Fli1 Acts Downstream of Etv2 to Govern Cell Survival and Vascular Homeostasis via Positive Autoregulation

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Rationale: Cardiovascular health depends on proper development and integrity of blood vessels. Ets variant 2 (Etv2), a member of the E26 transforming–specific family of transcription factors, is essential to initiate a transcriptional program leading to vascular morphogenesis in early mouse embryos. However, endothelial expression of the Etv2 gene ceases at midgestation; therefore, vascular development past this stage must continue independent of Etv2. Objective: To identify molecular mechanisms underlying transcriptional regulation of vascular morphogenesis and homeostasis in the absence of Etv2.

Methods and Results: Using loss- and gain-of-function strategies and a series of molecular techniques, we identify Friend leukemia integration 1 (Fli1), another E26 transforming–specific family transcription factor, as a downstream target of Etv2. We demonstrate that Etv2 binds to conserved Ets-binding sites within the promoter region of the Fli1 gene and governs Fli1 expression. Importantly, in the absence of Etv2 at midgestation, binding of Etv2 at Ets-binding sites in the Fli1 promoter is replaced by Fli1 protein itself, sustaining expression of Fli1 as well as selective Etv2-regulated endothelial genes to promote endothelial cell survival and vascular integrity. Consistent with this, we report that Fli1 binds to the conserved Ets-binding sites within promoter and enhancer regions of other Etv2-regulated endothelial genes, including Tie2, to control their expression at and beyond midgestation.

Conclusions: We have identified a novel positive feed-forward regulatory loop in which Etv2 activates expression of genes involved in vasculogenesis, including Fli1. Once the program is activated in early embryos, Fli1 then takes over to sustain the process in the absence of Etv2. (Circ Res. 2014;114:1690-1699.)

Key Words: apoptosis ▪ developmental biology ▪ embryonic development ▪ gene expression ▪ homeostasis ▪ transcription factors

Endothelial dysfunction is a cardinal feature of several human cardiovascular diseases.1 During embryogenesis, growth and survival of the vertebrate embryo rely critically on proper morphogenesis and homeostasis of the organs, tissues, and cell types that comprise the circulatory and cardiovascular systems.2 Endothelial and blood progenitor cells of the circulatory system derive from a common ancestor known as the hemangioblast.3,4 Progenitor cell survival, specification, and differentiation require precisely orchestrated-interacting action of numerous transcription factors. However, details of the transcriptional network that governs endothelial cell (EC) function and homeostasis during embryogenesis are still being defined. Elegant work using gene disruption and mutation strategies has demonstrated that diverse transcription factors are involved in vascular development.5 Among them, the importance of the E26 transforming–specific (ETS) family of transcription factors has been highlighted.3

The ETS family of transcription factors is conserved in metazoans and first identified in avian erythroblastosis virus, E26.6,7 They are highly conserved in their DNA-binding ETS domain and govern a plethora of biological processes, including, but not limited to, development, cellular growth, differentiation, cell–cell contact, and viability.3,4 To date, 29 different Ets genes have been identified in mammals. Each binds to a core GGAA/T DNA-binding element,8 suggesting that aspects of their functions overlap. Global inactivation or mutation of numerous Ets genes either in mouse or in zebrafish indicates that despite high conservation of the DNA-binding motif and recognition of a similar cis-regulatory element, certain ETS factors play an essential role in vascular morphogenesis and homeostasis, which cannot be compensated by other family members.5

Recently, we and others have reported that the ETS family transcription factor Etv2 (also called Etsrp71/ER71) is...
essential for the genesis of hemato-endothelial progenitor cells in mice.9–11 Mice lacking Etv2 die in utero around embryonic day 9.5 with complete loss of vasculature.9,11 Similarly, knockdown of the Etv2 ortholog Ets-related protein in zebrafish provokes a profound impairment of vasculogenesis.12 Furthermore, recent studies demonstrate that loss of the Etv2/Ets-related protein in mice and zebrafish redirects endothelial progenitor cells to a myogenic fate,13,14 highlighting a central role for this transcription factor in endothelial specification and vascular development in the early embryo. Intriguingly, endothelial expression of Etv2 in wild-type (WT) mice is undetectable at midgestation,9–11 thus raising 2 major questions: (1) how are vascular morphogenesis and integrity maintained throughout the remainder of gestation? and (2) what controls the continued expression of known Etv2 target genes, such as Tie2 (also known as Tek),9 in the absence of Etv2?

Genome-wide sequence analyses identified Ets-binding sites (EBSs) in the promoter and enhancer regions of many endothelial-expressed genes, including ones encoding other ETS proteins.5,15 Therefore, we postulated that once expression of an Ets gene is activated by Etv2 early in the development, an Etv2-targeted Ets gene may function to maintain its own expression as well as that of additional endothelial genes involved in EC survival, vascular morphogenesis, and homeostasis. Recently published studies demonstrate that compared with WT embryos and embryonic stem cells (ESCs), transcript levels of several transcription factors involved in hematopoietic and vascular development during early embryogenesis are attenuated in Etv2 knockout embryos and ESCs.11,16,17 However, the identity of a specific Etv2 target and its role in vascular morphogenesis in the absence of Etv2 remain elusive.

The primary purpose of this study was to identify a specific Etv2 target, which can activate a positive autoregulatory feedback mechanism that persists beyond midgestation (and in the absence of Etv2) to govern critical elements of embryonic vasculogenesis. We demonstrate that Friend leukemia integration 1 (Fli1) has such properties, being dependent on Etv2 to initiate embryonic expression early in the development and then acting to regulate its own expression, as well as that of additional Etv2 target endothelial genes involved in EC survival, vascular morphogenesis, and homeostasis, at and beyond midgestation.

### Methods

**Lentivirus Production, Purification, and Infection**

Hemagglutinin-tagged mouse Fli1 cDNA was subcloned into a lentiviral expression vector (Clontech) and cotransfected with pCD/NL-BH and pMD2-VSVG constructs into human embryonic kidney (HEK) 293T cells according to the manufacturer’s instructions (Clontech). Lentivirus was harvested from the culture supernatant and concentrated using ultracentrifugation for 2 hours at 22,000 rpm (41,000g) using SW28 rotor. Expanded protocols for viral infection, gene expression, and histological and immunohistochemical analyses using isolated mouse embryos are provided in the Online Data Supplement.

**Results**

**Excessive EC Death Leads to Hemorrhage in Fli1-Null Embryos at Midgestation**

We and others have reported that mice lacking Fli1 die between embryonic days 12.0 and 12.5 because of widespread hemorrhage at midgestation.18,19 However, the underlying cause of this perturbation is unknown. To gain insights into mechanisms underlying vascular leakage in Fli1-null embryos, we mated Fli1 heterozygous mice19 and isolated WT and Fli1-null embryos from timed pregnant females at distinct developmental stages. Consistent with previously reported studies,18,19 Fli1-null embryos isolated at embryonic day 10.5 (Figure 1A) and embryonic day 11.5 (Figure 1B) manifested hemorrhage within the embryo proper as well as in the extra-embryonic (eg, yolk sac) vasculature (data not shown) culminating ultimately in lethality by embryonic day 12.0 (Online Figure IA). This was further supported by the absence of erythrocytes within the vasculature (eg, the dorsal aorta and cardinal vein) and hemorrhage in the canal of the neural tube of Fli1-null, but not WT, embryos (Figure 1A). In addition, Fli1-null embryos also revealed diminished endocardial cushion formation (Online Figure IB), supporting a role of Fli1 in endocardial endothelium.

In contrast, Fli1-null embryos isolated at embryonic day 8.5 (data not shown) and embryonic day 9.5 (Online Figure IIA) are indistinguishable from WT littermates in terms of overall morphology, growth, and cardiovascular structures. The presence of erythrocytes within the dorsal aorta of Fli1-null embryos and immunohistochemical analyses for the endothelial/endocardial marker α-endomucin9 supported the notion that embryonic vascular structures were normal in early (between embryonic days 8.5 and 9.5) WT and Fli1-null littermates (Online Figure IIA and IIB). This conclusion was further supported by similar transcript levels of 2 endothelial genes in both genotypes at embryonic day 9.5 (Online Figure IIC). Collectively, these data support an essential role for Fli1 in vascular morphogenesis and homeostasis at midgestation.

Next, we analyzed whether vascular leakage in Fli1-null mice is associated with perturbation of endothelial proliferation and viability. Immunohistochemical analyses for Ki67 and isolated WT and Fli1-null littermates at embryonic day 10.5 (Online Figure IB), suggesting that disruption of vascular integrity was not because of lack of endothelial proliferation. Using terminal deoxynucleotidyl transferase dUTP nick-end labeling and immunohistochemical analyses for activated caspase-3 and endomucin, we tested for evidence of apoptotic cell death in WT and Fli1-null embryos. Compared with WT embryos, Fli1-null embryos isolated at midgestation manifested excessive EC death (Figure 1A and 1B). It is worth noting that elevated levels of cell death within the blood and cells of the neuroplil were also evident in Fli1-null embryos (Figure 1; Online
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Figure IB). Taken together, we conclude that Fli1 is essential for EC viability and homeostasis at and beyond midgestation.

**Etv2 Is an Essential Upstream Regulator of the Fli1 Gene**

Having identified an essential role for Fli1 in ECs at and beyond midgestation, we sought to determine molecular mechanisms governing embryonic expression of the Fli1 gene. Embryonic expression of Fli1 occurring after Etv2 expression coupled with significant attenuation of Fli1 transcript levels in Etv2-null ESCs and ECs together imply that Etv2 governs Fli1 expression in the early embryo. To test this concept, we purified RNA from embryonic day 8.0 WT and Etv2-null littermates and analyzed transcript levels of numerous Ets genes. Quantitative reverse transcriptase polymerase chain reaction (PCR) analyses showed that compared with WT, transcript levels of Fli1 and a closely related Fli1 family member Erg were significantly attenuated in Etv2-null embryos (Figure 2A). In contrast, the transcript levels of several other Ets genes, including Ets1, Ets2, Elf1, Elf2, and Etv6, were unaffected (Figure 2A). Transcript levels of the Tie2 gene, an established downstream target of Etv2 in early embryo, were also significantly decreased in mutant embryos (Figure 2A), which suggests that attenuation of Fli1 expression in the Etv2-null embryos was specific to the absence of Etv2. Based on these data, we hypothesized that Fli1 is a downstream target of Etv2 in the early mouse embryo.

It has been demonstrated that synthesis of 2 major isoforms of the Fli1 protein initiates from 2 distinct translation initiation sites (ATG) present within exons 1 and 2, respectively (Figure 2B). To confirm that Etv2-dependent Fli1 gene transcription initiates from exon 1, we used semiquantitative reverse transcriptase PCR techniques to analyze Fli1 transcripts using primers spanning the exon 1 untranslated region and exon 2 (Figure 2B). We noted that Fli1 transcripts detected in WT embryos were significantly attenuated in Etv2-null embryos (Figure 2B). These data suggest that marked reduction of Fli1 transcripts in the Etv2-null embryos was associated with Etv2-dependent regulation of Fli1 gene transcription from exon 1.

We, and others, have reported that endothelial expression of the Etv2 gene stops at midgestation (embryonic days 10.5–11.5). This observation raised the possibility that expression of the Fli1 gene in whole embryo and in ECs will also be undetectable or decline at midgestation. Using semiquantitative and quantitative reverse transcriptase PCR, we analyzed Etv2 and Fli1 gene expression in whole embryo and in green fluorescence protein–positive ECs isolated from Tie2-green fluorescence protein mice at distinct developmental stages. Consistent with our previously published study, the Etv2
gene was expressed in the early embryos (embryonic days 8.5–9.5; Online Figure IIIA) and ECs (Figure 2C), but its expression was undetectable at midgestation. In contrast, Fli1 transcripts were detected at all developmental stages and increased with age in whole embryos (Online Figure IIIA) and ECs (Figure 2C). Taken together, we conclude that Etv2 is required for endothelial Fli1 expression in the early embryo, whereas Fli1 expression at and beyond midgestation becomes Etv2 independent.

To complement our in vivo Etv2 loss-of-function data, we undertook in vitro approaches to validate Fli1 as a downstream target gene of Etv2. We fused a 0.7-kb Fli1 promoter region upstream of exon 1 harboring numerous conserved Ets-binding sites (EBSs) (Online Figure IIB) to a luciferase reporter cassette and conducted transcriptional analyses. Cotransfection of the reporter plasmid with increasing amounts of hemagglutinin-tagged Etv2 expression plasmid in primary human aortic ECs resulted in significant induction of luciferase activity (*P < 0.008 vs control). E, Transcriptional assays in COS1 cells elicited dose-dependent activation of luciferase activity by WT, but not mutant (lack of DNA-binding domain, ΔE26 transforming specific [ETS]) Etv2. F and G, Fold activation of luciferase activity in the absence (−) and presence (+) of Etv2 (250 ng) from full-length (FL) and indicated deletion mutant (Dm) reporter plasmids are shown in F. Note the specific and significant induction of Etv2 occupancy of the Fli1 promoter (ΔP < 0.001 vs control), HA indicates hemagglutinin.
COS1 cells with a mutant Etv2 that lacks the DNA-binding ETS domain failed to activate luciferase activity (Figure 2E), suggesting that DNA binding by Etv2 is essential for the transcriptional activation of the Fli1 gene.

To test for the presence of EBSs in the Fli1 promoter critical for Etv2 activity, we generated a series of deletion constructs of the 0.7-kb promoter fragment (Online Figure IVA). In transcriptional assays using each reporter plasmid in the presence or absence of Etv2, we identified 3 conserved EBSs, residing between 200- and 250-bp upstream of the ATG, that were essential and sufficient for Etv2-dependent activation of luciferase activity (Figure 2F; Online Figure IVA). Mutation of these EBSs disrupted Etv2-dependent activation of luciferase activity (Figure 2G; Online Figure IIIB). Using quantitative analyses of chromatin immunoprecipitation (quantitative PCR) assays in C2C12 cells expressing hemagglutinin-tagged Etv2, we confirmed specific occupancy of Etv2 at this Fli1 promoter region, and that Etv2 occupancy was not detected of an intronic region of the Fli1 gene, which does not harbor a conserved EBS (Figure 2H; Online Figure IVB). These data suggest that the binding of Etv2 to these conserved EBSs is essential for the activation of Fli1 expression. Indeed, ectopic Etv2 expression in C2C12 cells induced endogenous Fli1 transcript levels in a dose-dependent manner (Online Figure IVC). Collectively, these data suggest that Etv2 is an important upstream regulator of the Fli1 gene and that ectopic Etv2 expression will activate Fli1 expression in a cell autonomous manner.

**Fli1 Promotes Its Own Expression in the Absence of Etv2**

Having established Fli1 as an essential downstream target of Etv2 in early embryos, we set out to unveil molecular mechanisms underlying Etv2-independent transcriptional regulation of Fli1 expression at and beyond midgestation. Normal transcript levels of selected Ets genes in Etv2-null embryos (Figure 2A) along with the absence of vascular abnormalities at midgestation in mice lacking these factors suggest that they are not required for Fli1 expression at midgestation. Moreover, significant attenuation of transcript levels of Etv2 target genes, Erg and Gata2, in Fli1-null embryos at embryonic day 10.5 (Online Figure VA) suggests that they are also not involved in Fli1 expression at midgestation. Based on these data, we hypothesized that Fli1 regulates its own promoter activity in the absence of Etv2.

To test this hypothesis, we analyzed the N-terminal transcript levels of Fli1 in Fli1-null embryos, which express little Fli1 protein that lacks the C-terminal activation domain encoded by the exon 9.19 We detected Fli1 transcripts spanning the exon 1 untranslated region and exon 2 in embryonic day 9.5, but not in embryonic days 10.5 and 11.5. Fli1-null embryos (Figure 3A), whereas Fli1 transcripts at these developmental stages were readily detected in WT embryos (Online Figure IIIA). These data suggest that Fli1 is dispensable for Fli1 expression in early embryos but required for sustained expression at midgestation. To complement this observation, we performed chromatin immunoprecipitation assays using WT embryos isolated at distinct developmental stages and confirmed increased Fli1 occupancy of its own promoter in embryos isolated at embryonic day 11.5 (Figure 3B and 3C). Surprisingly, such occupancy was not detected in embryos isolated at embryonic day 9.5 or in the Fli1 intronic region in embryos isolated at embryonic day 11.5 (Figure 3B and 3C). Collectively, these data lend strong support to our hypothesis that Fli1 occupancy of its own promoter region was associated with sustained endothelial Fli1 expression at midgestation in the absence of Etv2 (Figure 2C).

To complement these in vivo data, we performed transcriptional assays using an Fli1 reporter construct. In COS1 cells, we detected robust and dose-dependent activation of luciferase activity by Fli1 from an Fli1 reporter plasmid harboring WT (Figure 3D), but not mutated (Figure 3E), EBSs. Using chromatin immunoprecipitation quantitative PCR, Western blot, and reverse transcriptase PCR analyses using primers designed to amplify only endogenous Fli1 transcripts, we
further demonstrated that Fli1 occupancy of its own promoter region in C2C12 cells can induce endogenous Fli1, but not En2, transcript levels (Online Figure VB–VD). These data further support our hypothesis that Fli1 acts downstream of Etv2 and can activate its own promoter activity. Interestingly, the activation of Fli1 reporter activity by Etv2 or Fli1 was indistinguishable from that of other ETS factors, including Elf1 and Elf2 (Figure 3E). However, we found that unlike Etv2 and Fli1, ectopic expression of Elf1 and Elf2 in C2C12 cells did not induce endogenous Fli1 expression and that expression of Ets1 showed only a modest effect (Figure 3F). Together, these data suggest that the failure of Elf1/Elf2 to induce endogenous Fli1 gene expression was secondary to an inability to access the EBS sites in native chromatin but not because of an inability to recognize the EBSs in the Fli1 reporter plasmid. Based on our in vitro and in vivo data, we conclude that in the absence of Etv2 at midgestation, Fli1 binds to conserved EBSs within the Fli1 promoter to induce its own expression via positive autoregulation.

**Fli1 Is Required for Endothelial Fli1 Expression for the Remainder of Gestation**

To determine whether feed-forward autoregulation of Fli1 is essential for sustained Fli1 expression during the remainder of gestation, we undertook Fli1 loss- and gain-of-function strategies in adult primary HAECs. Consistent with our recent findings of Fli1 expression in adult mouse ECs, we detected marked reduction of Fli1 transcript (Online Figure VE) and protein (Figure 4A) levels in HAECs using 2 independent Fli1-specific small interfering RNAs. We also noted that compared with COS1 cells, baseline Fli1 reporter activity was significantly higher in HAECs (data not shown), suggesting that endogenous Fli1 might activate its own reporter activity. To test this concept, we analyzed Fli1 reporter activity in HAECs and found that knockdown of Fli1 significantly attenuated Fli1 reporter activity (Figure 4B). Using chromatin immunoprecipitation quantitative PCR, we detected Fli1, but not Erg, occupancy of the Fli1 promoter region in HAECs (Figure 4C). Taken together, we conclude that Fli1 is required to maintain Fli1 expression in adult ECs.

To further test our model, we undertook Fli1 gain-of-function approaches finding that cotransfection of an Fli1 reporter construct and hemagglutinin-tagged Fli1 expression plasmids in HAECs resulted in significant and dose-dependent induction of luciferase activity (Figure 4D). Furthermore, lentivirus-mediated overexpression of mouse Fli1 in HAECs resulted in marked induction of endogenous Fli1 transcript levels (Figure 4E). Collectively, these data lend additional support to our hypothesis that Fli1 is essential for sustained Fli1 expression at and beyond midgestation.

**Fli1 Is an Upstream Regulator of Selected Etv2-Regulated Endothelial Genes in Developing and Adult Endothelium**

Finally, we set out to investigate whether Fli1 is essential for sustained expression of selected Etv2 target endothelial genes involved in EC viability and vascular homeostasis. For example, mice lacking Tie2, an endothelial receptor tyrosine kinase, and vascular endothelial cadherin manifest excessive EC death, abnormal vascular remodeling, hemorrhage, and embryonic lethality. Consistent with previously reported studies, transcript levels of Tie2, Cdh5, and Cdh3 were specific to the loss of Fli1. Immunohistochemical analyses for Tie2 and Fli1 also supported the notion that significant attenuation of Tie2 protein levels in vascular ECs of Fli1-null littermates was associated with EC-specific loss of Fli1 (Online Figure VIA). Given that Tie2 transcripts in Fli1-null embryos declined to a similar extent in Etv2-null embryos (Figure 2A), we reasoned that the regulation of endothelial genes abundance in midgestational endothelium occurred at the level of gene transcription.
It has been reported that an upstream promoter fragment (UPF) and a distal first intronic enhancer fragment are essential for sustained endothelial Tie2 expression at mid- and late gestation.\(^{29}\) Moreover, both the UPF and the intronic enhancer fragment have been reported to harbor several conserved EBSs.\(^9\) Therefore, we examined whether immunoprecipitation of Fli1–DNA complexes from embryos (Figure 3B) harvested regulatory elements of Tie2 and a known Fli1 downstream target Cdh5.\(^{25}\) Consistent with our previous report,\(^{23}\) we detected Fli1 occupancy of the Cdh5 promoter in vivo (Figure 5B). In fact, we also noted Fli1 occupancy of conserved EBSs within the UPF (Figure 5B) and intronic enhancer fragment (Online Figure VIB) of the Tie2 gene in embryos isolated at embryonic day 11.5, but not at embryonic day 9.5. These data suggest that Fli1 binds to the conserved EBSs within the promoter of selected Etv2 target endothelial genes, including Tie2, to govern their expression at and beyond midgestation.

To complement our in vivo data, we undertook Fli1 loss- and gain-of-function strategies and analyzed transcript levels of Tie2 and Cdh5 in vitro. We found that small interfering RNA–mediated knockdown of Fli1 in HAECs resulted in marked reduction of transcripts levels of Tie2 and Cdh5 (Figure 5C). Furthermore, cotransfection of the Tie2 reporter with increasing amounts of Fli1 expression plasmid in COS1 cells revealed marked and dose-dependent induction of luciferase activity, whereas mutation of the EBSs in a 2.1-kb UPF of the Tie2 gene\(^6\) significantly attenuated the induction of reporter activity (Figure 5D). Importantly, ectopic Fli1 expression in C2C12 cells (Online Figure VIC) and HAECs (Figure 5E) resulted in significant induction of endogenous Tie2 expression. Thus, we conclude that in the absence of Etv2 at midgestation, Fli1 governs EC survival and vascular integrity by regulating expression of selected Etv2 target transcription (Online Figure VA) and signaling (eg, Tie2) factors at and beyond midgestation.

**Discussion**

The overall goal of this study was to decipher the Etv2-mediated transcriptional network that integrates vascular morphogenesis from early to late gestation in the developing mouse embryo. Our study reports 3 important findings. First, using a combination of molecular and Etv2 loss- and gain-of-function experiments, we have identified Fli1 as a downstream target of Etv2 in the early embryo and defined the underlying regulatory mechanisms. Second, we have uncovered a previously unrecognized positive autoregulatory mechanism controlling Fli1 gene expression at and beyond midgestation, thereby providing insight into mechanisms governing vascular morphogenesis and homeostasis in the absence of Etv2. We demonstrate that when Etv2 expression is extinguished, Fli1 acts to regulate its own expression, as well as that of selected other Etv2 target genes, at and beyond midgestation. In doing so, it governs EC viability and vascular integrity, which accounts for the vascular leakage and ultimate embryonic lethality reported in Fli1 mutant mice.\(^{18,19}\) Third, we have identified Fli1 as an upstream regulator of Tie2 and demonstrated that Fli1 binds to the conserved EBSs within the UPF and intronic enhancer fragment of the Tie2 gene to regulate endothelial Tie2 expression at and beyond midgestation. Collectively, our findings identify Etv2 and Fli1 as a classic example of a feedforward autoregulatory feedback loop that initiates and maintains vascular morphogenesis, homeostasis, and subsequent fetal growth and survival during development.

Since the discovery of Etv2 as an essential regulator of endothelial fate of progenitor cells and its temporal and spatial expression pattern in the EC of early mouse embryo,\(^6,11\) the general consensus has been that Etv2 activates a downstream transcriptional network in early embryo, which in turn regulates EC homeostasis and vascular morphogenesis in the absence of Etv2. Indeed, compared with WT ESCs\(^{16,17}\) and
ECs,\textsuperscript{13,16} transcript levels of several transcription factors, including \textit{Flil}, were significantly attenuated in \textit{Etv2}-null cells. Reciprocally, ectopic expression of \textit{Etv2} in zebrafish embryo\textsuperscript{29} and differentiating ESCs\textsuperscript{30} induces endogenous \textit{Flil} transcript levels. Although these data are consistent with our hypothesis that \textit{Etv2} can induce \textit{Flil} expression in a cell autonomous manner, the specificity of this response and the underlying mechanisms of \textit{Flil} gene expression regulation by \textit{Etv2} were incompletely understood. A recently published study describes modest activation of the \textit{Flil} gene by \textit{Etv2} through binding to a conserved EBS located 11.4 kb downstream of the \textit{Flil} translation initiation site in exon 1, but mutation of that site did not significantly attenuate \textit{Flil} reporter activity,\textsuperscript{16} highlighting the physiological significance of \textit{cis}-regulatory motifs within the \textit{Flil} promoter region for transcriptional regulation of \textit{Flil} expression by \textit{Etv2}.

Although all ETS factors recognize a similar \textit{cis}-regulatory element,\textsuperscript{6,7} attenuation of \textit{Flil} expression in \textit{Etv2}-null mice was not complemented by several other ETS factors, which are known to be expressed in ECs.\textsuperscript{31,22} However, all of them, including Elf1 and Elf2, induced \textit{Flil} reporter activity in vitro. These data suggest that the binding specificity of ETS factors to a \textit{cis}-regulatory element is uniquely regulated in native chromatin, which is often indistinguishable in naked DNA. It is also plausible that the transcriptional regulation of \textit{Flil} and Tie2 genes by other ETS factors in endothelial and non-ECs is context dependent or dependent on their cooperative action with other family members. Indeed, cooperative actions among numerous ETS factors, including \textit{Etv2}, \textit{Flil}, and \textit{Erg}, and other transcription factors are known to play essential roles in endothelial gene expression during development.\textsuperscript{30,31} Consistent with these studies, a cooperative action between \textit{Flil} and \textit{Erg} is required for hematopoiesis in mice,\textsuperscript{32} and mice lacking \textit{Flil} and endothelial isoforms of \textit{Erg} (isoforms 5–7)\textsuperscript{33} manifest vascular and cardiac malformations at midgestation. Therefore, it is conceivable that a cooperative action between \textit{Flil} and other ETS factors governs endothelial gene expression and vascular morphogenesis in the absence of \textit{Etv2}.

Autoregulation of gene expression is a common theme among transcription factors that govern expression of a large number of genes during embryogenesis.\textsuperscript{34} Although autoregulatory mechanisms can be positive or negative, the significance of autoregulation relates to maintenance of cell fate and cellular homeostasis. Positive autoregulation has been described for numerous developmental transcription factors, including, but not limited to, paired box protein 6 (Pax6),\textsuperscript{35} homeobox (Hox) proteins, and several myogenic factors, MyoD and myocyte enhancer factor 2 (Mef2),\textsuperscript{34,36,37} which are essential for cardiac and skeletal myogenesis. For example, the basic helix-loop-helix transcription factor \textit{Twist}, which governs mesodermal specification and myogenesis,\textsuperscript{36} and Mef2 proteins are coexpressed during early phases of mesodermal development. However, \textit{Twist} expression is extinguished before muscle cell differentiation, whereas Mef2 expression persists throughout mesodermal development and subsequent muscle differentiation.\textsuperscript{38} Of interest is that positive autoregulatory mechanisms related to \textit{Twist} and Mef2 in skeletal myogenesis parallel those reported here for \textit{Etv2} and \textit{Flil} in vascular morphogenesis.

Our model suggests that in the early embryo, \textit{Etv2} activates a transcriptional network,\textsuperscript{16,17} involving \textit{Flil} and \textit{Tie2} to govern the endothemo-endothelial fate of progenitor cells and subsequent fetal growth and survival (Figure 6). At midgestation, when \textit{Etv2} expression turns off, \textit{Flil} autoregulates its own expression as well as that of selected \textit{Etv2} target genes (such as \textit{Erg}, \textit{Cdhl5}, and \textit{Tie2}) for the remainder of gestation to govern EC survival and vascular integrity (Figure 6). Consistent with our model, endothelial expression of a \textit{lacZ} reporter driven by the native \textit{Flil} promoter is significantly diminished at midgestation and undetectable at embryonic day 14.5 in \textit{Flil}-null embryos, whereas reporter expression in ECs persists in \textit{Flil}-heterozygous embryos during these developmental stages.\textsuperscript{18,19} In contrast, endothelial expression of a \textit{lacZ} reporter driven by the native \textit{Erg} promoter is not reduced in \textit{Erg}-null embryo at midgestation,\textsuperscript{33} suggesting that \textit{Flil}, but not \textit{Erg}, is required to govern its own promoter activity at midgestation. Although embryonic lethality of \textit{Etv2}-null mice is not associated with excessive EC death,\textsuperscript{30} our data along with previously published studies\textsuperscript{24–27} suggest a model in which attenuation of endothelial \textit{Tie2} and \textit{Cdhl5} expression is associated with excessive EC death in \textit{Flil}-null embryos. However, we cannot rule out the possibility that additional factors might be involved in this process. Previously, we demonstrated that \textit{Etv2} governs \textit{Tie2} expression in early embryonic ECs,\textsuperscript{8} yet the molecular mechanisms underlying transcriptional regulation of \textit{Tie2} expression at and beyond midgestation were unknown. Our study is the first to report that \textit{Flil} is an important regulator of endothelial \textit{Tie2} expression at and beyond midgestation.

In conclusion, we have uncovered a unique and previously unrecognized positive autoregulatory transcriptional circuit whereby coordinated transcriptional activity of \textit{Etv2} and \textit{Flil} regulates vascular morphogenesis and homeostasis at distinct stages of embryogenesis. Given the essential role of \textit{Flil} in cardiovascular morphogenesis and vascular inflammation,\textsuperscript{6,7} it is conceivable that \textit{Flil} may be intimately involved in vascular and cardiovascular diseases. Moreover, \textit{Flil} is associated with thymus development and cancer pathogenesis.\textsuperscript{38–40}
Looking to the future, development of small molecules that positively or negatively modulate FlI activity will provide molecular insights into a novel mechanism with potential clinical relevance.

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**Disclosures**

None.

**References**


What Is Known?

• The E26 transforming–specific family transcription factor Ets variant 2 (Etv2) governs endothelial fate of the progenitor cells and vascular morphogenesis in early development.
• In early mouse embryo, the Etv2 activates numerous endothelial genes, including the E26 transforming–specific family transcription factor Friend leukemia integration 1 (Fli1) gene.
• Endothelial expression of the Etv2 gene stops at midgestation; hence, molecular mechanisms underlying transcriptional regulation of vascular morphogenesis and homeostasis beyond this stage remain incompletely understood.

What New Information Does This Article Contribute?

• We have identified the mechanism underlying Etv2-dependent regulation of the Fli1 gene expression in early mouse embryo.
• At midgestation, when Etv2 expression turns off, Fli1 regulates its own expression as well as that of selected Etv2 target endothelial genes at and beyond midgestation.
• This positive autoregulatory mechanism of Fli1 gene expression is critical for endothelial cell survival and vascular integrity.

Novelty and Significance

Mice lacking Etv2 die in utero around embryonic day 9.5 with complete loss of vasculature. Etv2 is an essential upstream regulator of numerous endothelial genes and governs endothelial fate of the progenitor cells. Intriguingly, in mice endothelial expression of the Etv2 gene ceases at midgestation. This study was designed to identify a specific Etv2 target that controls its own expression as well as that of additional Etv2-regulated genes and maintains vascular morphogenesis and integrity through the remainder of gestation. We demonstrate that Fli1 has such properties, being dependent on Etv2 to initiate embryonic expression early in development and then acting to regulate its own expression as well as that of selected Etv2 target endothelial genes. In doing so, Fli1 governs endothelial cell viability and vascular integrity for the remainder of gestation critical for fetal growth and survival. Our study identifies the Etv2 and Fli1 axis as a classic example of a feed-forward autoregulatory feedback loop in the endothelium that initiates and maintains cardiovascular health at distinct developmental stages.
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SUPPLEMENTAL MATERIALS

Fli1 Acts Downstream of Etv2 to Govern Cell Survival and Vascular Homeostasis via Positive Autoregulation

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Methods

Plasmids:
The *Fli1* reporter plasmid was generated essentially as described previously. Briefly, the full-length (FL) 0.7-kb promoter fragment of the *Fli1* gene was PCR-amplified from mouse tail genomic DNA using the following (forward-TTTTTCGCGTTGACTCCGGCTGGAAAAAGC) and (reverse-TTTTTCTCGAGTTGGCCAAGTCTGCAGCGACGACG) set of primers, cloned into MluI and XhoI sites of the pGL3 reporter plasmid (Promega) and sequence confirmed. The deletion mutant-1 (Dm-1) was generated as described for FL except that the following forward (ATGCTACGCGTGAGTGATGCGAAAAGCAGGGC) primer was used. The Dm-1 reporter was treated with MluI and ApaI, blunted and forced ligated to generate Dm-3. Generation of Dm-2 harboring WT and mutated Ets-binding sites (EBSs) were described previously. The 2.1-kb upstream promoter fragment of the *Tie2* gene harboring WT or mutated EBSs were both previously described. The plasmid pCMV-HA-Fli1 was generated by in-frame fusion of an HA tag to the N-terminus of a FL murine Fli1 cDNA and sequence verified. Expression plasmids for Etv2, Ets1 and Elf1 and Elf2 were described. Etv2 mutant plasmid, lacking the DNA-binding ETS domain, was generated by serial PCR amplification of upstream N-terminus and downstream C-terminus region of Etv2-ETS domain, cloned into Not I and Xho I sites and sequence confirmed as described.

Mouse and embryo isolations:
Generation of *Etv2* and *Fli1* knockout and Tie2-GFP transgenic mice was described previously. Heterozygous male and female mice were intercrossed to isolate embryos at distinct developmental stages. The staging of embryos was performed by counting the presence of vaginal plug as day 0.5 after conception and by counting the number of somites. Genomic DNA, extracted from yolk sacs was used for genotyping as described. All embryos were harvested in ice-cold PBS, fixed in 4% paraformadehyde (PFA) overnight at 4°C and washed in PBS. The embryos were photographed under identical conditions and magnification before or after fixation as described, except that embryos at different developmental stages were photographed at different magnifications to fill the frame. All mice were maintained in the animal facility at University of Texas Southwestern Medical Center according to the guidelines of Institutional Animal Care and Use Committee and the Animal Resource Center.

Histological and Immunohistochemical analyses:
Histological and immunostaining analyses of transverse and saggital sections of paraffin-embedded embryos were described previously. Immunostaining analyses for Fli1, α-endomucin, Ki67 and Tie2 were described previously. Briefly, sections were deparaffinized before permeabilization with Triton (α-endomucin), citrate based microwave (Ki67) and trypsin (Tie2 and Fli1) for antigen retrieval, quenched for endogenous peroxidase, blocked with normal serum, and incubated overnight at 4°C with primary antibody. Bound α-endomucin, Ki67 and Tie2 primary antibodies were detected using species-specific biotinylated secondary antibodies, peroxidase-streptavidin, and 3,3′-diaminobenzidine (DAB) chromagen and fluorescein-avidin DCS, respectively.
TUNEL analyses:
Apoptotic cell death in WT and Fli1-null embryos was analyzed using the Promega DeadEnd Fluorometric TUNEL system (Promega) according to the manufacturer’s instructions as previously described\(^3\), \(^9\). Briefly, sections were deparaffinized, equilibrated in PBS, permeabilized with proteinase K, postfixed in 4% PFA, and incubated in TdT reaction mixture for 1 h at 37 °C in the dark. Slides were then washed in 2xSSC, counterstained with propidium iodide, and coverslipped with Vectorshield mounting medium.

Cell culture and treatment:
COS1 and C2C12 cells were cultured as described previously\(^3\), \(^9\). Primary human aortic endothelial cells (HAECs) (kindly provided by the Shaul lab of UT Southwestern Medical Center) were purchased and cultured according to the manufacturer’s instructions (Lonza). All siRNAs were purchased from Sigma and dissolved in opti-MEM (Invitrogen). A total of 100 pmol siRNA was used for each well of a 12-well plate, and transfection was performed with Lipofectamine RNAiMAX reagent (Invitrogen). Gene expression in HAECs was analyzed after 48 hrs.

Chromatin immunoprecipitation (ChIP) assay:
ChIP assays evaluating Fli1 and Tie2 promoter binding of Etv2 and Fli1 were performed as previously described\(^1\), \(^3\), \(^9\), \(^11\), except that the C2C12 myoblasts were transfected with HA-tagged Etv2 and Fli1 expression plasmids in a 15cm culture dish. Formaldehyde (1% final concentration) was added 24 hours post transfection and chromatin solution was prepared by sonication. Immunoprecipitation (IP) of diluted chromatin solution was carried out using anti-HA (Roche) and control IgG sera. Promoter-specific occupancy of Etv2 and Fli1 was analyzed by amplifying the DNA fragment corresponding to Fli1 promoter and intronic regions using specific primer sets. The following forward (a) gaggtatgcgaaagcagggc and reverse (b) ctgtgcagttgtagc primers were used to amplify 257-bp of mouse Fli1 promoter region, while forward (c) agcatgaccagcacatgaag and reverse (d) ttgaatgcccagagtcaca primers were used to amplify 229-bp of first intronic region (as control) of the Fli1 gene. On the other hand, forward-ggtgtctgccgaa aagcaggac and same reverse (b) set of primers were used to amplify 271-bp of human Fli1 promoter region. The binding of Fli1 of Cdh5 and Tie2 upstream promoter and Tie2 intronic enhancer regions was analyzed as described previously\(^3\), \(^12\). ChIP assays with isolated embryos were carried out as described\(^13\), \(^14\), expect that we isolated WT embryos in DMEM supplemented with 10% fetal bovine serum, minced the embryos before fixation for 15 min at room temperature. After washing with PBS, we used a Dounce homogenizer to disrupt the cell membrane. Protein-DNA complexes were IP’d using anti-Fli1 (Santa Cruz) and anti-Erg (Santa Cruz) (kindly provided by the Cleaver lab of UT Southwestern Medical Center) or control trinitrophenal (TNP) (BD Pharmingen)\(^1\) sera. Genomic DNA isolated before IP was diluted 100-fold and used (1-3μL) as input, and the undiluted IP’d DNA (1-3μL) were used for the PCR reaction, while 1μL was used for quantitative PCR analyses using SYBER master mixture (Roche).

Lentivirus production, purification, and infection
HA-tagged mouse Fli1 cDNA was subcloned into a lentiviral expression vector (Clontech) and co-transfected with pCD and VSVG constructs into HEK293T cells according to the manufacturer’s instructions (Clontech). Lentivirus was harvested from the culture supernatant and concentrated using ultracentrifugation for 2 hrs at 22k rpm using SW28 rotor. The viral titer
was determined by qRT-PCR (Clontech) and stored at -80°C until use. To infect HAECs, an MOI of 3-10 of GFP (as control) and Fli1-expressing lentivirus was added in culture medium containing polybrene (8µg/ml). RNA was extracted for gene expression analyses after 48 hrs post-infection.

Semi-quantitative and quantitative RT-PCR analyses:
Total RNA from C2C12 myoblasts and WT and Etv2- and Fli1-null embryos (n=3-4) was extracted using TRIzol (Invitrogen) according to the manufacturer’s instructions as previously described3,9,10. Equal amounts (0.3-1.0µg) of RNA were used to prepare cDNA using iScript cDNA synthesis kit according to manufacturer’s instructions (Bio-Rad). All cDNA was diluted 5-10 fold and employed for quantitative RT-PCR (qRT-PCR) analyses for Ets and endothelial-specific genes using gene-specific Taqman probes where 18S or Gapdh RNA was used as a loading control as described previously3,9. Transcript levels of mouse endothelial specific Erg isomers, Gata2, Cdh5 and Etv6 in WT and null embryos were analyzed using SYBER green master mix (Roche) as described14,15. To prepare cDNA from endothelial cells, we extracted RNA from Tie2-GFP+ cells isolated from E9.0 and E12.0 Tie2-GFP embryo by FACS (fluorescence activated cell sorting), amplified according to the manufacturer’s instructions (Ambion) and employed for semi-quantitative and qRT-PCR analyses as previously described3,9. In addition, cDNA of WT and null embryos and C2C12 cells was used for semi-quantitative RT-PCR analysis of Etv2 using the following set of primers (forward-ATCACCAAGGCCCATCGA GAGC) and (reverse- ACTGGTTGTGGCCATGGACGC) as described previously14,15. Transcript levels of human Fli1, Tie2, and Cdh5 were analyzed as described previously12. To assess activation of endogenous Fli1 promoter activity, we used (forward-GCTCGGCTGCAGACTTGGC) and (reverse-GCTTGACATTGACTCTCACTGGC) primers to analyze mouse endogenous Fli1 transcript levels spanning exon 1 UTR and exon 2 in C2C12 myoblasts and embryos.

Reporter gene assays
Transcriptional assays using Fli1 and Tie2 reporter constructs, harboring WT or mutated Ets-binding sites (EBSs), were performed as described previously3,9,16, except that Fugene-HD (Roche), Lipofectamine plus and Lipofectamine 2000 (Invitrogen) were used to transfect DNA into COS1, C2C12 and HAE cells, respectively. Luciferase activity was measured using the dual-luciferase kit (Promega), and luciferase activity of pTK-renilla (control) was used to normalize firefly luciferase activity. Each assay was performed in triplicate and repeated 2-3 times. Luciferase activity in the absence of Etv2 and Fli1 was normalized to one to determine Etv2- and Fli1-dependent fold activation of luciferase activity. Error bars represent mean ± SD, and p values were calculated by Student’s t test.

Western blot analyses:
Western blot analyses were carried out as described previously3,9,16, except that whole cell extracts from HAECs, control C2C12 myoblast cells or cell expressing HA-tagged Etv2 and Fli1 were separated on a 10% SDS-PAGE gel and the proteins were transferred to a nylon membrane. The membrane was incubated with anti-HA (1:3000, Roche) and anti-GAPDH (1:7500, Santa Cruz), washed and then processed as described17,18.
Statistical analyses:
Error bars represent mean ± SD, and p values, calculated by Student’s t test, less than 0.05 were considered to be statistically significant.
References


Supplemental Fig. I: Embryonic hemorrhage and lethality of Fl1-null mice are associated with increased cell death. (A) Morphological appearances of WT and Fl1-null littermates isolated at the indicated developmental stages. Vascular leakage within the embryo proper is evident only in Fl1-null embryos. Initiation of tissue disintegration and absence of heart beat (i.e. embryonic lethality) were noted in Fl1-null embryos at E12.0. (B) Routine histology (H&E), IHC analyses for proliferative marker, ki67 and TUNEL assays revealed hemorrhage in neural tube of null embryos and significant increased cell death in endothelial and blood cells as well as cells of the neural tube (also see Figure 1), but cellular proliferation was comparatively normal (bar=200µM). Neural tube (NT), dorsal aorta (DA), cardiac cushion (CC) and cardinal vein (CV) are indicated.
Supplemental Fig. II: Vascular morphogenesis is normal in early Fli1-null embryos. (A) Morphological appearances of WT and Fli1-null littermates are grossly indistinguishable at E9.5. Histologic (H&E) and IHC (α-endomucin) analyses revealed normal vascular and cardiovascular development in both WT and Fli1-null littermates. HT (heart), DA, PHV (primary head vein), EC (endocardium), MC (myocardium). (B) H&E close-ups of DA clearly illustrate blood within the DA of Fli1-null mice (red arrow). (C) qRT-PCR analyses of the indicated endothelial genes in WT and Fli1-null embryos. Note that expression of both genes is essentially similar in WT and Fli1-null littermates (n=3).
Supplemental Fig. III: Conserved EBSs within Fli1 promoter region confer Etv2-mediated Fli1 gene expression. (A) Semi-quantitative (left) and qRT-PCR (right) analyses of Etv2 and Fli1 transcripts in whole embryo isolated at the indicated developmental stages. Note the transient co-expression of the Etv2 and Fli1 genes in early embryos, while that expression of the Fli1, but not Etv2, gene persisted beyond mid-gestation. Transcript levels of each gene at E8.5 were normalized to 1. (B) Nucleotide sequence of 0.7-kb Fli1 promoter region. Evolutionarily conserved Ets-binding sites (EBSs) (highlighted), 5' untranslated region (UTR) (upper case), translation initiation site (ATG) and primer sequence in 5' UTR (see Figure 2) are indicated. Schematic alignment of nucleotide sequence of the indicated species for three essential EBSs (highlighted green for mouse) in reverse orientation is shown. Mutated nucleotides in the EBSs are indicated blue. (C) Transcriptional assays in C2C12 cells reveal that co-transfection of Fli1 reporter and the indicated amounts of Etv2 expression plasmids resulted in dose-dependent and significant induction of luciferase activity (*p<0.002 vs. control).
Supplemental Fig. IV: Etv2 governs *Fli1* gene expression by binding to specific conserved EBSs within the *Fli1* promoter. (A) Schematic of the full-length (FL) and deletion mutant (Dm) *Fli1* reporter plasmids. Numbers indicate the position of the conserved Ets-binding sites (EBSs) (black circles) upstream from the translation initiation site (ATG). White bar indicates the 5’ untranslated region (UTR). (B) ChIP assays revealing Etv2 occupancy of the *Fli1* promoter in vivo. Chromatin solution of HA-tagged Etv2 expressing C2C12 myoblasts was IP’d with anti-HA and control IgG sera. Genomic DNA (gDNA) purified before IP was diluted 100 fold and used as input. Specific primer sets and indicated volume (μL) of the gDNA were used to PCR amplify the promoter (a+b) and intronic (c+d) region (schematized top) of the *Fli1* gene. Note that DNA sample IP’d with anti-HA, but not IgG, sera amplified only the *Fli1* promoter harboring EBSs. (C) Western blot (top) and semi-quantitative RT-PCR (bottom) analyses demonstrating increased Etv2 protein levels in C2C12 myoblasts induces endogenous *Fli1* expression in a dose-dependent manner. PCR negative (-), protein (Tub) and PCR (actin) loading controls are indicated.
Supplemental Fig. V: Fli1 is required for Fli1 expression at and beyond mid-gestation. (A) qRT-PCR analyses for transcript levels of the indicated genes using RNA from E10.5 WT and Fli1-null embryos. Relative gene expression in WT embryo was normalized to 1. Significant attenuation of Erg and Gata2 expression was observed in Fli1-null (*p<0.005 vs. WT and (#p<0.005 vs. WT) embryos. NS: not significant. (B and C) Western blot (B) and RT-PCR (C) analyses demonstrate increased Fli1 protein levels and dose-dependent induction of endogenous Fli1 transcripts by Fli1 in C2C12 cells. Tubulin (B) and α-actin (C) were used as loading control. (D) ChIP-qPCR analyses for Fli1 occupancy of the Fli1 promoter in C2C12 cells were performed as described in panel B of Supplemental Fig. IV, except that HA-tagged Fli1 expression plasmid was transfected. (E) Quantitative RT-PCR analyses of Fli1 transcripts in primary HAECs following transfection of the indicated siRNAs. Note the marked reduction of Fli1 transcripts by two independent Fli1-specific siRNAs (*p<0.05 vs. control).
Supplemental Fig. VI: Fli1 is an upstream regulator of endothelial Tie2 gene expression at mid-gestation. (A) Enlarged view of histology (H&E) and IHC analyses for Tie2 and Fli1 corresponding to vasculatures (green bracket) of panel A of Figure 1 are shown. Note the marked reduction of Tie2 levels in Fli1-null vascular endothelium, such as dorsal aorta (DA) and cardinal vein (CV), was specific to the loss of Fli1 in vascular endothelium. (B) ChIP assays revealing Fli1 occupancy of the intronic enhancer fragment (IEF) of Tie2 gene (schematized) in mouse embryos isolated at E11.5 but not at E9.5. (C) qRT-PCR analyses of RNA from C2C12 cells transfected with indicated amounts of the Fli1 expression plasmid reveal significant and dose-dependent induction of endogenous Tie2 transcript levels (*p<0.002 vs. control).