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ETS Transcription Factors in Embryonic Vascular Development

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Abstract

At least thirteen ETS-domain transcription factors are expressed during embryonic hematopoietic or vascular development and potentially function in the formation and maintenance of the embryonic vasculature or blood lineages. This review summarizes our current understanding of the specific roles played by ETS factors in vasculogenesis and angiogenesis and the implications of functional redundancies between them.

Keywords

ETS transcription factor; vasculogenesis; angiogenesis

Introduction

The E26 transformation-specific (ETS) family of transcription factors are named for the avian erythroblastosis virus E26 transforming gene which was the first of the group identified [1]. There are 11 subfamilies of ETS factors classified based on putative structural domain content (Fig. 1). ETS factors are conserved in multiple species including worms, flies, zebrafish, mice and humans. They are involved in cell proliferation, migration, and differentiation during embryonic development and can act as transcriptional activators or repressors. All ETS-family members share an 85 amino acid conserved “ETS domain” which is a winged helix-turn-helix motif, often located in the C-terminal half of the protein. The ETS domain binds to a low-complexity target GGA(A/T) motif in conjunction with one or more unrelated transcription factors such as AP1, MafB and CBP [2, 3]. Putative ETS factor binding sites (EBS) and FOX:ETS binding sites are present in many of vascular endothelial-specific gene promoters and enhancers [4], although ETS-binding sites are not restricted to endothelial-expressed genes [4, 5]. The widespread occurrence of low-complexity EBS suggests the possibility of functional redundancy among the ETS factors. In fact, dissecting the individual roles served by these ETS factors has proven difficult due to

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

large overlap in their expression patterns and the lack of observable phenotypes associated with single ETS gene knockdown. For example, mouse *Ets1* or *Ets2* mutants do not have vascular defects and only double mutant embryos display angiogenic defects [6]. However, the extent to which functional redundancy plays a role in ETS-mediated vascular development remains incompletely understood.

At least thirteen ETS genes are expressed in the hematopoietic or endothelial cells during early vertebrate embryogenesis and are thus potentially involved in hematovascular development: *Ets1*, *Ets2*, *Etv2*, *Etv6*, *Fli1*, *Erg*, *Fev*, *Gabpa*, *Elf1*, *Elf2*, *Spi1*, *Spib* and *Elk4* [7, 8]. *Etv2*/ER71 sits atop this hierarchy during embryonic development, directly inducing *Fli1* in mouse and zebrafish embryos [9–11] and potentially many of the other vascular ETS genes.

In the following discussion, we summarize the current understanding of ETS factors in embryonic vascular development, discuss the potential redundancy between these factors, and speculate on the potential consequences of this redundancy. Several factors such as *Fev*, *Gabpa*, *Spi1*, *Spib* are only expressed in the hematopoietic and not vascular endothelial cells, while *Elk4* has no vascular function reported yet, therefore they are not discussed in this review.

ETS factors in vascular development

Etv2 (ETS variant 2)

The ETS transcription factor *Etsrp/Etv2/ER71* was initially identified as a gene expressed in mouse testes [12]. Later, its vascular endothelial expression was reported during microarray analysis of genes downregulated in the zebrafish *cloche* mutant [13]. The zebrafish *etv2* gene encodes a single protein isoform which has 26% identity (37% similarity) to the human ETV2 protein and 26% identity (36% similarity) to the mouse *Etv2* protein. The zebrafish *Etv2* protein shares 71% amino acid homology within the ETS domain region and only limited homology throughout the rest of the sequence.

In mouse and zebrafish embryos, *Etv2* expression labels early hematopoietic and vascular endothelial progenitors. In mouse embryos, the earliest *Etv2* expression is observed in the yolk sac blood islands while its later expression is localized to vascular endothelial progenitors of the intraembryonic vasculature [14–17]. In zebrafish, *etv2* expression is observed as early as the 1-somite stage in the anterior and posterior lateral plate mesoderm, and it appears earlier than any other reported vascular endothelial marker [7, 18]. In addition to vascular endothelial cells, *etv2*-expressing cells in zebrafish give rise to the primitive myeloid lineages, largely macrophages and also some neutrophilic granulocytes [19, 20]. Interestingly, *etv2* is not expressed in the primitive erythroid progenitors in zebrafish or frog *Xenopus laevis* embryos [21]. While zebrafish *etv2* is highly expressed during early vasculogenesis stages, its expression is downregulated shortly after the initiation of circulation [22]. Interestingly, *Etv2* expression is induced following injury in the mouse ischemic injury model and is required for vascular regeneration [23].

Loss of Etv2 function results in the most dramatic vascular phenotype as compared to other ETS transcription factors [18, 24]. Loss of murine Etv2 is embryonically lethal due to the inhibition of hematopoietic and endothelial development [16, 17], although Etv2 appears to bias the developmental program to favor the endothelial lineage as evidenced by induction of an endothelial phenotype in adult mouse hematopoietic cells constitutively expressing Etv2 [14]. Similarly, Etv2 null mutant or morpholino knockdown zebrafish embryos display nearly complete loss of vasculogenesis. Expression of multiple vascular endothelial genes including VEGF receptors *flk1/vegfr2* and *flt4/vegfr3* is downregulated in Etv2 loss of function embryos, and endothelial cells undergo apoptosis (Fig. 2) [18, 24]. It is thought that Etv2 directly regulates expression of multiple vascular endothelial genes by binding to FOX:ETS consensus sequence together with FOXC2 family of transcription factors [4]. Many direct Etv2 transcriptional targets have been identified in vitro and include genes expressed in hemangioblast and vascular endothelial cells [11]. The mouse Etv2 ortholog has been shown to interact with FoxC2 and Sox17 to induce expression of endothelin-converting enzyme-1 (Ece-1), a component of the endothelin signaling pathway which is an important regulator of vascular tone during embryonic development [25]. Interestingly, some Etv2+ progenitors undergo a fate switch and differentiate as cardiomyocytes when Etv2 function is inhibited [26, 27]. This illustrates that cells within the lateral plate mesoderm (LPM) have multilineage potential during early developmental stages. In addition to vascular defects, primitive myelopoiesis, in particular macrophage lineage, which originates from Etv2-positive progenitors, is absent in Etv2-deficient zebrafish embryos [20]. In contrast, primitive erythroid lineage is not significantly affected in Etv2 knockdown zebrafish or *Xenopus* embryos while it is largely absent in mouse embryos [16, 18, 19, 21]. It is apparent that zebrafish *etv2* is not expressed in erythroid progenitors, therefore it is possible that a different ETS factor may be involved in zebrafish erythropoiesis while in mouse both vasculogenesis and hematopoiesis are controlled by a single Etv2 factor. Hematopoietic stem cell-specific Etv2 knockout mouse shows defects primarily in myelopoiesis, somewhat similar to the myelopoiesis defects observed in zebrafish embryos [28]. Despite dramatic defects in early vasculogenesis, vascular development partially recovers even in *etv2* null mutant zebrafish embryos at later stages, and only aberrant angiogenesis is observed during later stages. This later recovery is mediated by related ETS transcription factors such as Fli1b which functions redundantly with Etv2 during later stages of vasculogenesis and during angiogenesis [10]. Overexpression of Etv2 results in dramatic expansion of hemangioblast and vascular endothelial marker expression in either zebrafish or *Xenopus* embryos, demonstrating that a single transcription factor is sufficient to induce vascular endothelial fate in different cell types [18, 21]. Intriguingly, Etv2 overexpression in somitic progenitors is sufficient to reprogram them into vascular endothelial progenitors during early muscle development, although it is unable to do that during later stages [29].

Experiments *in vitro* using ES cells indicate that *Etv2* expression is regulated by BMP, Notch and Wnt signaling pathways [16, 30]. *etv2* expression is lost in zebrafish *cloche* mutants, the molecular identity of which is still debatable [13]. Several other transcription factors including Nkx2.5, FoxC1, Gata5/6, CREB and the Vegf signaling pathway have been implicated in regulating Etv2 expression [17, 31–34], although in many cases their role in

regulating *Etv2* expression *in vivo* is still unclear. For example, *etv2* expression is still present in either *Foxc1* or *Vegfa* knockdown zebrafish embryos [18, 31].

While *etv2* is highly expressed during embryonic vasculogenesis, its expression greatly diminishes during angiogenesis stages in part due to *let-7* microRNA binding to the *etv2* 3' UTR in zebrafish embryos [22]. Mouse *Flk1:Cre* induced conditional *Etv2* knockdown embryos do not have any functional defects [35], therefore it has been argued that *Etv2* does not play any functional role during the later stages of vascular development. In contrast, a separate study demonstrated that *Flk1:Cre; Etv2* conditional mouse knockout embryos displayed disorganized vasculature and reduced hematopoietic progenitors cells and died at 10.5 [11]. Conditional *Etv2* knockdown in zebrafish embryos using photoactivatable caging strand hybridized to a morpholino also demonstrated lack of vascular phenotype when *Etv2* function is inhibited at 18-somite or later stages after the initial vasculature has formed [10, 22, 36]. However, recent studies show that *Etv2* functions redundantly with other ETS transcription factors during later angiogenesis stages [10], which may explain the absence of the phenotype in mouse *Flk1:Cre* conditional *Etv2*-knockout embryos. Inducible *Etv2* inactivation at the 18-somite stage resulted in angiogenic defects in *fli1b*^{-/-} zebrafish mutant embryos, which otherwise were phenotypically normal (Fig. 3). More recently, it was shown that murine *Etv2* expression was readily observed in endothelial cells after ischemic injury and that neovascularization after injury occurred via *Etv2*-dependent induction of *Flk1*, thus identifying an epistatic interaction between *Etv2* and *Flk1* in vascular regeneration [23].

Fli1 (Friend leukemia integration 1)

FLI1 encodes an ETS-family transcription factor proto-oncogene which is expressed in early hematopoietic and vascular endothelial progenitors and later restricted to the vascular endothelium [37, 38]. *FLI1* homologs exist across a wide range of taxa including insects, amphibians, reptiles, birds, and mammals. Translocations of the human *FLI1* are found in patients with Ewing's sarcoma, a highly malignant, metastatic small cell cancer of the bone and soft tissue which affects children and adolescents [39, 40].

Like *Etv2*, *Fli1* function is conserved across many vertebrates. Gain- and loss-of-function experiments in mice indicated that *Etv2* induces *Fli1* expression during early mouse embryogenesis by binding to an ETS-binding site located in the proximal *Fli1* promoter [9, 11]. During mid-gestation when *Etv2* expression is dramatically reduced, *Etv2* binding at this ETS-binding site is replaced by *Fli1* itself, thereby serving to maintain its own expression through autoregulatory feedback [9]. Targeted disruption of *Fli1* in mice leads to aberrant hematopoiesis and brain hemorrhages at around E11 and is embryonically lethal by E12 [41], suggesting that *Fli1* serves a role in maintaining vascular integrity. Endothelial cells in *Fli1*-null mice exhibit excessive cell death indicating that it is essential for endothelial cell viability and homeostasis at and beyond mid-gestation [9]. Similarly, morpholino knockdown of *Fli1* in *Xenopus* embryos led to a greatly reduced number of hemangioblasts through apoptotic cell death and a resulting loss of blood and endothelial lineages [42].

Zebrafish have two vascular-specific FLI1 homologs, *fli1a* and *fli1b*. These genes are located in pairs with other ETS factors, *ets1-fli1a* on chromosome 18 and *etv2-fli1b* on chromosome 16 [18].

Fli1a—The zebrafish *fli1a* gene is expressed in the anterior and posterior lateral plate mesoderm shortly after the onset of *etv2* expression [7, 18, 37]. While hemangioblast formation is dependent on *etv2*, *fli1a* knockdown in zebrafish does not affect the early hemangioblast program (i.e. *flk1*, *scl* and *pu.1*) [7] suggesting that Fli1a is not absolutely required at this early stage. Expression of *fli1a* is reduced in *cloche* mutants which lack hematopoietic and endothelial cells [7, 37] suggesting that *fli1a* is downstream of *cloche*. Further, it has been reported that Fli1a (but not Fli1b) is able to restore angioblasts in *etv2* morpholino (MO) knockdown embryos as evidenced by recovery of *flk1* expression [43], suggesting that *fli1a* is also downstream of *etv2*. However, *runx1* expression was not rescued by Fli1a unless it was co-injected with *scl-α* and *scl-β* mRNAs, suggesting that Fli1a is insufficient to initiate hematopoietic stem cell (HSC) specification, but that it is capable of mediating the endothelial program [43]. However, in two separate studies, Fli1a overexpression failed to induce vascular endothelial markers in zebrafish embryos [10, 42]. Rather, Fli1a likely plays an important later role in the maintenance of the endothelial lineage. *Fli1a* morphants, like *erg* morphants, display hemorrhages in the head around 2.5 days-post-fertilization (dpf) [7] due to an apparent loss of vessel integrity, although this phenotype was not recapitulated in *fli1a* genetic mutant embryos which developed normally into adulthood with no observable vascular defects [10].

Fli1b—Fli1b, a paralog of Fli1a, has predicted orthologs in many bony fishes. The zebrafish Fli1b protein has 54% amino acid identity to Fli1a in zebrafish (<http://www.ensembl.org>) but shares limited homology with other zebrafish ETS factors. *fli1b* is located just upstream of *etv2* on zebrafish chromosome 16, with only 4.2 kb separating the open reading frames, thus opening the possibility that the genes share distal regulatory elements and potentially function in a partially redundant manner as a result of this shared regulation. *Fli1b* is expressed in 4 isoforms with the primary isoform encoding a 458 amino acid long protein with an N-terminal SAM / pointed protein binding domain and a C-terminal ETS DNA-binding domain. Expression of *fli1b* is first detected at 5–6 somites in the anterior and posterior lateral plate mesoderm (somewhat later than *etv2* or *fli1a*) and is subsequently restricted to vascular tissues throughout embryogenesis [7, 24]. *Fli1b* is induced by *etv2* overexpression [44] suggesting that *fli1b* is downstream of *etv2*, although it remains unclear whether *fli1b* is a direct binding target of ETV2. Like *fli1a*, expression of *fli1b* is reduced in *cloche* mutants [7], suggesting that it (like *etv2*) is downstream of *cloche*. We recently showed that ETV2 and Fli1b function in a partially redundant manner to support sprouting angiogenesis in zebrafish embryos (Fig. 3) [10].

A recent study found that maternal zebrafish p21-activated kinase (*pak4*) knockdown resulted in an increase of *fli1b* in the posterior domain and *etv2* in the rostral domain at the 5-somite stage [45]. Pax4 is involved in the regulation of microfilament dynamics and morphants exhibit normal erythropoiesis but have primitive myeloid deficiencies attributed to aberrant migration of progenitor cells (similar to *etv2* morphants). While the connection

between *pax4* and *fli1b* or *etv2* remains unclear, the observation that both ETS factors are affected by *pax4* knockdown suggest that *etv2* and *fli1b* might share upstream regulators.

Erg (ETS-related gene)

ERG function has been implicated in regulating hematopoiesis [46, 47]. ERG is highly expressed in white blood cells and is required for platelet adhesion. Consistent with this function, depletion of human ERG is associated with acute myeloid leukemia and Ewing's sarcoma [48–50]. ERG also appears to have a role, either directly or indirectly, in early vascular development. Very similar to Fli1, Erg is expressed beginning at around tailbud stage and later in the vascular endothelium, endocardium and neural crest-derived mesenchyme cells of the branchial arches [51].

At least five ERG proteins are encoded by the human *ERG* gene as a result of differential splicing and alternate start codon usage [9, 52, 53]. The human ERG proteins are able to form homoiso-dimeric complexes *in vitro*, which may modulate ERG transcriptional activity [54]. Human ERG proteins are able to form heterodimers with the vascular-specific FLI1 and are expressed in a similar spatiotemporal manner, thus raising the possibility that FLI1 may also participate in the regulation of transcriptional activity of ERG.

Conditional ablation of Erg in mice is embryonically lethal by mid-gestation and angiogenesis and vascular integrity are highly compromised suggesting that Erg has a cell autonomous role in endothelial cell development and blood vessel maintenance [55, 56]. Inducible endothelial cell knockout of Erg in mice results in pathological retinal angiogenesis resulting from a disruption of Wnt / β -catenin signaling [56]. Further, loss of Erg resulted in a loss of vascular stability and tumor formation. Conversely, overexpression of Erg reduced vascular permeability to low molecular weight dextrans and increased vascular stability, potentially by promoting pericyte recruitment [56]. In a separate study, conditional TMPRSS2-Erg knock-in mice which displayed robust ERG expression throughout the prostate were combined with homozygous PTEN loss resulting in highly invasive prostate cancer and significantly increased androgen receptor expression. ERG overexpressing mice exhibited a substantial increase in the expression of androgen receptors and an upregulation of genes involved angiogenesis, including CYR61, ANGPT2, PLXND1, SMO, NOS3, and EGFL7 [57].

In *Xenopus*, ERG appears to regulate the vascular-specific FLK1 by forming a regulatory complex with KLF2 [58]. *In vitro* binding assays suggest that ERG binds directly to the VE-cadherin promoter and inhibits its transcription [59], thus suggesting a role for ERG in mediating endothelial cell migration. Overexpression of VE-cadherin rescues apoptosis in ERG-deficient HUVECs [60]. Studies in *Xenopus* also suggest that X1 Erg may be involved in development of the circulatory system, as *XI Erg* mRNA induced ectopic endothelial cell accumulation [51].

Zebrafish have one copy of ERG, which is downstream of *cloche*, *tal1/scl* and *etv2* during early vascular development. Zebrafish ERG morpholino knockdown embryos do not have any vascular defects, although combinatorial knockdown experiments have indicated a possible redundant role with Etv2 and Fli1a factors [61, 62].

Ets1 (E-twenty six gene 1)

In adult humans, ETS1 is highly expressed in immune tissues and is known to mediate differentiation of B and T cells [57]. Ets1 knockout mice are characterized by T-cell apoptosis and increased terminal B-cell differentiation [63], but develop normally despite an increase in perinatal mortality [64]. *Ets1* is transiently expressed in vascular endothelial cells (ECs) during angiogenesis or re-endothelialization after denuding injury [65]. Ets1 appears to elicit its effects through interactions with multiple genes in the VEGF signaling cascade. Ets1 is induced by FGF, EGF and VEGF in ECs, and itself induces VEGF in a positive feedback loop [66, 67]. In fact, bFGF inoculated into murine subcutaneous tissue led to *in vivo* ETS-1 protein expression in neo-vessels invading the matrigel [66]. Antisense oligonucleotides directed against Ets1 inhibit endothelial cell migration and VEGF-induced cell proliferation [68]. VEGF and hypoxia both serve to increase Ets1 expression in bovine retinal endothelial cells, while a dominant negative Ets1 was found to inhibit retinal angiogenesis in a mouse model of proliferative retinopathy [69]. An analysis of Flk1 promoter occupancy in Ets1 transgenic mice provided some mechanistic insight by identifying tandem Hif1-alpha and ETS binding sites which are functionally required for full transcriptional activation of Flk1 and its endothelial-specific expression [70]. VEGFR1 (Flt-1) induction is also associated with ETS1 and HIF-2alpha recruitment to the VEGFR1 locus in differentiating mouse embryonic stem cells [71].

Ets1 also mediates angiogenesis through the regulation of genes involved in apoptosis and cell invasion. Apoptosis of endothelial cells occurs at vessel branch points and in regressing vessels, thus facilitating angiogenic patterning [72]. Overexpression of Ets1 in HUVEC cells induced apoptosis in serum-deprived conditions by increasing the expression of pro-apoptotic genes such as Bid, cytochrome p450, caspase-4, p27 and p21. Exogenously supplied VEGF served to inhibit apoptosis and enhanced Ets-1 binding [73]. Ets1 also modulates endothelial cell invasion by inducing angiogenesis-related genes such as matrix metalloproteinases (MMPs) and integrins [73–76]. Elevated ETS-1 expression correlated with increased levels of MMP-1, MMP-3, and MMP-9 in MSS-31 murine endothelial cells [77]. Interestingly, ETS1 was found to lower capillary endothelial cell density at confluence and induce the expression of VE-cadherin [78], while estrogen-induced Ets1 promotes capillary density in an *in vitro* tumor angiogenesis model [79].

Expression of a dominant negative Ets1 in a mouse ear model of growth factor and tumor angiogenesis led to reduced FGF-2-mediated angiogenesis [80]. Further, adenovirus-mediated delivery of a trans-dominant mutant Ets1 significantly inhibited angiogenesis in matrigel plugs injected under the skin of C57BL mice [76], thus suggesting the therapeutic potential of DN-Ets1 in blocking angiogenesis. However, despite all the data supporting the role for Ets1 in angiogenesis, mouse Ets1 knockout embryos do not have any apparent defects due to its functional redundancy with a related Ets factor Ets2 [6].

Ets2 (E-twenty six gene 2)

A role for Ets2 in mediating cell proliferation and differentiation of myeloid cells is well established, and overexpression of Ets2 in myeloid progenitor cells stimulates the

development of mature macrophages [81]. However, Ets2 also appears to have a secondary role in supporting developmental, tumor and lymphatic angiogenesis.

Ets2 knockout mice die prior to E8.5 due to defects in extraembryonic tissues related to a failure of trophoblast migration [82]. Ets2 is sufficient to promote blood vessel formation as observed in an *in vivo* angiogenesis assay performed in the absence of tumor cells [83], and co-expression of Ets1 and Ets2 induces expression of murine VEGFR-2/*flk1* [84]. In PTEN-deficient stromal fibroblasts, Ets2 is upregulated, phosphorylated and recruited to the promoters of target genes known to be involved in ECM remodeling and angiogenesis [85]. Accordingly, Ets1 and Ets2 double knockout embryos exhibit greatly reduced levels of Mmp9, Bcl-XL, and cIAP2 versus controls [6]. Further, overexpression of miR-320, an upstream negative regulator of Ets2, led to a decrease in Ets2 in mouse cardiac endothelial cells leading to impaired endothelial migration and tube formation [86] suggesting that Ets2 might also be involved in cardiovascular development. Nonetheless, the targeted disruption of either murine Ets1 or Ets2 alone is not associated with vascular defects, and only a combined knockdown of both genes concurrently yielded pronounced defects in vascular branching due to apoptosis of vascular endothelial cells suggesting that the two genes act in a combinatorial fashion to support angiogenesis [6].

Ets2 also appears to have a role in lymphatic vessel development as mouse Ets2 has been shown to interact with the lymphatic specific transcription factor Prox1 to directly induce VEGFR3 expression resulting in enhanced inflammatory lymphangiogenesis and endothelial migration towards VEGF-C [87]. By contrast, Ets1 is not expressed in lymphatic vessels [88].

Elf1 (E74-like factor 1)

The ELF1 ETS factor is primarily expressed in lymphoid tissues and is a known regulator of the IL-2, GM-CSF and CD4 genes [89–91]. While Elf1 is not expressed in the embryonic vascular endothelium or in endothelial progenitor cells, it is however enriched in the extraembryonic blood vessels found within the chicken chorioallantoic membrane [92], and thus may play a role in the formation of the extraembryonic vascular network, although a direct role has not yet been demonstrated.

Elf1 may affect endothelial function through several mechanisms. First, Elf1 is a strong transactivator of the Tie1 and Tie2 receptors through binding to ETS sites in the promoters of these genes [92]. Tie1 and Tie2 are the cognate receptors for the Angiopoietins which serve as growth factors required for the formation of blood vessels. Targeted disruption of the Tie1 gene leads to leaky blood vessels and disruption of Tie2 leads to venous malformations, vessel dilation and abnormal sprouting and branching defects [93]. Second, Elf1 forms a transcriptional complex with Sp1, Sp3 and MAZ to transcriptionally activate the human endothelial nitric oxide synthase (eNOS) [94], and may thus serve as an indirect regulator of vascular tone. In addition, along with Ets1, Elf1 acts as a strong enhancer of endothelial cell Ang-2-promoter activity in mouse fibroblasts [95].

Elf2 (E74-like factor 2, NERF-2)

ELF2 was first isolated from human spleen, fetal liver and brain samples [96]. ELF2 shares 44% amino acid homology with the human ELF1 and is highly expressed in vascular endothelial cells and their progenitors as well as B and T cells. Elf2 deficiency yields a phenotype similar to those observed with Fli1 disruption which causes abnormalities in extraembryonic large vessel defects (esp. vitelline vein) while capillaries appear largely normal [97].

ELF2 enhances transcription in humans, but the chick Elf2 homologue acts as a competitive inhibitor of Elf1, suggesting Elf1 and Elf2 may act as positive and negative regulators of the same gene targets in the chicken [98]. Like Elf1, Elf2 is enriched in the developing blood vessels of the chicken chorioallantoic membrane. Chicken Elf2 was shown to bind and transactivate the Tie1 and Tie2 genes via a cluster of ETS sites in their respective promoters [96]. *In vitro* translated cElf1 and cElf2 can bind equally well to conserved ETS binding sites in the promoters of the Tie1 and Tie2 genes, but cElf1 binds preferentially to these promoters during the early stages of vascular development, suggesting that cElf2 binding may dominate during later stages of vessel development and play a role in vessel remodeling [98].

Etv6 (ETS-variant 6, Tel)

ETV6 (TEL) encodes an evolutionary conserved ETS repressor protein and is expressed throughout embryonic development and in adults [99]. The *ETV6* gene is frequently rearranged in myeloid leukemias [100]. ETV6 regulates endothelial sprouting by binding to the generic co-repressor CtBP.

In mice *Etv6* potentially plays a role in the development of extraembryonic vessels. *Etv6* mutant mice exhibit defective yolk sac angiogenesis and die by E10.5, although vasculogenesis in the yolk sac and embryo appear normal [97]. Extra-embryonic branching vessels are observed at E9.5, but not at E10.5 suggesting that *Etv6* is not required for initiating sprouting angiogenesis, but is required for maintaining the integrity of the forming branched network. Mutant mice have pronounced extraembryonic mesenchymal apoptosis in the larger vessels while the capillaries appear largely normal. It is unclear whether the observed extraembryonic vascular defects are related to a role for *Etv6* in preventing apoptosis [97]. *Etv6* knockdown led to a marked decrease in filopodia formation with a coordinated increase in *dll4* (by both qPCR and *in situ*). Expression of Tie-2, Flk-1, Flt-1 and GAP mRNAs is not significantly affected in mouse *Etv6* mutants suggesting that *Etv6* may be a downstream player in the tyrosinase signaling pathway in yolk vascularization [97]. Conditional knockout studies also revealed that *Etv6* is essential for adult hematopoietic stem cell survival [101].

Morpholino knockdown of *Etv6* in zebrafish resulted in a reduction in intersegmental vessel number, aberrant patterning, and gaps in the dorsal longitudinal anastomotic vessel (DLAV) in a 70–80% of morphants while the primary vasculature remained largely normal, suggesting a role for TEL in angiogenesis [102]. In addition, *Etv6* zebrafish morphants display reduced numbers of erythrocytes and macrophages but increased numbers of

differentiated heterophils and enhanced lymphopoiesis [103]. In *Xenopus* Etv6 has been implicated in hematopoietic stem cell development and arterial specification and regulates VEGFA transcription [104].

Taken together, the data suggest that Etv6 may play a role in priming endothelial cells for sprouting by controlling the threshold of angiogenesis inhibitors that is optimal for sprouting in response to VEGFR signaling [102].

Redundancy between vascular ETS factors

It is apparent that there are multiple levels of redundancy among ETS transcription factors during vascular development and angiogenesis. Only double mouse *Ets1*^{-/-};*Ets2*^{-/-} mutant embryos show defects in angiogenesis and cell apoptosis, while individual mutants do not show vascular phenotypes [6]. The vascular ETS factors *erg* and *fli1* have been reported to cooperatively regulate expression of the endothelial cell junction molecule VE-cadherin to support angiogenesis [7]. Similarly, *erg* and *fli1a* zebrafish morphants give mild phenotypes individually, but double morphants exhibit a more severe phenotype with added intersegmental vessel patterning and DLAV defects and an elevated incidence of hemorrhage as well as arterial specification defects [7, 62]. Zebrafish *fli1b* mutants or Etv2 conditional knockdown embryos do not show any defects in angiogenesis, while only double *etv2*; *fli1b* loss of function embryos show profound defects in angiogenesis [10]. A greater level of redundancy has been argued for zebrafish *fli1a*, *fli1b*, *ets1* and *etv2* factors based on combinatorial morpholino knockdown experiments [24]. However, genetic *fli1a* and *fli1b* mutants do not show angiogenesis defects previously reported in the individual *fli1a* or *fli1b* MO knockdown embryos, potentially due to off-target effects caused by MOs [10], therefore further analysis of genetic mutant phenotypes is needed to fully determine the extent of functional redundancy. It is likely that many ETS binding sites can be shared different ETS transcription factors [9–11], however further research is needed to fully understand functional overlap between multiple ETS factors.

Given the potential for widespread functional redundancy between vascular ETS factors, it is intriguing to consider the possibility that the failure of anti-angiogenic therapies and the associated tumor escape is due, in part, to a functional compensation by ETS factors which are expressed in VEGF-independent manner. An improved understanding of the extent to which ETS redundancy mutes the effectiveness of single-target therapeutic strategies will aid in the development of the multi-target strategies likely required to minimize therapeutic breakthrough.

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Fig. 1. Homology tree of all human ETS transcription factors. ETS factors with known hematopoietic or vascular-specific expression pattern are shown in red. ClustalW algorithm and MacVector 13.0 software was used for the protein sequence alignment.

Fig. 2.

Etv2 morpholino knockdown, similar to *etv2* mutants, results in the nearly complete absence of zebrafish vascular endothelial specific transgenic reporter *flk1:GFP* (A,B) and strong downregulation of vascular endothelial *fli1a* (C,D) expression at 24–26 hpf [18].

Fig. 3.

Etv2 functions redundantly with Fli1b during angiogenesis and late vasculogenesis. (A,B) As observed in the trunk region of zebrafish embryos at 48 hpf, vasculogenesis and angiogenesis partially recovers in *etv2* morpholino knockdown embryos (A), while nearly complete absence of vascular development is observed in the *etv2*; *fli1b* double deficient embryos (B). (C,D) Zebrafish *fli1b*^{-/-} single mutant embryos are normal and do not show any vascular defects at 42 hpf, while inducible *etv2* MO knockdown by photoactivatable morpholino, which is uncaged at the 18-somite stage in *fli1b*^{-/-} background results in a specific inhibition of angiogenesis of intersegmental vessels (arrows) without affecting embryonic vasculogenesis. This argues for a redundant function between Etv2 and Fli1b during embryonic angiogenesis which is separate from the earlier Etv2 role during vasculogenesis [10].