Regulation of endothelial and hematopoietic development by the ETS transcription factor Etv2

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Purpose of review
Vasculogenesis and hematopoiesis are essential for development. Recently, the ETS domain transcription factor Etv2 has been identified as an essential regulator of vasculogenesis and hematopoiesis. Here, we review the recent studies that have established the critical role of Etv2 in the specification of mesoderm to blood and endothelial cells.

Recent findings
Loss and gain-of-function studies have demonstrated the conserved role of Etv2 in endothelial and hematopoietic development. Recent studies have placed Etv2 at or near the top of the hierarchy in specification of these lineages and have begun to dissect the upstream regulators and downstream effectors of Etv2 function using multiple model organisms and experimental systems.

Summary
Etv2 is essential for the specification of endothelial and hematopoietic lineages. Understanding the mechanisms through which Etv2 specifies endothelial and blood cells by defining upstream transcriptional regulators and cofactors will lead to greater insight into vasculogenesis and hematopoiesis, and may help to identify therapeutic targets to treat vascular disorders or to promote or inhibit vessel growth.

Keywords
endothelial, ER71, ETS, etsrp, Etv2, hematopoietic, mesoderm, vasculogenesis

INTRODUCTION

The vascular system is essential for embryogenesis and is one of the first organ systems to develop. Blood vessels initially form through the process of vasculogenesis, which first occurs in the extra-embryonic yolk sac and subsequently in the embryo proper [1]. After primitive vessels have formed, the vascular plexus expands by endothelial sprouting and intussusceptive vessel growth in a process known as angiogenesis. Maturation of the vascular network also involves recruitment of mural cells for stabilization, and remodeling of blood vessels into arteries, veins and capillaries [2,3].

During vasculogenesis, the initial formation of endothelial progenitors (angioblasts) from the extra-embryonic mesoderm and hematopoietic cells also appear. These angioblasts and hematopoietic cells coalesce into blood islands, which fuse and form the primitive vascular plexus [4]. The close appearance of hematopoietic cells and endothelial progenitors in the extraembryonic mesoderm led to the hypothesis that they develop from a common progenitor, the hemangioblast. In embryonic stem cell differentiation experiments, researchers identified a mesodermal precursor called the blast colony-forming cell (BL-CFC), characterized by expression of Brachyury and Flk1, which gives rise to both endothelial and hematopoietic cells and represents the *in vitro* equivalent of the hemangioblast [5,6]. Within the embryo, hemangioblasts have been shown to arise within the posterior primitive streak [7], and recent studies established that hemangioblasts give rise to hematopoietic cells through a hemogenic endothelial intermediate [8,9].

Several major signaling pathways, including vascular endothelial growth factor (VEGF), bone morphogenetic protein (BMP), Notch, Wnt, and fibroblast growth factor (FGF) are required for induction of endothelial and hematopoietic lineages [7], and much has been learned about the nonautonomous signals involved in vasculogenesis and

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KEY POINTS

- The ETS domain transcription factor Etv2 is essential for endothelial and hematopoietic development.
- Etv2 is sufficient to induce ectopic endothelial gene expression in zebrafish, frogs, and embryonic stem cells.
- Etv2 cooperates with the Forkhead transcription factor FoxC2 to activate numerous endothelial genes through a composite binding motif, the FOX:ETS motif.
- Etv2 promotes endothelial and hematopoietic development and inhibits cardiac development, possibly through an antagonistic relationship with the cardiac-restricted homeodomain protein Nkx2–5.

angiogenesis. By comparison, much less is known about the transcription factors involved in vascular development. Recent studies have identified the ETS (E26 transformation specific) domain transcription factor Etv2 as an essential regulator of vasculogenesis and hematopoiesis. In this review, we highlight the role of Etv2 in the development of endothelial and blood lineages and discuss what has been learned about this transcription factor's function, lineage contributions, targets and cofactors.

THE ETS TRANSCRIPTION FACTOR Etv2 IS ESSENTIAL FOR ENDOTHELIAL AND HEMATOPOIETIC DEVELOPMENT

Members of the ETS family of transcription factors play important roles in endothelial development [1,10]. There are approximately 30 members of the ETS family in vertebrates [11]. All ETS proteins share a highly conserved winged helix–turn–helix ETS DNA-binding domain and bind to a consensus GGAA/T-binding motif [11]. Many ETS proteins are expressed within hematopoietic and endothelial cells and are important in the development of these tissues. For example, *erg* is essential for definitive hematopoiesis in mice and is required for angiogenesis in zebrafish [12,13]. The ETS transcription factor Etv6 controls angiogenic sprouting [14], and Fli1 acts early in hemangioblast development by functioning upstream of many early endothelial and hematopoietic genes, including *Flk1*, *Scl* and *Gata2* [15]. Loss of Fli1 function in *Xenopus* causes a severe decrease in the number of hemangioblasts [15]. However, in mouse and zebrafish, loss of Fli1 does not result in severe vascular defects [15–17], suggesting that other ETS factors may compensate or that Fli1 is not absolutely required for hemangioblast specification. It is plausible that other ETS factors may function redundantly during embryogenesis to compensate for the loss of Fli1.

Recently, the essential role of Etv2 in endothelial and hematopoietic development has been clearly demonstrated in multiple vertebrate species. In contrast to other members of the ETS family, mouse knockouts for this gene die early in gestation and fail to develop any endothelial or hematopoietic cells [18,19]. Fli1 has been proposed to act upstream of *Etv2* [15], but other studies show that Fli1 is downstream of Etv2 in mice and zebrafish [20▪,21,22]. Additionally, Etv2 overexpression is sufficient to up-regulate *fli1* expression in zebrafish [23,24] (discussed below). *Etv2*-null mouse embryonic stem cells fail to differentiate into $Flk1^{+}$ vascular mesoderm from which endothelial and blood cells are derived [20▪]. Mutation or knockdown of the zebrafish or *Xenopus* orthologs of *Etv2* also causes severe vascular and endocardial defects [22,25–27,28▪,29▪,30▪▪,31]. In contrast to the requirement for Etv2 during early hematopoiesis in mice, only the myeloid lineage is affected in zebrafish *Etv2* mutants, whereas erythroid lineage development occurs normally [22,25,26,30▪▪,31]. However, Ren *et al.* [21] have shown that *Etv2* is also required for the initiation of hematopoietic stem cell (HSC) development from hemogenic endothelium, demonstrating a requirement for *Etv2* in definitive hematopoiesis in zebrafish.

In the mouse, definitive HSCs arise from hemogenic endothelium at E10.0 [32–34]. However, $Etv2^{-/-}$ mouse embryos die prior to this stage [18,19], precluding an analysis of hemogenic endothelium formation in *Etv2* germline-null mice. Recent work by Lee *et al.* [35▪] demonstrated that when Etv2 function is removed exclusively from the hematopoietic system in adult mice, there was a significant decrease in HSCs. The authors of that study found that Etv2 was required for maintenance of HSCs, that HSC progenitors isolated from $Etv2^{-/-}$ bone marrow exhibit reduced capacity to differentiate into granulocyte-monocyte progenitors, and that the number of myeloid lineage cells in the bone marrow was significantly reduced [35▪]. These results are consistent with the role of Etv2 in zebrafish and *Xenopus* myeloid development. Thus, in addition to its requirement for endothelial cell specification, Etv2 is also required for HSC development, differentiation and maintenance (Fig. 1), although further work needs to be done to clarify its role during mouse hematopoietic development.

Etv2 IS SUFFICIENT TO INDUCE ENDOTHELIAL AND HEMATOPOIETIC GENE EXPRESSION

In addition to the loss-of-function experiments described above, which demonstrate the requirement

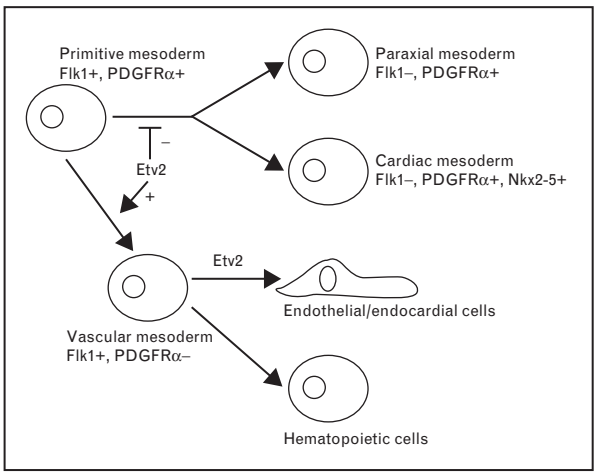

FIGURE 1. The role of Etv2 in mesodermal differentiation. Etv2 promotes the differentiation of primitive mesoderm into vascular mesoderm and endothelial and hematopoietic lineages, while inhibiting differentiation into paraxial and cardiac mesoderm.

of Etv2 for endothelial and hematopoietic development, gain-of-function experiments performed in zebrafish and *Xenopus* embryos demonstrate that Etv2 is also sufficient to induce ectopic endothelial and myeloid marker expression [22–24,27,29▪,31]. Overexpression of the ortholog of Etv2 in zebrafish embryos induces expression of endothelial markers *flk1*, *scl* and *fli1* and the myeloid marker *pu.1* [22–24,31]. Likewise, injection of *Etv2* mRNA into *Xenopus* embryos at an early stage into cells normally fated to be avascular endoderm results in ectopic induction of endothelial-specific markers *flk1* and *cd31* and the myeloid marker *runx1* [27,29▪]. This activity of Etv2 is profoundly enhanced by co-injection of mRNA encoding the Forkhead transcription factor FoxC2 ([27]; discussed below). Similarly, overexpression of Etv2 in embryonic stem cells strongly induces the formation of hematopoietic and endothelial progenitor cells, and Etv2 expression is sufficient to rescue formation of $Flk1^+$ mesoderm after inhibition of BMP, Notch, and Wnt signaling [19].

EXPRESSION AND LINEAGE ANALYSES DEMONSTRATE AN EARLY ROLE FOR Etv2 IN ENDOTHELIAL AND HEMATOPOIETIC SPECIFICATION

Etv2 is essential for normal hematopoietic and endothelial development, but it is only expressed transiently in early progenitors in the mesoderm. In the mouse, *Etv2* is expressed at the earliest site of endothelial cell development within the extraembryonic mesoderm in E7 mouse embryos [19,20▪]. *Etv2* expression persists in early progenitors and in newly formed endothelial cells, but expression rapidly fades and is essentially absent by E11.5 [18,19,20▪,36▪▪]. In zebrafish, the *Etv2* ortholog *etsrp* is also expressed transiently within angioblasts and the early vasculature but is down-regulated as blood vessels mature [22,37,38▪]. Etv2 is enriched in embryonic stem cell-derived hemangioblast-like BL-CFCs and is only briefly expressed during embryonic stem cell differentiation to mature endothelium and hematopoietic lineages, similar to the early and transient expression seen in mouse and zebrafish embryos [19,20▪].

The early expression of *Etv2* in the mesoderm at sites where endothelial and hematopoietic progenitors arise, combined with the critical requirement for Etv2 during development, suggests that Etv2 is expressed and functions in the progenitors that give rise to the mature vasculature and to the hemogenic endothelium from which hematopoietic stem cells arise. Indeed, recent lineage tracing studies using an Etv2 enhancer to direct expression of Cre recombinase demonstrated that Etv2-expressing progenitors contribute to the vasculature of the developing embryo and to hematopoietic cells including $CD45^+$ nonerythroid and $Ter119^+$ erythroid cells [36▪▪]. Similarly, $Flk1^+$, $Etv2^+$ cells isolated from E7.5 or E8.5 embryos are enriched for cells that differentiate into hematopoietic lineages, including $Mac1^+$, $CD45^+$ granulocytes and $Ter119^+$ erythroid cells [20▪]. It is still unclear whether Etv2-expressing progenitors give rise to the entire vasculature or to what extent hematopoietic stem cells and hematopoietic lineages are derived from Etv2-expressing cells. A more detailed characterization of the lineages derived from $Etv2^+$ cells is still needed to gain a complete understanding of the origins of the vascular system.

Rasmussen *et al.* [36▪▪] also described a contribution of $Etv2^+$ cells to other mesodermal compartments, suggesting the possibility that Etv2 may play additional roles outside of the cardiovascular system. These extra-mesodermal contributions by Etv2-expressing cells need further investigation and confirmation using additional techniques, but raise the intriguing possibility of additional developmental roles for Etv2.

The differentiation of multipotent mesodermal progenitors requires tight control of cell fate, which recent studies demonstrate is achieved through reciprocal control of cardiac versus hematopoietic and endothelial fate (Fig. 1). In zebrafish, the cardiac-restricted homeodomain protein Nkx2–5 [39,40] acts downstream of FGF signaling to restrict hemangioblast fate, causing down-regulation of *Etv2* and *Scl*, while promoting cardiac fate [41].

Similarly, in mice, Nkx2–5 acts to repress mesodermal differentiation to hematopoietic lineages and to promote cardiomyocyte development [42], suggesting an antagonistic relationship between cardiomyocyte and endothelial/hematopoietic lineage commitment. In support of this notion, Etv2 has recently been shown to modulate these mesodermal cell fate decisions in favor of hematopoietic and endothelial differentiation at the expense of the cardiomyocyte lineage [30▪▪,36▪▪].

In *Etv2*-null mouse embryos, mesodermal cells normally fated to become endothelial and hematopoietic cells differentiate into cardiac and other mesodermal lineages [36▪▪]. These studies in mice are complemented by studies in zebrafish demonstrating that loss of Etv2 causes down-regulation of endocardial markers and expansion of myocardial gene expression [30▪▪]. Furthermore, induction of Etv2 both in embryonic stem cells and zebrafish is sufficient to inhibit cardiac gene expression [19,36▪▪]. Together, these observations emphasize the importance of Etv2 in the specification and differentiation of endothelial and hematopoietic cells from multipotent mesodermal progenitors, and suggest that it acts as a master regulator of mesoderm specification to the endothelial/hematopoietic lineage, possibly in an antagonistic relationship with the cardiac regulatory factor Nkx2–5 (Fig. 1).

TRANSCRIPTIONAL TARGETS OF Etv2

To better understand how Etv2 controls development of hematopoietic and endothelial lineages, it is essential to identify the direct targets of Etv2 during early mesoderm development, and several studies have sought to identify Etv2 targets using genome-wide expression analyses. Microarrays have been carried out on zebrafish embryos and mouse embryonic stem cells overexpressing Etv2 and on *Etv2::YFP*, Flk1^{+}, Pdgfrα^{+} cells isolated from *Etv2*-null mouse embryos [20▪,23,24,36▪▪]. A common conclusion that can be drawn from these studies is the important role that Etv2 plays in inducing the expression of essential endothelial and hematopoietic genes. Some of the direct targets of Etv2, which may mediate its downstream effects, include *Flk1*, *Scl*, *Fli1*, *Gata2* and *Tie2* [18,19,20▪,27,35▪]. Importantly, all of these targets are required for normal hematopoietic or vascular development [1,43]. These microarray analyses also led to the identification of novel vascular and hematopoietic-restricted genes [23,24]. Additionally, *nfatc1*, which encodes a REL domain transcription factor important in endocardial development, is also a transcriptional target of Etv2 in the endocardium of zebrafish [30▪▪], suggesting that a distinct Etv2-dependent transcriptional program may function in the endocardium compared to the vascular endothelium.

Another interesting outcome of recent genome-wide transcriptional analyses performed in *Etv2*-null cells and embryos is the observation that expression of genes associated with other mesodermal lineages, including the cardiac lineage, is increased in the absence of Etv2, further supporting the notion that Etv2 function promotes mesodermal specification to the endothelial/hematopoietic lineage and represses cardiomyocyte specification [30▪▪,36▪▪]. This raises an interesting question as to whether cardiac genes are repressed directly or indirectly by Etv2. Further work needs to be carried out to determine whether Etv2 may act as a transcriptional repressor, potentially through interactions with cofactors.

As an alternative approach for identification of Etv2 targets, our lab identified a novel FOX:ETS motif [27], which is strongly associated with endothelial genes and provides predictive power for endothelial enhancers (Fig. 2). The FOX:ETS motif is bound by Etv2 in combination with Forkhead transcription factors, including FoxC2 [27]. Etv2 and FoxC2 bind to the FOX:ETS motif and cooperatively activate endothelial enhancers containing that motif (Fig. 2, [27]).

Regardless of the experimental or bioinformatic methodology used, the identification of Etv2 targets represents an important starting point for understanding the genetic networks and mechanisms governing endothelial and hematopoietic development.

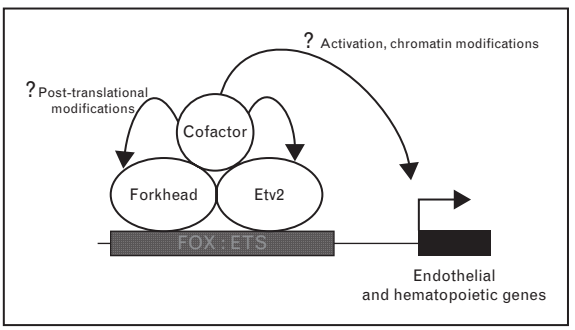

FIGURE 2. A model for the synergistic activation of endothelial and hematopoietic genes by Etv2 and Forkhead transcription factors. Binding of Etv2 and Forkhead factors, such as FoxC2, to the FOX:ETS motif could allow recruitment of cofactors which cause posttranslational modifications to Etv2 and Forkhead factors. Alternatively, cofactors could induce histone modifications and alter DNA methylation to activate downstream gene targets.

REGULATION OF Etv2: SIGNALING PATHWAYS AND TRANSCRIPTIONAL REGULATION

As described in this review, Etv2 plays an essential role in endothelial and hematopoietic development and numerous studies have suggested that it acts at or near the top of the hierarchy in the specification of those lineages [19,20▪,30▪▪,36▪▪]. Furthermore, *Etv2* exhibits very dynamic and transient expression during endothelial development ([18,19,20▪,36▪▪]; K.L. and B.L.B., unpublished observations). Thus, it is important to understand how *Etv2* is activated in the early mesoderm. The rapid extinction of *Etv2* expression and function also suggests a highly regulated transcriptional shut off as well. Transcriptional regulators, post-translational modifications, and epigenetic mechanisms appear likely to play major roles in Etv2 regulation.

To understand how *Etv2* is transcriptionally regulated during development, several groups have identified conserved *Etv2* promoter and enhancer elements that are active at sites of endogenous *Etv2* expression, leading to the identification of *trans*-acting factors that bind to and regulate *Etv2* expression. Recently, Veldman and Lin [38▪] identified a conserved *cis*-acting element in zebrafish *Etv2* (*etsrp*) that directs expression to angioblasts and the developing vasculature. They showed that FoxC1a and FoxC1b bind to this enhancer region and are required for *Etv2* expression in endothelial progenitors [38▪]. Although *Etv2* expression is reduced, some expression is retained, suggesting that other factors are required for the initial induction of *Etv2*. Furthermore, mouse *Foxc1* and *Foxc2* mutants do not exhibit as severe defects as *Etv2* mutants [44], suggesting that critical upstream regulators are still unknown.

A 3.9-kb promoter/enhancer element from the mouse *Etv2* locus has also been identified. This element directs expression to the developing vasculature and endocardium [18]. Nkx2–5, which is a critical regulator of myocardial and endocardial development [39,40], acts upstream of *Etv2* via the 3.9-kb promoter/enhancer in the endocardium [18]. However, it is unclear whether Nkx2–5 regulates the expression of *Etv2* within endothelial progenitors in the early mesoderm. Notably, the expression of *Etv2* precedes expression of Nkx2–5 in the early mesoderm ([36▪▪]; K.L. and B.L.B., unpublished observations), suggesting that factors other than Nkx2–5 are likely to be responsible for the initial activation of *Etv2* in mesodermal progenitors. Furthermore, loss of Nkx2–5 in mice or overexpression in embryonic stem cells does not affect endothelial differentiation [42]. On the other hand, ectopic overexpression of Nkx2–5 causes down-regulation of *Etv2* and other hemangioblast markers in zebrafish and affects hematopoietic development in embryonic stem cells [41,42].

In addition to transcriptional regulators, some of the signaling pathways that induce *Etv2* expression have been identified. BMP, Notch and Wnt signaling are required for induction of *Etv2* expression in BL-CFCs and the subsequent formation of $Flk1^+$ mesoderm and endothelial and hematopoietic cell differentiation [19]. Importantly, other studies have demonstrated that Etv2 is not essential for the generation of all $Flk1^+$ mesoderm. Rather, Etv2 is essential for the generation of $Flk1^+$, $Pdgfr\alpha^-$ vascular mesoderm from primitive $Flk1^+$, $Pdgfr\alpha^+$ mesoderm, and VEGF signaling was shown to be a robust inducer of *Etv2* expression within primitive mesoderm [20▪]. The SHH–VEGF–Notch signaling axis has also been reported to be important in the proliferation of $Fli1^+$, $Etv2(etsrp)^+$ hematopoietic/endothelial progenitors in zebrafish [37]. However, it is unclear whether this pathway is required for specification or only for proliferation of these progenitors.

Regulation of Etv2 function by cofactors

Transcription factors often interact with other proteins to increase target specificity or modulate function. As previously mentioned, Etv2 interacts with members of the Forkhead family, including FoxC2, to synergistically activate endothelial-specific gene expression [27]. Etv2 and FoxC2 act combinatorially to induce ectopic endothelial gene expression in *Xenopus* embryos, but the mechanism by which these two factors promote synergistic activation is still unknown. It is possible that Etv2–FoxC2 interaction facilitates recruitment of additional proteins that provide strong activation or induce post-translational modifications. Interestingly, Etv2 also interacts with the histone demethylase Jmjd1a to repress transcriptional activation of the Etv2 target gene, *Mmp1* [45]. Interestingly, Jmjd1a alters the self-renewal of embryonic stem cells through demethylation and positive regulation of pluripotency-associated genes [46]. Thus, Jmjd1a represents a good candidate for the modification of Etv2 function during vasculogenesis, possibly by affecting the balance between progenitor maintenance and differentiation, but its effect on Etv2 targets during endothelial and hematopoietic development are unknown.

Chromatin modifications play an integral role in the regulation of embryonic stem cell differentiation to multiple lineages. Further studies should determine how histones at the *Etv2* locus are modified during its induction and repression. Other ETS

transcription factors are subject to post-translational modifications, which alter their function. Phosphorylation, acetylation, sumoylation, and ubiquitination have all been shown to affect the transcriptional activity of numerous ETS factors [10]. A detailed characterization of the post-translational modifications of Etv2 and how those modifications affect transcriptional activity is needed to properly understand how Etv2 functions in endothelial specification.

CONCLUSION

Although much remains to be learned about the regulation and function of Etv2 in endothelial and hematopoietic development, studies over the past few years have clearly established its role as an essential regulator of specification of these lineages from the early mesoderm. Future studies will define the upstream transcriptional activators of Etv2 in the early mesoderm and will identify cofactors that modulate Etv2 activity, and it will be interesting to determine if Etv2 has roles in other mesodermal lineages or if it is reutilized in response to physiological or pathological stimuli in the postnatal vasculature.

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Conflicts of interest

There are no conflicts of interest.

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- ■■ of outstanding interest

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