Key Transcriptional Regulators of Early Vascular Development

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Abstract—The formation of the vasculature depends on the precise spatial and temporal control of gene expression to define endothelial cell identity and to ensure the correct distribution and structure of the forming vessel network. This review provides an overview of the establishment of the vascular system, accompanied by a detailed discussion of the transcription factors involved in regulating endothelial gene expression during vasculogenesis and early vessel formation in both fish and mammalian systems. We also review the transcriptional pathways lying both upstream and downstream of key vascular transcription factors. (Arterioscler Thromb Vasc Biol. 2011;31:1469-1475.)

Key Words: angiogenesis ■ endothelium ■ molecular biology ■ transgenic models ■ vascular biology

The development of the vasculature begins before the initiation of the embryonic circulation with the process of vasculogenesis. This de novo formation of endothelial cells occurs as mesodermal precursors differentiate into endothelial cell progenitors (also known as angioblasts) and assemble together to form the early primitive vessel network, which provides the basis for the entire mature blood, lymphatic, and cardiac vascular system (Figure 1).

The Establishment of the Vascular System

The Origin of Endothelial Cells

Vasculogenesis and hematopoiesis (the formation of blood cells) are closely related processes, occurring at similar locations in the developing embryo and using related, and often overlapping, arrays of transcription factors and signaling molecules. Consequently, it has long been hypothesized that blood and endothelial cells may share a common progenitor, known as the hemangioblast. A number of animal models, including cloche zebrafish and Etv2-null mice, display severe defects in both blood and vascular development, indicating a shared requirement for these factors and suggesting a common progenitor lineage.1,2

In vitro studies in human and mouse embryonic stem cells identified a putative hemangioblast-like population, often referred to as blast colony–forming cells, that display both hematopoietic and endothelial potential.3 In addition, a single-cell-resolution fate map demonstrated that cells in the early zebrafish were capable of giving rise to both hematopoietic and endothelial cells, although few did, suggesting that not all endothelial cells come from a shared precursor.4 Significantly, endothelial cell progenitors can be detected in mouse embryos earlier than hematopoietic cells, and lineage tracing in multichimeric mice has demonstrated that the yolk sac blood islands (often cited as a site of the hemangioblast) do not share clonal precursors.5,6 Consequently, although there is considerable evidence demonstrating that endothelial and hematopoietic cells have the capacity to originate from a single precursor cell, the degree to which these lineages originate from shared versus separate origins in vivo, and the factors dictating this decision, are still not fully understood.

Vasculogenesis and Early Vessel Formation

In zebrafish, vasculogenesis occurs in 2 distinct intraembryonic populations of mesodermal cells: progenitors that give rise to the head endothelium migrate from the anterior lateral plate mesoderm, whereas endothelial progenitors from the posterior lateral plate mesoderm migrate to the midline and coalesce to form a precursor vascular cord.7 In contrast, the establishment of endothelial cells in mammals occurs via both extraembryonic and intraembryonic vasculogenesis. In the yolk sac, endothelial progenitors are first detected within the mesodermal layer as a component of blood islands, which consist of an inner mass of hematopoietic cells surrounded by an outer layer of endothelial cells. In combination with independently differentiated endothelial cells elsewhere in the yolk sac, these blood islands coalesce together to form a primitive vascular plexus, which ultimately becomes the vitelline circulation.7,8 Extraembryonic vasculogenesis also occurs independently in the allantois and contributes to the formation of the umbilical vessels. Intraembryonic vasculogenesis occurs slightly later, and in the mouse is first detected in the endocardium.8 Endothelial specification then occurs throughout the embryo, with progenitor cells arising throughout the intraembryonic mesoderm as loose cell aggregations.
endothelial cells (the hemogenic endothelium). These later hematopoietic stem cells emerge directly from ventral aortic pharyngeal and coronary vasculature form. Additionally, hematopoietic precursors differentiate into endocardium, arteries, veins and capillaries, and the lymphatic and coronary vasculature form. As the vasculature matures, it becomes differentiated into distinct arteries and veins, through the expression of the venous fate marker Efnb2 and the arterial fate marker Ephb4, respectively. In the zebrafish, these arterial and venous-fated cells are both initially located in the precursor vascular cord formed via vasculogenesis, from which ventral migration of venous-fated endothelial cells occurs to form the cardinal vein. Although not yet clear whether similar selective cell sprouting occurs in mammals, ablation of Efnb2/Ephb4 signaling in the mouse affects cell distribution between the aorta and cardinal vein, with the resultant enlarged dorsal aorta containing venous-fated endothelial cells that have failed to migrate.

Figure 1. Schematic depiction of early mammalian vascular formation, with the transcription factors likely required for each step marked in blue text. Gray text and lines indicate the related processes of hematopoiesis.

These aggregations then come together, forming a primary vascular plexus, bilateral vascular cord, and endocardial primordial. The specification of endothelial cells to an arterial or venous fate is now thought to occur before the formation of distinct arteries and veins, through the expression of the ligand Efnb2 and the receptor Ephb4, respectively. In the zebrafish, these arterial and venous-fated cells are both initially located in the precursor vascular cord formed via vasculogenesis, from which ventral migration of venous-fated endothelial cells occurs to form the cardinal vein. Although it is not yet clear whether similar selective cell sprouting occurs in mammals, ablation of Efnb2/Ephb4 signaling in the mouse affects cell distribution between the aorta and cardinal vein, with the resultant enlarged dorsal aorta containing venous-fated endothelial cells that have failed to migrate.

Later Events in Vascular Development

Further vascular development occurs through the process of angiogenesis, in which new blood vessels are formed from existing ones through endothelial sprouting, branching, and intussusception. Alongside angiogenesis, smooth muscle cells and pericytes are recruited to provide structural support. As the vasculature matures, it becomes differentiated into endocardium, arteries, veins and capillaries, and the lymphatic and coronary vasculature form. Additionally, hematopoietic stem cells emerge directly from ventral aortic endothelial cells (the hemogenic endothelium). These later events in vascular differentiation are reviewed in detail elsewhere and are not the topic of this review, which concentrates on the regulatory events controlling vasculogenesis and early vascular formation.

Transcriptional Regulation of Early Vascular Development

Although endothelial gene expression has been extensively studied, no single transcription factor has yet been identified as its master regulator. Additionally, all transcription factors so far known to play a role in endothelial regulation have an expression domain that extends outside of the vascular system. Consequently, it is hypothesized that the very precise spatial and temporal control of gene expression during vasculogenesis and early vessel development is achieved through the actions of multiple transcription factors working together in concert.

Etv2/Etsrp Is Required for Vasculogenesis

Members of the Ets transcription factor family, which share a highly conserved ETS DNA-binding domain, have long been known to be crucially important for endothelial gene expression. All identified endothelial promoter and enhancer regions contain essential clusters of Ets binding motifs, and multiple Ets family members can directly bind these regions. As many as 12 different Ets factors are expressed in zebrafish endothelial and hematopoietic progenitors, and 19 of the 27 known mammalian Ets factors are expressed in human endothelial cells. The presence of multiple Ets factors, combined with conservation of binding domain sequences and a shared preferences for binding motifs, has contributed to the difficulties in ascertaining specific roles for individual Ets proteins in vessel growth. However, Etv2/Etsrp is increasingly recognized as the primary Ets transcription factor required for vasculogenesis.

First identified through a screen for genes downregulated in mutant cloche zebrafish, a mutation in the Etsrp gene was subsequently demonstrated to be the cause of the N-ethyl-N-nitrosurea-induced zebrafish vascular mutant y. Etsrp expression is detected in the presumptive vascular cells from early somitogenesis and is specifically expressed at high levels throughout the vasculature as it forms. Knockdown of Etsrp can result in a complete absence of endothelial cells, with a corresponding loss of endothelial markers, whereas forced expression of Etsrp leads to ectopic upregulation of vascular marker expression. The role of Etsrp in zebrafish is not limited to endothelial cells, as it is also both required and sufficient for the formation of the myeloid lineage and possibly erythroid differentiation. Phylogenetic analysis identified the mammalian Etv2 transcription factor (also known as ER71 and Etsrp71) as the potential orthologue of zebrafish Etsrp. Although they share relatively little sequence similarity, Etv2 behaves as a functional orthologue of Etsrp in zebrafish ectopic expression studies, and they share similar expression patterns and required functions. Etv2 expression in the hemangioblast-like blast colony–forming cells precedes that of Flk1, the vascular endothelial growth factor receptor required for blood and endothelial cell development, and enforced Etv2 increases expression of endothelial and hematopoietic lineage markers. Expression of Etv2 in mouse embryos is transiently
detected in Flk1+ hematopoietic and endothelial precursors but does not persist in the vasculature once endothelial cells are formed.\textsuperscript{2,21} No endothelial or blood cells can be detected in Evt2-null mice embryos, which die at midgestation, suggesting that Evt2 is absolutely required for vasculogenesis and hematopoiesis.\textsuperscript{2,21} In keeping with an essential role in vasculogenesis, Evt2 can directly transactivate many genes required for the establishment of the early endothelium, often in combination with Forkhead factors (see discussion below).\textsuperscript{22}

Transcriptional regulation of the Evt2/Etsrp gene itself has so far been studied only in mammalian endothelial cells within and around the developing heart. The absence of Evt2 promoter activity in Nkx2.5-null hearts, combined with the identification of a functional Nkx2.5 binding site within the Evt2 promoter, has suggested a potential role for the Nkx2.5 transcription factor upstream of Evt2.\textsuperscript{21} Nkx2.5, a member of the NK homeobox transcription factor family, is highly expressed in early heart progenitor cells and is essential for heart development.\textsuperscript{23} However, because Nkx2.5 is not expressed in endothelial progenitors outside of the heart, it is likely that other, currently undefined transcription factors contribute to the vasculogenic regulation of Evt2 and Etsrp\textsuperscript{21} (Figure 2).

**Etv2/Etsrp Can Regulate Endothelial Genes in Combination With Forkhead Factors**

Mammalian Evt2 expression can be detected elsewhere in the embryo at midgestation and at high levels in the testis of adult male mice.\textsuperscript{2,24} Furthermore, multiple Ets factors, including Evt2, appear to recognize similar DNA binding motifs with high affinity.\textsuperscript{25} Consequently, it has been hypothesized that Evt2/Etsrp may achieve endothelial-specific gene activation in combination with other vascular transcription factors. In particular, studies have demonstrated that Evt2 can bind a low affinity composite binding motif (known as the FOX:ETS site) in combination with the Forkhead transcription factor Foxc2.\textsuperscript{22} Similar FOX:ETS binding motifs can be identified within at least 11 different validated endothelial enhancer or promoter regions, including Flk1, Nrp1, Notch4, and Cdh5 (VE-cadherin). Evt2 and Foxc2 can synergistically activate endothelial gene expression in both in vitro and in vivo models, and combinatorial morpholino knockdown of Etsrp and Foxc1a in zebrafish, at individually subphenotypic doses, resulted in near-complete ablation of the vasculature without affecting the hematopoietic compartment.\textsuperscript{22} Additionally, the FOX:ETS motif is sufficient to locate endothelial gene enhancers from genomic sequences.\textsuperscript{22} Although not all identified endothelial promoters or enhancers contain a FOX:ETS motif, in examples where multiple endothelial regulatory regions have been identified for a single gene (including Mef2c, Tal1, and Tie2), at least 1 of these elements contains the motif.\textsuperscript{22} Consequently, it is likely that many genes expressed during early endothelial establishment and growth are at least partially under the combinatorial activation of Evt2/Etsrp and Forkhead factors via a FOX:ETS DNA binding motif.

Although it is clear that the FOX:ETS motif is a prevalent motif in endothelial gene regulation, endothelial cells can become specified in the absence of Foxc factors in both zebrafish and mammals\textsuperscript{22,26}(see discussion below). Additionally, mammalian Foxc factors also play an important role in later vascular differentiation events, when Evt2 is no longer expressed at detectable levels.\textsuperscript{26,27} Consequently, either the early events of vascular specification do not require this combinatorial regulation, or other Forkhead factors can contribute to Evt2/Etsrp-mediated endothelial cell specification. Of note, the Forkhead factor Foxo1 is able to bind the FOX:ETS motif and is also expressed during early vascular formation (see below).\textsuperscript{22,28,29}

**Tal1 Is Required for Hemangioblast Formation but Not Vasculogenesis**

Like Evt2, the basic helix-loop-helix transcription factor Tal1 (SCL) is expressed in both blood and endothelial progenitor populations.\textsuperscript{30,31} In zebrafish, forced expression of Tal1 induces the expression of many hematopoietic and vascular genes and can partially rescue the blood and vascular defects seen in Etsrp morphant and cloche mutant fish.\textsuperscript{31–33} Tal1 expression is absolutely required for the formation of mammalian blast colony–forming cells, and Flk1+ Tal1+ cell populations were enriched for this lineage.\textsuperscript{34,35} However, although ablation of Tal1 expression in both fish and mammals results in severe hematopoietic and vascular defects, endothelial cells can still be specified in both models.\textsuperscript{31,36,37} In fish, endothelial progenitors are formed but have decreased expression of vascular endothelial growth factor receptors, and dorsal aorta formation is severely compromised.\textsuperscript{31,36} Similar defects are seen in Tal1-null mice in which the hematopoietic expression of Tal1 has been rescued.\textsuperscript{37} Endothelial cells are formed and some arrange into plexus-like structures, but major vessels are absent, and death occurs around midgestation.\textsuperscript{37} Consequently, Tal1 appears essential for the establishment of the hemangioblast but not for vasculogenesis. Alternative routes to endothelial cell formation may account for this apparent contradiction. In embryonic stem cell studies, a subset of endothelial cells form from Flk1+ Tal1− cell populations, and as discussed above, not all endothelial cells in the zebrafish appear to develop from cells with hemangioblast capability. The fact that Tal1-null mice experience severe vascular defects after endothelial cell
formation may potentially indicate different roles, or differentiation status, for endothelial cells of alternative origins.34

In keeping with the latter role of Tal1 in vascular formation, evidence suggests that Tal1 transcription is primarily downstream of Etv2/Etsrp in endothelial cells (Figure 2). In zebrafish, forced expression of Etsrp results in ectopic expression of Tal1, whereas knockout of Tal1 has little effect on Etsrp expression.19,38 Additionally, the mammalian Tal1 downstream enhancer, directing expression to hematopoietic and endothelial compartments, contains an essential FOX:ETS binding motif.22,39 This enhancer also contains key Gata motifs, placing Tal1 downstream of Gata factors and potentially explaining the maintenance of some Tal1 in Etsrp-mutant zebrafish.19,39

Tal1 can directly bind E-box recognition motifs of DNA as an obligate heterodimer with E-proteins. In agreement with the vascular phenotype after Tal1 ablation, E-box motifs and direct Tal1 binding have been reported for some, although not all, known endothelial enhancer and promoter elements, including Fli1, Gata2, and CdH513 (Figure 2). However, direct DNA binding of Tal1 may be dispensable for early vascular development, because knock-in mice homozygous for a non-DNA-binding form of Tal1 survive significantly longer than mice lacking Tal1 in the vasculature.40 Although it is still unclear how Tal1 regulates endothelial development in the absence of direct DNA binding, data from hematopoietic lineages suggests that Tal1 can also be recruited to DNA indirectly.41

Gata Factors May Be Involved in Hemangioblast and Endothelial Development

The Gata2 transcription factor is essential for hematopoietic development in both zebrafish and mammals.32,43 Gata2 expression is enriched in the hemangioblast-like blast colony–forming cells, and enforced Gata2 expression in this system can induce Flk+ and Tal1+ cells, suggesting that Gata2 may be important for hemangioblast formation.44 The strong expression of Gata2 in endothelial cells has also implicated it in vascular development, although its precise role is still unclear, and separating the role of Gata2 in hematopoiesis from any putative involvement in endothelial cell development has remained a challenge. Most vascular transcriptional targets of Gata2 are also required for hematopoiesis, and depletion of Gata2 in fish and mammals has no reported effect on the vasculature.45,46 Gata2 is not the only Gata factor expressed in endothelial cells, and functional redundancy may explain the lack of vascular phenotype. However, much less is currently known about other vascular Gata factors, although recent data have suggested that Gata3 is the primary Gata in large blood vessels and can directly regulate the Tie2 gene.47

Gata2 expression is likely downstream of Evt2/Etsrp; Gata2 is not expressed in y13 Etsrp mutant zebrafish, and enforced expression of Evt2 during mesoderm formation results in increased Gata2 expression.16 However, neither known mammalian Gata2 enhancers contain a FOX:ETS motif, suggesting that Gata2 regulation may not be downstream of Evt2-Forkhead regulation.42,48 The position of Gata2 in the transcriptional hierarchy below Evt2/Etsrp has been harder to gauge. It has been hypothesized that Gata2, Tal1, and Ets factors, most notably Fli1, form a recursively wired transcriptional network during early hematopoietic development, in which they are each required for the others’ transcriptional regulation.49 Although evidence supporting such a model primarily comes from the hematopoietic lineage, this does not preclude the involvement of this regulatory network in endothelial cell gene regulation, because the regulatory elements targeted by this transcriptional network also direct expression to the endothelial compartment (Figure 2).

The Roles of Fli1, Erg, and Other Ets Factors in Early Vessel Formation

As previously discussed, potential redundancy of Ets in the vasculature has presented challenges when assigning a role to individual Ets proteins during vascular formation, and only Evt2/Etsrp has so far been shown as essential for vasculogenesis (see discussion above). However, it is becoming clear that other Ets factors beside Evt2/Etsrp are also specifically required during the early events of vascular development.

Studies from Xenopus have suggested that the Ets factor Fli1 acts at the top of the hematopoietic and endothelial transcriptional network.51 However, although expression is detected very early in zebrafish vascular development, knockdown of Fli1, both alone and in combination with related Ets factors, has failed to recapitulate the Xenopus data.51 Additionally, overexpression of Fli1 can stimulate ectopic endothelial gene expression only when a constitutively active Fli1-VP16 construct is used, likely reflecting a more general role for Ets factors.16,51 Similarly, mammalian Fli1 is detected at sites of vasculogenesis but is not required for endothelial cell formation.53 However, Fli1-null embryos die soon after midgestation, at least partially because of loss of vascular integrity, indicating that even in the presence of highly similar Ets factors, Fli1 expression is absolutely required for the vasculature after endothelial establishment.51 Unlike most other vascular regulators, Fli1 does not appear to lie downstream of Evt2/Etsrp (Figure 2). Fli1 is not downregulated in cloche mutant zebrafish, and considerable Fli1 expression is still detected after depletion of Etsrp19,51 Additionally, the mammalian Fli1 enhancer, directing both hematopoietic and endothelial expression, does not contain a FOX:ETS motif, instead requiring Ets, Gata, and E-box motifs, probably as part of the recursively wired Ets, Gata, and Tal1 transcriptional circuit.49 Direct Fli1 binding and transgene activation has also been reported for other hematopoietic and endothelial genes, including Erg and Lmo2.13

Transcriptional redundancy between Fli1 and the related Ets factor Erg, also highly expressed in endothelial cells, may go someway toward explaining the absence of a vascular phenotype in Erg-null mice. Erg is highly homologous to Fli1 and can bind the same Ets motifs in target genes.54,55 The homozygous ErgMid2 Mid2 mouse line, encoding a missense Erg that cannot transactivate downstream gene expression, has no overt vascular phenotype before death because of failure of definitive hematopoiesis.56 However, the vasculature of these mice appears unusually dilated, possibly reflect-
ing the known role of Erg in the transcriptional regulation of Cdh5 (VE-cadherin) via direct binding to the promoter.56,57

Etv6 (Tel) is another Ets factor essential for vascular development, although its primary role appears to be during later angiogenesis. In Xenopus, loss of Etv6 results in suppression of arterial markers, including Notch4 and Dll4.58 In mice, where loss of Etv6 results in death at embryonic day 12.5 with gross vascular defects, Etv6 is essential for angiogenic sprouting, regulating the expression of factors that constrain angiogenesis, including Dil4 and Spry4, through binding to the generic co-repressor CtBP.59,60

**Foxc, Foxo, and Other Forkhead Transcription Factors**

Similar to Ets, there are many different Forkhead (Fox) transcription factors involved in vascular development. Some appear to have specialized roles in particular regions of the vasculature, such as Foxm1 in pulmonary vessels and Foxo1 in the fetal testis vasculature; others, like Foxp1, are expressed in endothelial cells but as yet have no defined vascular function.61–63 However, evidence has clearly implicated members of the Foxc and Foxo families in vascular development.

In zebrafish, Foxc1a and Foxc1b are expressed in vascular progenitors and the dorsal aorta, although not in the inter somitic sprouts. Depletion of Foxc1a alone results in severe vascular defects, loss of blood flow, and hemorrhage, whereas combinatorial knockdown of Foxc1a with Etsrp leads to near-complete vascular loss (discussed above).22,64 Depletion of Foxc1 also causes defects in arteriovenous formation, suggesting a later role in vascular differentiation.64 It is likely the mammalian Foxc factors are also involved in the later differentiation of endothelial cells, in addition to their early role with Etv2 (discussed above). Compound Foxc1:Foxc2 homoygotes die at midgestation, and although endothelial cells are specified, severe defects are detected during vessel formation, and arterial marker expression is repressed.65 Although these later stages of vascular differentiation are discussed in detail elsewhere,65 the multiple different regulatory roles of Foxc during vascular development are reflected in the genes downstream of Foxc. In addition to regulation via the FOX:ETS motif, other direct transcriptional targets are more similar to consensus Fox binding sites. Both Dil4 and Hey2 promoters bind and are directly activated by Foxc2 in vivo, and chromatin immuno precipitation analysis in lymphatic endothelial cells identified Foxc2 sites within the Bmp4 and Nrp1 loci, in addition to the known FOX:ETS motif of Mef2c.66,67

Members of the Foxo protein subfamily are more generally associated with promoting cell cycle arrest and apoptosis and with counteracting oxidative stress.68 However, mouse models of Foxo1 demonstrated previously unsuspected roles in vascular development. Foxo1 is expressed at high levels in endothelial cells of the early vasculature, and Foxo1-null mice die at midgestation with severe vascular defects.28 Endothelial cells are specified, but the dorsal aorta is thin and disorganized, and few distinct vessels form.28,29 Additionally, both Foxo1 and Foxo3 can inhibit endothelial tube formation and migration in vitro, and somatic deletion of Foxo factors in the adult results in widespread hemangiomas with associated increases in endothelial cell levels, suggesting that Foxo factors are also important for adult vascular homeostasis.52,68

Although there have been many studies of the pathways upstream of Foxo activation, and posttranslational modifications, including phosphorylation, acetylation, and ubiquitination, are known to contribute to the level and activity of Foxo transcription factors within different cells, the transcriptional regulation of Foxo in endothelial cells is still not clear.68,69 Downstream, Foxo factors can function as both positive and negative regulators of their transcriptional targets, including a number of endothelial genes primarily involved in angiogenesis, such as Pdgfb, Nos3, and Spry2.68,70

**Mef2c May Be Required for Correct Vessel Formation**

Mef2 proteins, members of the MADS family of transcription factors, are expressed in both the endothelial and smooth muscle cells of the developing vascular system.71 Mef2c is the first Mef2 factor expressed in endothelial cells, and although it may be required for vascular development, it is dispensable for vasculogenesis.72,73 Mef2c-null mice die at midgestation; endothelial cells are specified and early vascular markers detected, but the major vessels are incorrectly formed and the dorsal aorta is abnormal.72,73 However, the severe cardiac defects also observed may explain much of the vascular phenotype, whereas the continued expression of the Mef2a transcription factor may partially compensate for the lack of Mef2c. Further hindering our understanding of the role of Mef2 factors in endothelial cells is the fact that only a few downstream targets of Mef2 transcription factors, such as Klf2, have yet been identified in the vascular system, although the endothelial regulation of Mef2c itself is directly downstream of Etv2/Etsrp via an essential FOX:ETS motif within a vasculogenesis-specific enhancer.22,74,75

**Concluding Remarks**

Understanding the regulation of vascular growth provides insights not only into the developmental process but also into potential mechanisms to fight diseases reliant on adequate vascularization, most importantly cancer. Whereas the development of the tumor vascular system has traditionally been thought to occur via angiogenesis from surrounding regions, recent research has demonstrated that in certain tumor types, endothelial cells can be derived from the tumor itself. This observation, currently only verified in glioblastoma and potentially linked to cancer stem cell–like populations within the tumor, raises the tantalizing prospect that vasculogenesis itself occurs during tumor vessel formation.76,77 It is consequently ever more important that we clearly understand the mechanisms controlling gene expression during these very early stages of endothelial specification and development.

**Disclosures**

None.

**References**


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