin upstream promoter transcription factor II</td>
</tr>
<tr>
<td>CREB1</td>
<td>cAMP response element-binding protein</td>
</tr>
<tr>
<td>dab2</td>
<td>disabled homolog 2</td>
</tr>
<tr>
<td>Dll1/4</td>
<td>delta-like 1/4</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>EphrinB2</td>
<td>ephrin type-B receptor 2</td>
</tr>
<tr>
<td>EphB4</td>
<td>ephrin type-B receptor 4</td>
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<tr>
<td>Erg</td>
<td>ets related gene</td>
</tr>
<tr>
<td>Er71</td>
<td>ets-related 71</td>
</tr>
<tr>
<td>Ets1/2</td>
<td>e26 avian leukemia oncogene 1/2</td>
</tr>
<tr>
<td>etsrp</td>
<td>ets1-related protein</td>
</tr>
<tr>
<td>Fgfr3</td>
<td>fibroblast growth factor receptor 3</td>
</tr>
<tr>
<td>Flk1</td>
<td>fetal liver kinase 1</td>
</tr>
<tr>
<td>Fli1</td>
<td>friend leukaemia integration-site 1</td>
</tr>
<tr>
<td>flt4</td>
<td>fms-related tyrosine kinase 4</td>
</tr>
<tr>
<td>Fox</td>
<td>forkhead transcription factor</td>
</tr>
<tr>
<td>Gata</td>
<td>GATA binding protein</td>
</tr>
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<td>Gja4/5</td>
<td>gap junction alpha 4/5</td>
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<tr>
<td>grl</td>
<td>gridlock</td>
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<tr>
<td>Hand2</td>
<td>heart- and neural crest derivatives-expressed protein 2</td>
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<tr>
<td>hCD4</td>
<td>human cluster of differentiation 4</td>
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<tr>
<td>Hey1/2</td>
<td>hairy/enhancer-of-split related with YRPW motif protein 1/2</td>
</tr>
<tr>
<td>Id</td>
<td>Inhibitor of differentiation and DNA binding 1</td>
</tr>
<tr>
<td>Klf4</td>
<td>krüppel-like factor 4</td>
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<tr>
<td>lycat</td>
<td>lysocardiolipin acyltransferase</td>
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<tr>
<td>Lyve1</td>
<td>lymphatic vessel endothelial hyaluronan receptor 1</td>
</tr>
<tr>
<td>Nfatc1</td>
<td>nuclear factor of activated T-cells, cytoplasmic 1</td>
</tr>
<tr>
<td>NICD</td>
<td>notch intracellular domain</td>
</tr>
<tr>
<td>Nkx2-5</td>
<td>nk2 homeobox 5</td>
</tr>
<tr>
<td>Np1</td>
<td>neuropilin 1</td>
</tr>
<tr>
<td>Oct4</td>
<td>octamer-binding transcription factor 4</td>
</tr>
<tr>
<td>Pdgfra</td>
<td>platelet-derived growth factor receptor α</td>
</tr>
<tr>
<td>Prox1</td>
<td>prospero-related homeobox</td>
</tr>
<tr>
<td>Rbpj</td>
<td>recombination signal binding protein for immunoglobulin kappa J region</td>
</tr>
<tr>
<td>Scl (Tal1)</td>
<td>stem cell leukemia (T-cell acute lymphocytic leukemia protein 1)</td>
</tr>
<tr>
<td>SMAD</td>
<td>sma/mad homology</td>
</tr>
<tr>
<td>Smc-α</td>
<td>smooth muscle actin alpha</td>
</tr>
<tr>
<td>Sox</td>
<td>sry-related HMG box</td>
</tr>
<tr>
<td>Su(H)</td>
<td>suppressor of hairless</td>
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<tr>
<td>Tel</td>
<td>translocated ets leukemia</td>
</tr>
<tr>
<td>Tgfβ</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>Timp</td>
<td>tissue inhibitor of metalloproteinase 1</td>
</tr>
<tr>
<td>Vcam1</td>
<td>vascular cell adhesion molecule 1</td>
</tr>
<tr>
<td>VEcadherin</td>
<td>vascular endothelial cadherin</td>
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<tr>
<td>Vegfr2/3</td>
<td>vascular endothelial growth factor receptor 2/3</td>
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Table 2. Phenotypes of mice and zebrafish deficient in genes critical for cardiovascular development

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<th>Genes</th>
<th>Experiments</th>
<th>Findings</th>
<th>References</th>
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<td>spontaneous mutants</td>
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<tr>
<td>Scl</td>
<td>MO study in zebrafish knockout mice</td>
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<td>86-88</td>
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<tr>
<td>Er71</td>
<td>knockout mice</td>
<td>lethal by E10.5 due to complete lack of vessel and hematopoietic cells</td>
<td>55,56</td>
</tr>
<tr>
<td></td>
<td>MO study in zebrafish</td>
<td>deficient in endothelial cell development and definitive hematopoiesis</td>
<td>17,90</td>
</tr>
<tr>
<td></td>
<td>MO study in xenopus</td>
<td>defects in vessel formation, hematopoiesis was not affected</td>
<td>67</td>
</tr>
<tr>
<td>Gata2</td>
<td>knockout mice</td>
<td>lethal by E10.5 due to defects in primitive erythropoiesis</td>
<td>100</td>
</tr>
<tr>
<td>Id1/Id3</td>
<td>knockout mice</td>
<td>lethal by E13.5, vascular defects in the forebrain, absence of branching of blood vessels into the neuroectoderm</td>
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</tr>
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<td>FoxO1</td>
<td>knockout mice</td>
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<td>135,136</td>
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<tr>
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<td>knockout mice</td>
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<td>136,139</td>
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<tr>
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<td>knockout mice</td>
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<td>foxc1b</td>
<td>MO study in zebrafish</td>
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<tr>
<td>foxc1a/1b</td>
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<td>no intersomitic vessel was detected at 24hpf, decreased formation of axial vessels</td>
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<td>Sox7/Sox18</td>
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<tr>
<td>Prox1</td>
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<td>175</td>
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<tr>
<td>Coup-TFII</td>
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<td>Tie2 conditional knockout</td>
<td>loss of vein identity, expression of arterial markers in vein</td>
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<tr>
<td>Ets1</td>
<td>knockout mice, no vessel defect, 50% of lethality by 4 weeks.</td>
<td>125</td>
<td></td>
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<tr>
<td>Ets1</td>
<td>MO study in zebrafish, reduced number of intersomitic vessel sprouting, defects in trunk circulation</td>
<td>250</td>
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<tr>
<td>Ets2</td>
<td>knockout mice, lethal by E8.5 due to impaired development of the extra-embryonic</td>
<td>251</td>
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<tr>
<td></td>
<td>Knockout Mice/Treatment</td>
<td>Description</td>
<td>Reference</td>
</tr>
<tr>
<td>----------</td>
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<td>------------------------------------------------------------------------------</td>
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<tr>
<td><em>Ets1/Ets2</em></td>
<td>knockout mice</td>
<td>Lethal during E11.5-15.5 due to dilated vessels, edema and hemorrhage</td>
<td>126</td>
</tr>
<tr>
<td><em>Erg</em></td>
<td>ENU mutagenesis</td>
<td>Embryonic lethal by E13.5, defects in definitive hematopoiesis, development of the embryonic vasculature unaffected</td>
<td>130</td>
</tr>
<tr>
<td><em>Fli1</em></td>
<td>knockout mice</td>
<td>Lethal by E12.5 due to the defects in hematopoietic cell differentiation and hemorrhage</td>
<td>129</td>
</tr>
<tr>
<td></td>
<td>MO study in zebrafish</td>
<td>Reduction in the number of intersomitic vessel sprouting</td>
<td>250</td>
</tr>
<tr>
<td><em>Tel</em></td>
<td>knockout mice</td>
<td>Lethal between E10.5 and E11.5, defective yolk sac vessel remodeling</td>
<td>122</td>
</tr>
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* morpholino knock-down
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