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Fli1 Promotes Vascular Morphogenesis by Regulating Endothelial Potential of Multipotent Myogenic Progenitors

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Abstract

Rationale: Fetal growth and survival depend critically on proper development and integrity of the vascular system. Fli1 (Friend leukemia integration 1), a member of the Ets family of transcription factors, plays critical roles in vascular morphogenesis and homeostasis at mid-gestation, the developmental stage at which expression of its upstream regulator, Etv2, ceases. However, molecular mechanisms of Fli1 action in vascular morphogenesis remain incompletely understood.

Objective: To dissect molecular mechanisms of vascular morphogenesis governed by Fli1.

Methods and Results: Utilizing *Fli1* promoter-driven lineage-specific *LacZ* expression, Fli1 loss-of-function strategies, and a series of molecular techniques, we demonstrate that *Fli1* expression in multipotent myogenic progenitor cells (MPCs) occurs independent of Etv2, and

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AUTHOR CONTRIBUTIONS

A.F. and J.A.H. designed research; A.F., S.S., Y. L., J.M.A., and N.J. performed research; C.E.P. and B.M.E. performed histology and IHC analyses; M.K. and M.T. contributed new reagents and mouse model; A.F. and T.G.G. analyzed data; and A.F. and J.A.H. wrote the paper; and M.K., M.T. and T.G.G. edited it.

DISCLOSURES

The authors declare no competing interests.

SUPPLEMENTAL MATERIALS

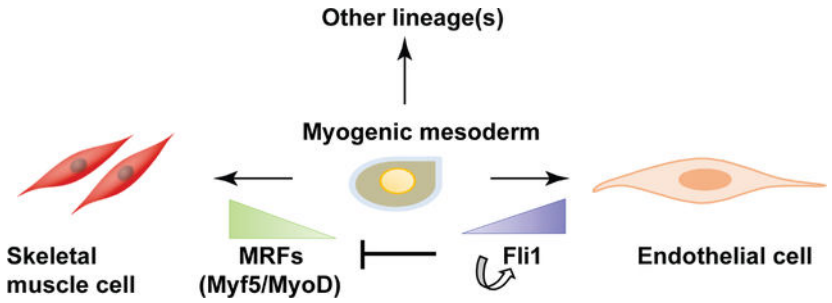
Supplementary Information includes:

- Expanded Materials and Methods,
- Major Resources Table
- Online Figures I–V and figure legends
- Unedited immunoblots and gels
- Additional references (#66–#70) included in main reference list.

loss of *Fli1* expression results in a significant increase in LacZ$^+$ cells in mesoderm within somites and limb buds, leading to reciprocal regulation of the expression of several key endothelial and myogenic genes and vascular abnormalities. Conversely, embryos with conditional Fli1 gain-of-function in MPCs manifested aberrant vasculogenesis with lack of myogenesis. Mechanistically, elevated Fli1 activity in myoblasts and in adult MPCs (also called satellite cells) of X-linked muscular dystrophic *mdx* mice markedly induced endothelial, but attenuated myogenic, gene expression and differentiation. Importantly, ectopic expression of Myf5 or MyoD, two key myogenic regulators, in Fli1-expressing myoblasts restored their differentiation potential, indicating that levels of Fli1 and myogenic regulators in MPCs inversely regulate their endothelial versus myogenic potential.

Conclusions: Fli1 governs vascular morphogenesis by regulating endothelial potential by inversely regulating endothelial versus myogenic programs in MPCs. Our data uncover an important and previously unrecognized mechanism of vascular morphogenesis governed by Fli1 and highlight the physiological significance of the fine tuning of Fli1 activity in multipotent progenitors for proper vascular and muscle morphogenesis during development and disease.

Graphical Abstract



Keywords

Developmental Biology; Vascular Biology; Basic Science Research

INTRODUCTION

Establishment of a normal vascular system following gastrulation sets the stage for two fundamental processes in vertebrate embryos, cardiogenesis, development of a beating heart [1–3], and somitogenesis, sequential subdivision of unsegmented paraxial mesoderm (PM) into paired somites for skeletal muscle morphogenesis [4, 5]. Therefore, endothelial dysfunction can have catastrophic consequences on growth and survival of the vertebrate embryo, as well as the pathophysiology of cardiovascular diseases during postnatal and adult life [6].

Accumulating evidence suggests that hemangioblasts, a common progenitor for endothelial and blood cells, contribute to vascular morphogenesis within the embryo proper and within the extraembryonic yolk sac at embryonic day 7.5 (E7.5) [7]. Additional studies using lineage-tracing and embryoid body differentiation strategies have identified Flk1$^+$ multipotent progenitors that give rise not only to endothelial and blood cells but also to other Flk1$^+$ and Flk1$^-$ mesodermal lineages for the genesis of cardiomyocytes and skeletal muscle,

respectively [8, 9]. These studies indicate that the precisely orchestrated and overlapping action of numerous transcription factors and signaling molecules is essential for progenitor cell growth, cell fate decisions, and cellular differentiation to develop specific organs/tissues. However, detailed understanding of the transcriptional network that governs muscle and endothelial cell fate during embryogenesis is lacking. Elegant work using lineage-tracing, gene disruption and mutation strategies has identified essential roles for Pax3/7 (paired-homeobox 3 and 7) and the bHLH (basic helix-loop-helix) transcription factors, Myf5 and MyoD, [10–12] for skeletal muscle development, and the ETS (E26-specific) family of transcription factors for vascular development [13].

Skeletal muscle morphogenesis involves a series of molecular and morphogenetic events and the precisely orchestrated action of numerous MRFs (myogenic regulatory factors) [10–12]. In particular, Pax3 expression in paraxial and hypaxial mesoderm of the dermomyotome, the site of residence of MPCs (multipotent myogenic progenitors), governs the “stemness” of MPCs in early embryos [11, 12]. Subsequent induction of *Myf5* and *MyoD* expression by Pax3 in MPCs dictates their migration to somites and subsequent growth and myogenic differentiation into myotubes, the multi-nucleated myofiber [14, 15]. Finally, expression of several other bHLH transcription factors, including Myog (myogenin) in the myotube initiates limb and trunk muscle morphogenesis [11, 12, 16]. Besides these myogenic factors, embryos with compound loss of Fox (Forkhead box) proteins, FoxC1 and FoxC2, are marked by attenuated endothelial fate of $Pax3^+$ MPCs and manifest abnormal vascular and somite morphogenesis [17, 18], supporting the role of these molecules in both vessel and muscle morphogenesis.

Among the 29 different *Ets* genes identified in mammals, the essential roles of ETS family transcription factors Etv2 (also called Etsrp71/ER71) [19–22] and Fli1 (Friend leukemia integration 1) [23–25], in vascular morphogenesis have been highlighted. Mice lacking Etv2 die in *utero* around E9.5 with complete disruption of vasculature [19, 22]. Furthermore, cooperative action of Etv2 and FoxC2 is required for expression of numerous endothelial genes in mice and zebrafish [20]. Importantly, global loss of Etv2/Ets-related protein in mice and zebrafish redirects endothelial progenitor cells in early embryos to a myogenic fate [26, 27], highlighting its central role in endothelial fate of the progenitors at the expense of their myogenic potential. Intriguingly, expression of *Etv2* in wild-type (WT) mice ceases at mid-gestation [19–21], suggesting that an Etv2 downstream target contributes to regulating endothelial potential of the progenitors, vascular morphogenesis, and homeostasis at and beyond mid-gestation.

Indeed, we previously demonstrated that Fli1 has such properties, being dependent on Etv2 to initiate early embryonic expression and then acting to regulate its own expression as well as that of select Etv2-regulated endothelial genes involved in vascular morphogenesis and homeostasis [28]. However, mechanisms whereby Fli1 governs vascular morphogenesis in the absence of Etv2 remain incompletely understood. The existence of a common progenitor for endothelial and muscle cells [29–31], spatiotemporal expression of *Etv2* in MPCs of early embryos (~E7.75), and the myogenic fate of endothelial progenitors in *Etv2*-null embryos [26] support the hypothesis that Etv2-dependent expression of *Fli1* in MPCs in early embryos

plays an important role in regulating the endothelial potential of MPCs in the absence of Etv2.

In this study, we have utilized *Fli1* promoter-driven lineage-specific reporter gene expression and a series of molecular techniques combined with Fli1 loss- and gain-of-function strategies to show that Fli1 promotes vascular morphogenesis by regulating the endothelial potential of MPCs at the expense of their myogenic fate, which coincides with reciprocal regulation of endothelial versus myogenic programs in MPCs. As such, elevated *Fli1* expression in MPCs of WT and dystrophic mice triggered aberrant vascular morphogenesis and marked attenuation of myogenic gene expression and differentiation. Importantly, ectopic expression of MyoD or Myf5 in Fli1-expressing C2C12 myoblasts restored their differentiation potential. We conclude that Fli1 governs vascular morphogenesis by inversely regulating endothelial versus myogenic potential of MPCs at and beyond mid-gestation, and fine tuning of Fli1 levels in progenitors is essential for proper vascular and muscle morphogenesis.

METHODS

Data Availability.

The authors will make their data, analytic methods, and study materials available to other researchers based on reasonable request. Please see the Major Resources Table in the Data Supplement.

Engineering of Fli1-lacZ knockin and doxycycline-inducible Fli1 transgenic mice.

Fli1-lacZ knockin chimera mice were generated using a *Fli1* targeting vector purchased from the KOMP repository (UC Davis, Sacramento, CA, Clone name: HTGR06010_A_1_C02, (Online Figure IA) [32]. Insertion of the KOMP vector into a 129 agouti embryonic stem cell line was performed at the transgenic mouse core of Harvard Medical School (Boston, MA, United States) and used for blastocyst injection into pseudo-pregnant females to generate chimeric mice, which were then selected for germline transmission. All experimental procedures were approved by the Boston University Animal Care and Use Committee and conducted in accordance with the guidelines of the National Institutes of Health.

Fli1 chimera mice were crossed with global Cre-expressing *CAG*-Cre^{+} mice (Jackson Laboratory) (Provided by Dr. Olson of UTSW) at the Animal Care facility of the University of Texas Southwestern Medical Center (UTSW). Tail genomic DNA and specific primer sets were utilized to identify offspring with specific Cre-mediated recombination of loxP sites located immediately downstream of the *LacZ* cassette and exon 3 (Online Figure IA). The resulting chimeric mice (lacZ^{+}/Neo^{-}/Δexon3/CAG-Cre^{+}) were then crossed with WT C57BL/6 mice to generate *CAG*-Cre^{-}/*Fli1*-lacZ^{+} *(Fli1$^{+/LacZ}$)* chimera mice. *Fli1$^{+/LacZ}$* chimera mice were backcrossed with C57BL/6 for another six generations before using for experiments.

Doxycycline-inducible *Fli1* transgenic mice were generated as described [33]. Briefly, PCR-amplified HA-tagged *Fli1* cDNA was subcloned into the pTRE-Tight vector (Clontech) with a rabbit β-globin 3′ UTR. The C57BL/6 TRE-Fli1 transgenic

mice were generated by the transgenic core facility at UTSW. Tail genomic DNA and transgene-specific primer sets (forward-GTTTAGTGAAC CGTCAGATCGCC, and reverse-GCTTGACATTGACTCTCACTGGC) were used to identify transgene-positive founder mice. Homozygous C57BL/6 Rosa26-loxP-STOP-loxP-rtTA transgenic (Jackson Laboratory) (Provided by Dr. Scherer of UTSW) and Myo-Cre (Provided by Dr. Olson of UTSW) [34] mice were crossed with each founder mouse line to generate a Fli1-expressing founder line. Transgene negative and very low transgene-expressing male mice were excluded from analyses. Mice were maintained on a 12-hour dark/12-hour light cycle from 6 a.m. to 6 p.m. with unlimited access to water and chow (2916, Teklad) and for defined periods of Dox-containing (200 mg/kg) chow diets (Bio-Serv). All mice were maintained at the UTSW under protocols approved by the Institutional Animal Care and Use Committee and Research Animal Resources. Please see the Major Resources Table in the Data Supplement. Detailed protocols for viral transduction, gene expression, FACS analyses, histological, immunohistochemical and immunocytochemical analyses using isolated mouse embryos, genetically modified mouse tissues and cells are provided in the Online Data Supplement.

RESULTS

Fli1 is expressed in endothelium and myogenic progenitors of early embryos.

To gain insight into molecular mechanisms whereby Fli1 promotes vascular morphogenesis at and beyond mid-gestation, we engineered a *Fli1-LacZ* knockin reporter mouse model, in which expression of β-galactosidase is under the control of the native *Fli1* promoter (Figure 1A and Online Figure IA). We crossed *Fli1*-heterozygous [*Fli1*-het (*Fli1*$^{+/LacZ}$)] males with *Fli1*-WT (*Fli1*$^{+/+}$) or heterozygous females and isolated embryos from timed pregnant females at distinct developmental stages (Online Figure IB). Whole-mount LacZ staining enabled us to detect lineage-specific *Fli1* expression. As expected, strong β-galactosidase activity (blue) was detected in the vasculature of the entire embryo proper in E9.5, E10.5 and E11.5 *Fli1*-het and -null (*Fli1*$^{LacZ/LacZ}$), but not WT, embryos (Figures 1B, 1C and Online Figures IC, ID). Unlike E9.5 *Fli1*-null embryos (Figure 1B), we noted diminished vascular β-galactosidase activity in *Fli1*-null embryos at E10.5 and E11.5 (Figure 1C and Online Figures ID, IF), supporting our previous report of positive autoregulation of vascular *Fli1* gene expression at and beyond mid-gestation [28]. On the other hand, compared with E9.5 and E10.5 *Fli1*-het embryos, β-galactosidase activity was more intense and widespread in *Fli1*-null embryos (Figures 1B and 1C), supporting the possibility of LacZ^{+} non-endothelial cells.

Histology of transverse sections of E9.5 *Fli1*-het and *Fli1*-null embryos through the heart and hind limb further confirmed robust and specific β-galactosidase activity (i.e. *Fli1* expression) in the vascular and endocardial endothelium, supporting a role for Fli1 in cardiovascular development (Figure 1B and Online Figure IE). In addition to vasculature, a subset of primitive mesoderm in dermomyotome and somites was LacZ^{+} in E9.5 and E10.5 *Fli1*-het embryos (Figures 1B, 1C and Online Figures IE, IF). Importantly, compared with *Fli1*-het embryos, the number of LacZ^{+} cells was significantly increased and extended in somites and limbs of *Fli1*-null embryos (Figures 1B, 1C and Online Figures IE, IF).

However, cardiomyocytes within the myocardium of E9.5 and E10.5 *Fli1*-het or *Fli1*-null embryos were not $LacZ^+$ (Online Figures IE, IF). These data suggest that beyond vascular and endocardial endothelium, *Fli1* is expressed in a subset of MPCs in dermomyotome and somites and that loss of *Fli1* expression enhances their myogenic potential. Absence of *Etv2* expression in dermomyotome and somites at this developmental stage [26] supports the hypothesis that Etv2-independent *Fli1* expression in MPCs governs vascular morphogenesis by promoting endothelial potential of MPCs at and beyond mid-gestation.

Reciprocal regulation of endothelial and myogenic genes in Fli1-null embryos.

To further validate Etv2-independent *Fli1* expression in MPCs, we crossed Myo-Cre transgenic mice [34] with a ROSA reporter line [35]. These animals will express fluorescent tomato protein in MPCs only upon Cre-mediated deletion of the stop codon upstream of the reporter cassette (Online Figure IIA) [36]. We isolated fluorescence labeled MPCs from dissociated somatic tissue of E10.5 embryos (see Figure 1D, dotted line) by fluorescence activated cell sorting (FACS) (Online Figure IIB). Purified RNA was used to analyze expression of selected *Ets* and myogenic regulatory genes by quantitative RT-PCR (qPCR). Although the expression of *Fli1*, *Pax3* and *Myf5* were readily detected in the isolated MPCs, expression of *Etv2* was undetectable (Online Figure IIC), supporting the notion that *Fli1* expression in a subset of MPCs occurs independent of Etv2.

Next, we examined the specificity of *Fli1* expression in MPCs. We crossed *Fli1*-het male and female mice and isolated WT and *Fli1*-null embryos at E10.5. Consistent with our previous report [28], *Fli1*-null embryos manifested hemorrhage within the embryo proper (Figure 1D), highlighting Fli1's role in vascular homeostasis. Immunohistochemical (IHC) analyses detected Fli1 in vascular endothelial cells (ECs) as well as in dermomyotome of WT, but not in null, embryos (Figure 1D). Co-staining analyses of Pax3 and Fli1 further confirmed co-expression of Fli1 and Pax3 in a subset of MPCs in hypaxial dermomyotome (Figure 1E). Based on these data and other reports [26, 28], we conclude that Etv2-dependent initiation of *Fli1* expression in MPCs of early embryos persists at and beyond mid-gestation even in the absence of Etv2.

To assess the physiological significance of *Fli1* expression in MPCs, we purified RNA from somatic tissue of WT and *Fli1*-null embryos (Figure 1D, dotted line) and analyzed the expression of selected myogenic and endothelial genes. Consistent with our prior report [28], we noted that compared with WT littermates, transcript levels of select endothelial genes were significantly attenuated in *Fli1*-null embryos (Online Figure IID), whereas the expression of several critical myogenic regulators, including *Pax3* and *Myf5*, was up-regulated (Figure 1E). However, mRNA levels of late differentiation markers, such as *Myog* and troponin I (*Tnni1*) were not induced (Figure 1F). Collectively, these data lend further credence to our hypothesis that Fli1 expression in a subset of MPCs plays an essential role in governing their endothelial versus myogenic potential.

MPC-specific Fli1 gain-of-function results in aberrant vasculogenesis and perturbs myogenesis.

To complement our Fli1 loss-of-function data, we next undertook conditional and MPC-specific Fli1 gain-of-function strategies. We engineered a doxycycline (Dox)-inducible Fli1-expressing transgenic construct (TRE-Fli1) by sub-cloning hemagglutinin (HA)-tagged Fli1 cDNA downstream of a tetracycline responsive element (TRE) (Online Figure IIIA). HA-tagged Fli1 expression exclusively in Dox-treated HEK239 cells, co-transfected with a reverse tetracycline transactivator (rtTA) expression plasmid, was confirmed by Western blot (Online Figure IIIB).

We used this plasmid to generate Fli1 transgenic (Fli1-Tg) mice and employed a triple transgenic model strategy to establish a Fli1-expressing transgenic line [19, 33]. Briefly, the ROSA-STOP-rtTA mouse does not express rtTA due to the presence of a stop codon upstream of the rtTA cassette (Online Figure IIIC) [37]. In order to induce rtTA expression in MPCs, we sequentially crossed the ROSA-rtTA ($rtTA^{+}$) mouse with the Myo-Cre line to drive Cre recombinase expression in somites of early (E9.5) embryos. Homozygous double transgene positive ($rtTA^{+/+}/Cre^{+/+}$) females were then mated with Fli1 transgene-positive founder males to generate triple-Tg^{+} ($Fli1^{+}/rtTA^{+}/Cre^{+}$) offspring. Triple-$Tg^{+}$ mice from each Fli1 transgene-positive founder were exposed to Dox (100μg/mL, Calbiochem) in the drinking water and chow (200μg/g, Bio-Serv) for 5 days. HA-Fli1 expression in the hind limb *tibialis anterior* (TA) muscle was confirmed by immunoblotting (Online Figure IIID). To confirm specificity of Dox-induced Fli1 expression, double ($rtTA^{+}/Cre^{+}$) and triple-Tg^{+} mice were fed normal chow or exposed to Dox, and HA-Fli1 expression in TA muscle of triple-Tg^{+} mice exposed to Dox was confirmed by semi-quantitative RT-PCR using transgene-specific primer sets (Figure 2A) and immunoblotting (Figure 2B).

Next, we assessed the effect of MPC-specific Fli1 expression on vascular and skeletal muscle morphogenesis in developing embryos. We crossed Fli1-Tg^{+} male and homozygous double-Tg^{+} ($rtTA^{+/+}/Cre^{+/+}$) female mice. After detecting a vaginal plug (E0.5), pregnant females were provided Dox-containing drinking water and chow at E8.5. We were able to detect live double-Tg^{+} ($Fli1^{-}/rtTA^{+}/Cre^{+}$), but no triple-$Tg^{+}$, offspring (data not shown), suggesting embryonic lethality of triple-Tg^{+} embryos. Therefore, pregnant females were provided Dox-containing drinking water and chow at E8.5 and embryos were isolated at E12.5 and E15.5 (Figure 2C). We noted that compared with control (double-Tg^{+}) littermates, the growth of the triple-Tg^{+} embryos was essentially normal at E12.5 (Figure 2D), whereas their growth was markedly retarded at E15.5 (Figure 2E). Importantly, triple-Tg^{+} embryos manifested aberrant vascular morphogenesis, which was evident by the presence of blood-filled vessels within the embryo proper, otherwise absent in control embryos (Figures 2D, 2E). Moreover, aberrant vascular morphogenesis in triple-Tg^{+} embryos also impacted the normal blood-filled vascular network observed in control littermates (Figure 2E), suggesting that aberrant vasculogenesis affects the normal embryonic vascular network.

Histology, along with IHC analyses for muscle troponin I, a marker of myofibers, revealed normal myofiber structures in double-Tg^{+} embryos at E12.5 and E15.5 (Figure 2D, 2E). By contrast, triple-Tg^{+} embryos manifested abnormal limb myofiber structure at E12.5 (Figure 2D), which was essentially undetectable at E15.5 (Figure 2E). These data suggest

strongly that increased Fli1 activity in MPCs had a detrimental, age-dependent effect on MPC fate and/or differentiation potential during skeletal muscle morphogenesis. Consistent with this notion, qPCR analyses of limb RNA revealed that compared with control double-Tg^+, but Fli1-Tg^- (NTg), littermates, mRNA levels of key myogenic factors, essential for MPC fate and differentiation, were markedly attenuated in triple-Tg^+ embryos (Figure 2F), which coincided with robust induction of *Fli1* and its known downstream endothelial targets (Figure 2G). Collectively, our data lend additional support to the notion that fine tuning of *Fli1* expression in MPCs is essential for proper vascular and skeletal muscle morphogenesis during development.

Conditional Fli1 expression in myoblasts induces endothelial, but attenuates myogenic, gene expression and differentiation.

To corroborate our *in vivo* data and elucidate mechanisms whereby Fli1 attenuates skeletal muscle morphogenesis, we utilized an isogenetically modified C2C12 (iC2C12) myoblast cell line [38–40] to engineer a Dox-inducible Fli1-expressing C2C12 cell line (Figure 3A). Expression of HA-tagged Fli1 in Fli1-inducible C2C12 (iFli1-C2C12) cells upon induction with increasing concentrations of Dox (Figure 3B) and duration of induction (Figure 3C) was confirmed by immunoblotting. Lack of Fli1 expression in the absence of Dox suggests that Fli1 expression is exclusively dependent on Dox-induction (Figure 3B), which was confirmed by immunocytochemical (ICC) techniques (Online Figure IVA). We further observed that Dox-induced Fli1 expression in iFli-C2C12 cells grown in growth or differentiation medium harbored a predominant cytosolic or nuclear localization, respectively (Online Figure IVA).

Next, we set out to investigate whether nuclear Fli1 impacts the myogenic potential of myoblasts. We exposed iC2C12 and iFli1-C2C12 cells to differentiation medium and cultured them for 5 days with or without Dox treatment. In the absence of Dox, we observed efficient differentiation of both iC2C12 and iFli1-C2C12 cells into multi-nucleated myotubes (Figure 3D) as judged by the expression of myosin heavy chain (MyHC), a myotube-specific marker (Figure 3E). However, addition of Dox completely blunted myogenic differentiation of iFli1-C2C12 cells, but not control iC2C12, cells (Figure 3D); MyHC-positive myotubes and protein were detected only in Dox-treated control, but not in iFli1-C2C12, cells (Figures 3E, 3F). Importantly, compared with untreated cells, Dox-treated iFli1-C2C12 cells manifested a cobblestone appearance (Figure 3D), a hallmark feature of dedifferentiation into endothelium-like cells [41, 42]. Together, these data provide additional credence to our *in vivo* data that aberrant Fli1 activity in MPCs results in significant alteration of endothelium-like morphology at the expense of myogenic potential.

To assess the levels of Fli1 sufficient to attenuate myogenic differentiation, we treated control and iFli1-C2C12 cells with increasing concentrations of Dox for 5 days. We found that Dox-treatment had essentially no effect on differentiation potential of control iC2C12 cells (Online Figure IVB), which corresponds with no statistically significant change in their fusion index (Figure 3G). By contrast, Dox-treated iFli1-C2C12 cells revealed a dose-dependent and marked attenuation of myogenic differentiation and fusion index (Figure 3G and Online Figure IVB).

We also isolated RNA from vehicle- and Dox-treated iFli1-C2C12 cells grown in differentiation medium and assessed the expression of selected myogenic and endothelial genes. We noted that compared with vehicle-treated iFli1-C2C12 cells, transcript levels of several myogenic genes, including *MyoD* and *Myf5*, were markedly attenuated in Dox-treated cells in a dose- (Figures 4A, 4B, 4C) and time-dependent (Figures 4D, 4E) manner. By contrast, we observed robust, dose- and time-dependent induction of expression of select endothelial genes, *Cd31* (Figures 4F, 4H) and *Tie2* (Figures 4G, 4I), respectively. These data support the notion that Fli1 activity is associated with reciprocal regulation of endothelial and myogenic gene expression. Collectively, we conclude that even a subtle change in Fli1 levels in MPCs promotes an endothelial program with negative consequences on their myogenic program and differentiation potential.

Over-expression of myogenic factors restores differentiation potential of Dox-treated iFli1-C2C12 myoblasts.

A growing body of literature suggests that cooperative and overlapping actions of myogenic (Pax3/Pax7, MyoD and Myf5) [11, 12] and endothelial (ER71, FoxC1/2) [26, 27] regulators in multipotent progenitors govern expression of lineage-specific markers to dictate their cell fate decision, growth, and the genesis of specific organs. On the other hand, gain-of-function of these lineage-specific regulators in progenitors and terminally differentiated cells has been reported to redirect them to an alternative fate, highlighting the plasticity of progenitor and differentiated cells to dedifferentiate into an alternative cell fate [42–46]. These studies, along with our current data, thus raised an intriguing question regarding whether attenuation of myogenic differentiation of the Fli1-expressing iC2C12 is irreversible and whether their differentiation potential can be restored by forced expression of defined myogenic factors.

To test this, we cultured iFli1-C2C12 cells in growth medium and transduced them with retrovirus expressing control [green fluorescence protein (GFP)], Myf5 or MyoD proteins, respectively. The next day, cells were washed and cultured in differentiation medium and treated with vehicle or 50ng/mL Dox. Myogenic differentiation was assessed after 4 days. Although vehicle-treated cells efficiently differentiated into myotubes, cells treated with Dox and transduced with control or either myogenic factor-expressing virus manifested little or no differentiation potential (data not shown, Online Figure IVC). To test the effects of the dose of Dox and whether sustained expression of myogenic factors is necessary to rescue Fli1's inhibitory effect, we repeated the experiment and treated the cells with vehicle or low and high dose Dox. We also transduced cells with control and myogenic factor-expressing virus for the first two days in differentiation medium (Figure 5A). Compared with vehicle-treated cells, myogenic differentiation of Dox-treated cells transduced with control virus was attenuated in a dose-dependent manner (Figure 5B, Online Figure IVB and IVC). By contrast, myogenic differentiation of iFli1-C2C12 cells treated with low dose Dox was markedly increased in cells transduced with Myf5- or MyoD-expressing virus (Figure 5B), which coincides with restoration of expression of select differentiation markers (Figure 5C). However, Myf5 or MyoD expression did not restore myogenic differentiation of iFli1-C2C12 cells treated with high dose Dox (Online Figure IVC), despite the fact that their mRNA levels were comparable in low and high dose Dox-treated cells (Figure 5D). Although the underlying reason is unknown, a precedent for such a phenomenon has been

reported for Dux4 proteins [38, 39]. Collectively, these data suggest strongly that reduced expression of these key myogenic regulators contributes to Fli1-dependent inhibition of myogenic differentiation of MPCs *in vitro* and *in vivo*.

Elevated Fli1 expression in MPCs of mdx mice correlates with diminished expression of myogenic regulators and muscle differentiation.

Aberrant Fli1 activity is critically associated with the pathogenesis of various diseases, including Ewing's sarcoma, systemic sclerosis, and lung cancer [47–49]. Indeed, compared with a normal lung cell line, qPCR analyses revealed increased *Fli1* mRNA levels in a lung cancer cell line (Online Figure VA). Unlike heart muscle of adult mice, skeletal muscle has the capacity for complete regeneration following severe injury/stress [50–53]. This unique feature of skeletal muscle is known to derive from a population of resident somatic MPCs (also called satellite cells). Exhaustion of MPCs or perturbation of their myogenic differentiation potential is thought to be intimately linked with muscular dystrophies, a group of inherited neuromuscular disorders characterized by progressive skeletal myopathy [10, 54–56]. Consistent with a previously reported study [57], histology of hind limb TA muscle of 4-week old WT mice revealed normal morphology and peripherally nucleated myofibers, whereas *mdx* mice manifested necrotic and centrally nucleated myofibers, a hallmark of persistent muscle degeneration and regeneration (Figure 6A).

Fli1-dependent repression of the myogenic program and differentiation of MPCs prompted us to test whether the dystrophic phenotype in *mdx* mice is functionally linked with aberrant *Fli1* expression in dystrophic MPCs. To test this, we isolated MPCs from hind limbs of 4–5 week old WT and *mdx* mice. Enrichment of MPCs >95% (Figures 6B, Online Figure VB) and Fli1 expression in satellite/MPCs (Figure 6C) was validated by ICC analyses of MyoD and Fli1 as well as semi-quantitative RT-PCR (Online Figure VC). However, *Fli1* expression was undetectable in isolated myofibers (Online Figure VC), further supporting the hypothesis that spatio-temporal expression of *Fli1* in MPCs is required for normal skeletal myogenesis. In addition, bromodeoxyuridine (BrdU) incorporation assays revealed that MPCs/satellite cells from WT and *mdx* mice harbored relatively similar growth potential (Online Figures VD, VE).

To assess their differentiation potential, MPCs were cultured in growth medium until 80% confluent and then switched to differentiation medium for 5 days. We noted myotube formation by both MPCs isolated from WT and *mdx* mice, yet compared with WT MPCs, differentiation potential of MPCs from *mdx* mice was markedly diminished (Figure 6D). To unveil the underlying cues, we analyzed selected myogenic gene and *Fli1* mRNA levels in MPCs isolated from WT and *mdx* mice. qPCR analyses revealed that relative expression of *Fli1* was lower than that of all other myogenic genes analyzed in WT myoblasts (Figure 6E), and that *Fli1* mRNA levels were further reduced in myotubes (Online Figure VF). On the other hand, expression of *Myf5*, but not *Pax3*, was increased in myotubes (Online Figure VF), supporting its role in differentiation. These data along with our *in vitro* and *in vivo* data support the notion that reciprocal expression of myogenic and *Fli1* genes in MPCs is required for efficient myogenic differentiation.

To test whether such reciprocal expression of myogenic and *Fli1* genes in MPCs from *mdx* mice was associated with their attenuated differentiation potential, we compared myogenic and *Fli1* gene expression in MPCs from WT and *mdx* mice. We found that mRNA levels of *Fli1* and its downstream target, *Cd31* [25, 28], were elevated in MPCs of *mdx* mice, whereas mRNA levels of several key myogenic factors, including *Pax3* and *Myf5*, were decreased in MPCs of *mdx* mice (Figure 6E).

Collectively, our report coupled with previously reported studies [8, 9, 13], support a model in which Etv2 governs *Fli1* expression in $Flk1^+$ multipotent progenitors in early embryos (around E7.75), which subsequently differentiate into $Etv2^-/Flk1^-/Pax3^+/Fli1^+$ mesoderm (known as MPCs). A gradient in myogenic regulatory factors and Fli1 levels in a subset of MPCs governs their myogenic versus endothelial potential by reciprocally regulating lineage-specific gene programs, which is essential for normal vascular and skeletal muscle morphogenesis at and beyond mid-gestation (Figure 7).

DISCUSSION

This study unveils a previously unrecognized mechanism of vascular morphogenesis governed by Fli1 (Figure 7). Specifically, we report four important findings. First, in addition to expression in vascular and endocardial endothelium, we demonstrate that *Fli1* expression occurs in MPCs of dermomyotome and somites. Second, utilizing Fli1 loss- and gain-of-function strategies in MPCs, we demonstrate that Fli1 activity is essential for reciprocal regulation of the expression of critical endothelial and myogenic genes. Third, we discovered a previously unrecognized effect of Fli1 activity on MPC differentiation and show that the Fli1-mediated inhibitory effect can be rescued by forced expression of essential myogenic regulators. Finally, and from a broader perspective, we detected aberrant Fli1 activity in MPCs harvested from muscular dystrophic mice, which was accompanied by significantly attenuated myogenic gene expression and differentiation. In aggregate, these data highlight the physiological significance of the fine tuning of *Fli1* expression in MPCs for proper vascular and muscle morphogenesis during development and disease.

Reciprocal control of endothelial versus myogenic cell fate.

Spatiotemporal expression of Etv2 is known to be essential for the genesis of hemato-endothelial progenitors [19–22]. Similarly, Pax3/Pax7, along with Myf5 and MyoD, are critical to the governance of fate, growth and differentiation of MPCs in developing embryos [10–12, 58]. As such, myogenic [18] and endothelial [26, 27] progenitors have been shown to adopt alternative fates in *Pax3* and *Etv2* mutant embryos, respectively, supporting the genesis of vascular and muscle cells from common progenitors. However, the mechanism whereby the endothelial potential of MPCs is maintained to promote vascular morphogenesis is unclear, given that expression of *Etv2* stops at mid-gestation. Having detected temporal expression of Etv2 in MPCs of early embryo [26], it is conceivable that a downstream target of Etv2 compensates this function. Our data suggest that Fli1 plays this role.

We have provided comprehensive evidence to support the notion that Fli1 expression in MPCs reciprocally governs vascular versus myogenic programs in MPCs to promote endothelial potential of the progenitors in the absence of Etv2. Although the expression of

double homeobox proteins DUX4 and DUX4c in dermomyotome has not been reported, ectopic expression of these proteins in myoblasts also attenuated myogenic gene expression and differentiation without inducing endothelial gene expression or transducing myoblasts to a cobblestone appearance [38, 39]. Unlike Fli1, induction of Dux4 in myoblasts triggers cellular toxicity in a dose-dependent manner. Therefore, it is conceivable that the molecular action of Fli1 in myoblasts is distinct from that of DUX4/DUX4c.

The precise mechanism whereby expression of select myogenic and endothelial transcription factors, including Fli1, governs lineage-specific cell fate decisions of progenitor cells is incompletely understood. It has been proposed that activation and overlapping action of specific signaling pathways results in a gradient of specific protein levels within progenitors that dictates progenitor cell fate decision from one lineage to another. Indeed, numerous signaling pathways from surrounding neural tube and notochord have been identified to modulate the expression and activity of lineage-specific transcription factors. For example, BMP (bone morphogenetic protein), Notch and Wnt signaling pathways act upstream of Etv2 to promote the genesis of Flk^+ hemato-endothelial mesoderm [21], whereas BMP and Notch signaling inhibit myogenesis by repressing myogenic gene expression in cardiomyocytes [59] and MPCs [60]. On the other hand, Wnt and sonic hedgehog (Shh) antagonize BMP signaling and promote myogenesis by increasing specification of muscle progenitors and MyoD expression [60–62]. Together, these studies suggest that cooperative and overlapping action of specific signaling pathways activates lineage-specific transcription factor(s) and their downstream targets in progenitors to dictate specific lineage commitment during development.

Consistent with this notion, genetic manipulation of the dose of Hand2/Nkx2–5 and Etv2/Scl in mouse and zebrafish embryo reciprocally modulates cardiomyocyte versus endocardial fate of progenitors in anterior lateral plate mesoderm [63]. Similarly, doses of Pax3 and FoxC2 reciprocally modulate myogenic versus endothelial fate of MPCs [18, 64]. As Etv2 does not express at mid-gestation, and vasculogenesis and myogenesis are relatively normal in *Foxc2*-null embryos at mid-gestation [17, 65], it is plausible that the balance between the dose of Fli1 and myogenic regulators in MPCs is critically associated with endothelial versus myogenic potential of MPCs in the absence of Etv2 (Figure 7). Given that the cooperative action of FoxC2 and Etv2 is essential for robust endothelial gene expression and that FoxC2 is required for endothelial fate of MPCs at mid-gestation [18, 64], it is conceivable that such cooperative action of Fli1 and FoxC2 might exist to modulate endothelial gene expression and endothelial potential of MPCs. Single and compound inactivation of *Fli1* and *FoxC2* in MPCs will be required to formally test that possibility. Regardless, our data demonstrate that Fli1 activity in MPCs is critically associated with endothelial potential of progenitors at the expense of a myogenic fate. Indeed, conditional and myogenic progenitor-specific expression of EWS-Fli1, a Ewing's sarcoma-causing fusion protein harboring Fli1 DNA binding and activation domains, in embryonic limb bud triggers a number of developmental defects in limbs [66].

Earlier studies revealed that global loss of Etv2 significantly increased Etv2 promoter-driven reporter gene expression in both somites and myocardium of early embryos [26], whereas our studies show that global loss of Fli1 increased only *Fli1* promoter-driven

reporter gene expression in somites but not in myocardium (Figure 1 and Online Figure I). These results suggest that the Etv2-Fli1 axis preferentially governs endothelial potential of the multipotent myogenic, but not cardio/hemato-endothelial, mesoderm (Figure 7). Therefore, future studies employing ectopic expression of Etv2 in *Fli1*-deficient fibroblasts or myoblasts and *vice versa* will be required to assess their independent contribution to reciprocal regulation of endothelial versus myogenic gene expression in progenitor cells and converting terminally differentiated non-endothelial cells into endothelial cell (EC)-like cells.

Potential therapeutic implications.

Direct conversion of postnatal cells into ECs, cardiomyocytes, or muscle cells has potential implications for regenerative medicine, cell therapy, and pathophysiological investigation. Ectopic expression of Pax3 and MyoD [44, 45] or Etv2 [42, 46] in terminally differentiated fibroblasts induces downstream gene expression and converts fibroblasts into muscle- or EC-like cells, supporting their role at the top of transcriptional hierarchy of myogenic and endothelial programs, respectively. However, induction of endothelial genes and conversion of fibroblasts into EC-like cells by ectopic expression of Etv2 requires long-term treatment with high dose Dox [42, 46]. By contrast, induction of Fli1 expression using a very low dose of Dox triggered robust induction of endothelial gene expression within hours and dedifferentiation of myoblasts into EC-like morphology within just 2 days. We have reported previously that spatiotemporal expression of Etv2 initiates *Fli1* expression in early embryo, and that Fli1 governs its own expression, as well as that of several other Etv2-regulated endothelial genes, in embryonic and adult endothelium [28]. Therefore, induction of endothelial genes and conversion of fibroblasts to EC-like cells by ectopic expression of Etv2 might be associated with Etv2-dependent induction of *Fli1* expression in fibroblasts.

Conclusion.

In conclusion, endothelial dysfunction is a cardinal feature of cardiovascular, diabetic, hypertensive, and cancer pathogenesis [6]. Elevated *Fli1* expression in MPCs of dystrophic mice raises an intriguing question regarding whether elevated *Fli1* expression in MPCs of dystrophic patients is also associated with muscular dystrophy pathogenesis. Conditional Fli1 loss- and gain-of-function in satellite cells will be necessary to uncover the physiological significance of Fli1 in vascular and muscle regeneration in WT and dystrophic mice following injury. Nevertheless, our results highlight the potential clinical benefit of discovering small molecules to fine tune Fli1 activity in progenitors and terminally differentiated cells as a therapeutic target for regenerative medicine to mitigate cardiovascular, metabolic, and muscle wasting diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms:

Fli1	Friend leukemia integration 1
ETS	E26 transformation-specific
E	Embryonic
EC	Endothelial cell
MPC	Myogenic progenitor cell
MRF	Myogenic regulatory factor
TRE	Tetracycline responsive element
rtTA	Reverse tetracycline transactivator
qRT-PCR	quantitative RT-PCR

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NOVELTY AND SIGNIFICANCE

What Is Known?

- The ETS (E26 transformation-specific) family transcription factor Etv2 and its downstream target Fli1 govern vascular morphogenesis at distinct developmental stages via a feed-forward autoregulatory loop.
- Etv2 initiates vascular morphogenesis in early embryo, whereas Fli1 is essential for vascular morphogenesis and homeostasis at mid-gestation, the developmental stage at which *Etv2* expression extinguishes.
- At mid-gestation, Fli1 promotes endothelial cell survival by regulating its own expression as well as the expression of several other Etv2 target endothelial genes. However, molecular mechanisms of Fli1 action in vascular morphogenesis remain incompletely understood.

What New Information Does This Article Contribute?

- We have identified Etv2-independent *Fli1* gene expression in multipotent myogenic progenitor cells (MPCs) in developing and adult mice and report that *Fli1* expression is absent in skeletal myofibers.
- Increased Fli1 activity in MPCs induced endothelial gene expression but attenuated critical myogenic gene expression and myogenic differentiation. Forced expression of select myogenic regulator(s) rescued the differentiation potential of Fli1-expressing MPCs, suggesting that Fli1 promotes vascular morphogenesis by inversely regulating endothelial versus myogenic potential in MPCs.
- Compared with wild-type mice, elevated *Fli1* expression was also detected in MPCs of X-linked muscular dystrophic *mdx* mice.

Mice lacking Fli1 die *in utero* around embryonic day 12.0 due to vascular hemorrhage and EC death, supporting its essential roles in vascular morphogenesis and integrity at and beyond mid-gestation. However, molecular mechanisms of Fli1 action in vascular morphogenesis remain unknown. This study was designed, therefore, to unveil mechanisms whereby Fli1 governs vascular morphogenesis. We demonstrate Etv2-independent *Fli1* expression in dermomyotome and somites, the site of residence of multipotent myogenic progenitor cells (MPCs), as well as in adult MPCs (also called satellite cells). Loss of Fli1 in MPCs markedly increased transcript levels of several critical myogenic regulators, including Pax3 and Myf5. Conversely, MPC-specific Fli1 gain-of-function or elevated *Fli1* expression in MPCs of *mdx* mice resulted in reciprocal regulation of endothelial versus myogenic gene expression, leading to attenuated myogenic differentiation and myofiber formation. Ectopic expression of select myogenic regulators (e.g. Myf5) rescued the myogenic potential of Fli1-expressing myoblasts. In aggregate, our data uncover an important and previously unrecognized mechanism of vascular morphogenesis governed by Fli1 and highlight the physiological significance of

the fine tuning of Fli1 activity in MPCs for proper vascular and muscle morphogenesis during development and disease.

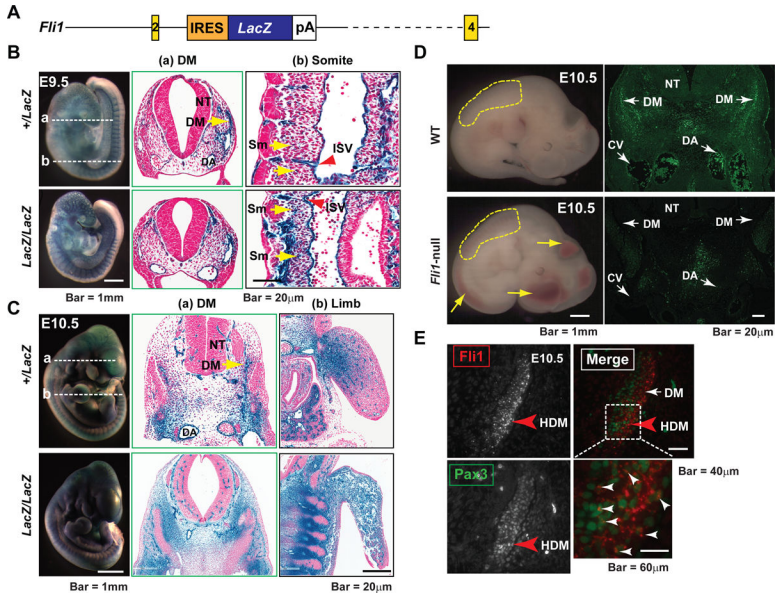

Figure 1. Loss of *Fli1* expression reciprocally governs endothelial versus myogenic gene expression at mid-gestation.

(**A**) Schematic of *Fli1-LacZ* knockin mouse model indicating that *LacZ* expression is under the control of the native *Fli1* promoter. Numbers within yellow rectangles denote exon. (**B, C**) Whole-mount LacZ staining of *Fli1*-het (+/LacZ) and *Fli1*-null (LacZ/LacZ) embryos at E9.5 (B) and E10.5 (C) revealing *LacZ* expression in entire embryo proper (bar = 1mm). Enlarged view of transverse section (see Online Fig. IE and IF) through heart (a) and posterior (b) (dashed lines) region reveals robust *Fli1* expression in the vasculature and dorsal aorta (DA) (bar = 20μm). Note a subset of $LacZ^+$ cells in mesoderm of dermomyotome (DM) (middle) and somites (right) in het embryos, which was significantly increased in E9.5 null embryo and extended into entire limbs of E10.5 null embryo (yellow arrows). Neural tube (NT), intersomatic vessel (ISV), somite (Sm) are indicated. (**D**) Whole-mount (left; bar = 1mm) and immunohistochemical (right; bar = 20μm) analyses for Fli1 of E10.5 *Fli1*-WT (WT) and *Fli1*-null embryos. Note hemorrhage (arrows) within embryo proper and lack of Fli1 expression in the DM and vasculature, DA and cardinal vein (CV) of null embryo. (**E**) An adjacent section of WT embryo was also immunostained for Pax3, a key marker of myogenic progenitors, to confirm Pax3 and Fli1 expression in DM (bar = 40μm), with higher intensity and co-localization in hypaxial DM (HDM) (bar = 60μm). (**F**) qRT-PCR analyses of mRNA levels of the indicated genes in somatic tissue of WT and *Fli1*-null embryos (yellow dotted line in panel D). Note the marked induction of critical myogenic gene expression in null embryo (n = 4). Data are depicted as mean ± SEM, and nonparametric statistical analyses using Mann-Whitney U test were performed. P=0.028 vs WT; NS indicates nonsignificant vs WT.

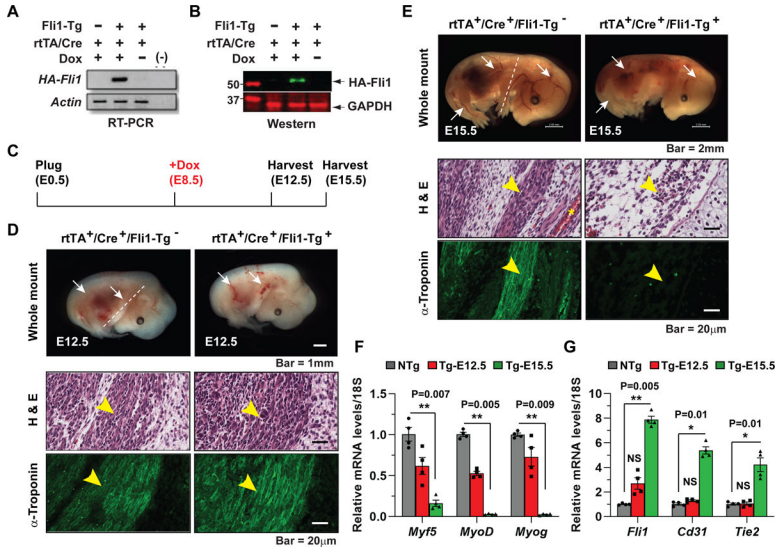

Figure 2. Conditional Fli1 gain-of-function in MPCs blunts myogenesis and reciprocally governs myogenic and endothelial gene expression in vivo.

(A, B) Analyses of Fli1 expression in Fli1 transgenic (Fli1-Tg) mice with (+) or without (−) doxycycline (Dox) treatment using semi-quantitative RT-PCR (A) and immunoblotting (B) techniques. Actin (A) and GAPDH (B) served as loading controls. Note the Fli1 expression only in the Dox-treated triple transgene-positive mice. (C) Schematic of Dox treatment of pregnant females to induce Fli1 expression in MPCs and isolation of embryos at the indicated developmental stages, where detection of a vaginal plug was counted as embryonic day 0.5 (E0.5). (D, E) Gross (whole mount) view of embryos with indicated genotypes and developmental stages are shown (E12.5, bar = 1mm and E15.5, bar = 2mm). Note the aberrant blood-filled vasculature (arrow) in E12.5 Fli1-Tg+ embryos, otherwise absent in control littermates (D), resulted in diminished normal blood-filled vasculature at E15.5 Fli1-Tg+ embryos (E). Serial and adjacent sections through forelimb (white dotted line) were used for hematoxylin and eosin (H&E) and IHC analyses of skeletal troponin, respectively (bar = 20μm). Note the abnormal and absence of myofibers in triple-Tg+ embryos at E12.5 and E15.5, respectively. (F, G) qRT-PCR analyses of mRNA levels of the indicated myogenic (F) and endothelial (G) genes in hind limbs of embryos with indicated genotypes and developmental stages. Relative mRNA levels in Fli1 non-transgenic (NTg) embryos were set to 1 (n = 4). Data are depicted as mean ± SEM, and nonparametric statistical analyses using the Kruskal-Wallis test with Dunn's multiple comparisons test were performed. Statistical significance (p values) for each gene vs NTg is shown. NS indicates nonsignificant vs NTg.

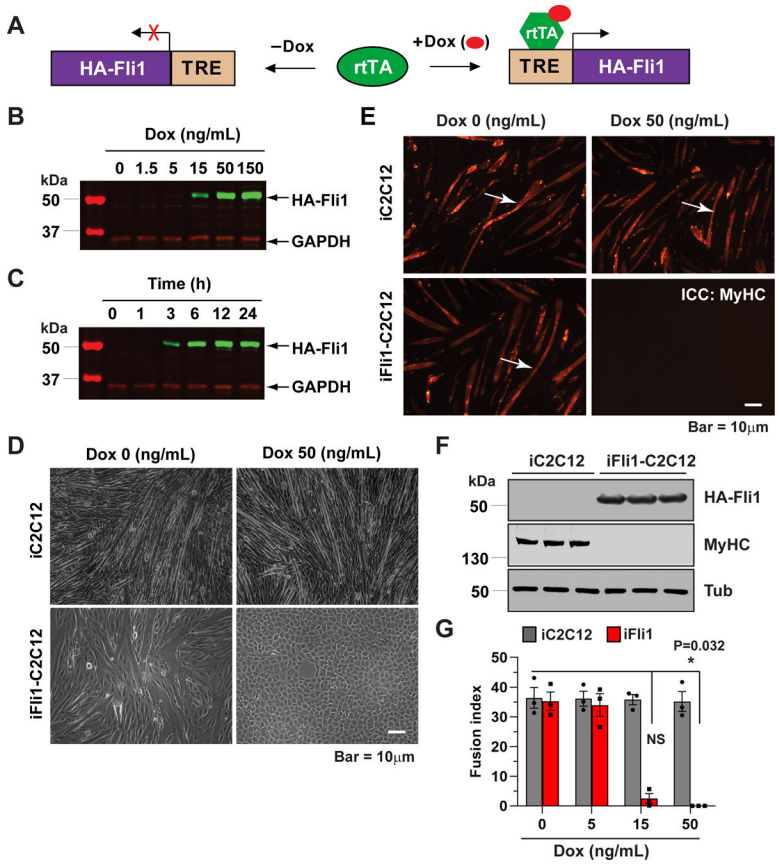

Figure 3. Conditional Fli1 gain-of-function in myoblasts blunts myogenic differentiation and myogenic gene expression in vitro.

(A) Schematic of Dox-inducible Fli1-expressing C2C12 cells. Note that the reverse tetracycline transactivator (rtTA) binds to the tetracycline responsive element (TRE) only in Dox-treated cells to induce *Fli1* expression. (**B, C**) Immunoblotting assays indicating dose- (B) and time-dependent (C) induction of HA-tagged Fli1 levels in Dox-treated Fli1-inducible C2C12 (iFli1-C2C12) cells. Fli1 expression was not detected in untreated cells (B). GAPDH was used as loading control and protein standard markers (kDa) are shown. (**D-F**) Myogenic differentiation of iC2C12 and iFli1-C2C12 cells was analyzed in the absence or presence of Dox (50 ng/mL) (bar = 10μm). Note that Dox treatment blunted myogenic differentiation of iFli1-C2C12, but not the parental iC2C12, cells (D). Marked attenuation of myogenic differentiation of Dox-treated iFli1-C2C12 was confirmed using immunocytochemical (ICC) (**E**) and immunoblotting (**F**) analyses of Fli1 and myosin heavy chain (MyHC), a marker of myotubes. Tubulin (Tub) was used as loading control. (**G**) Fusion index of iC2C12 and iFli1-C2C12 (iFli1) cells treated with indicated concentrations of Dox (n = 3). Note the dose-dependent reduction of fusion index in iFli1-C2C12, but not iC2C12, cells. Data are depicted as mean ± SEM, and nonparametric statistical analyses using the Kruskal-Wallis test with Dunn's multiple comparisons test were performed. *P*=0.032 vs control (iC2C12-Dox0) and NS indicates nonsignificant vs control.

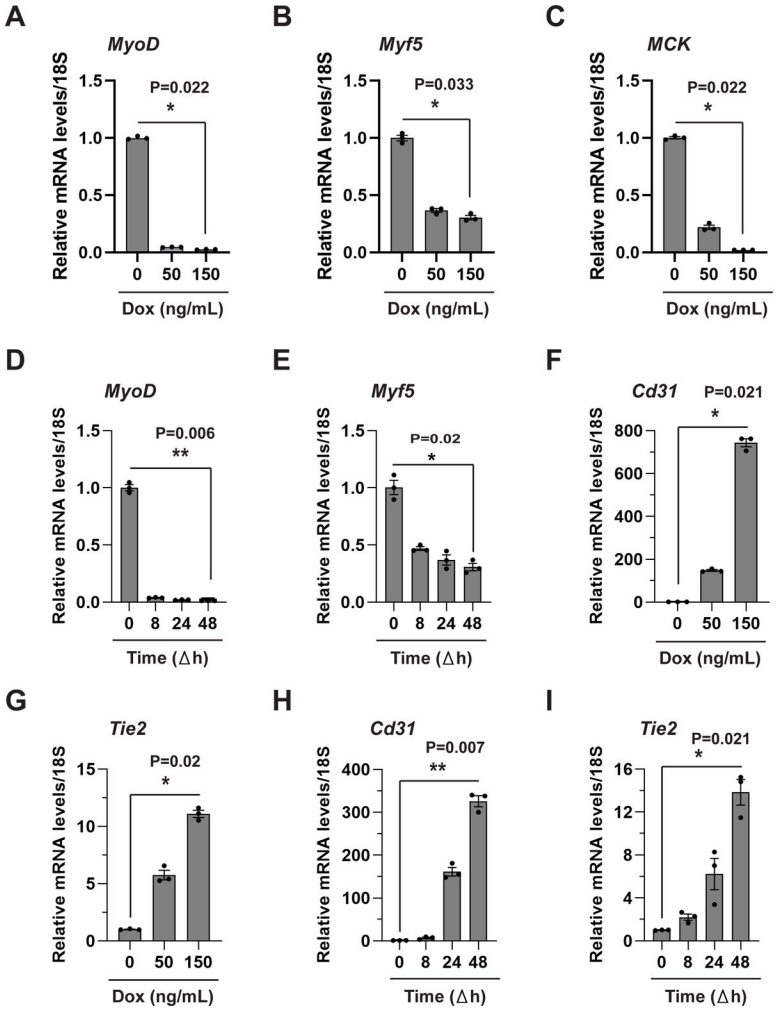

Figure 4. Reciprocal regulation of myogenic and endothelial genes in Dox-treated iFli1-C2C12 cells.

(**A-I**) qRT-PCR analyses of mRNA levels of the indicated myogenic (*MyoD, Myf5* and *MCK*) and endothelial (*Cd31* and *Tie2*) genes in Dox-treated and -untreated iFli1-C2C12 cells (n = 3). Note the dose- and time-dependent attenuation of myogenic genes (A-E), whereas dose- and time-dependent induction of endothelial genes (F-I) expression is seen in Dox-treated cells. Relative mRNA levels of each gene in untreated cells were set to 1. In all panels, data are depicted as mean ± SEM, and nonparametric statistical analyses using the Kruskal-Wallis test with Dunn's multiple comparisons test were performed. Statistical significance (p values) for each gene vs control is shown.

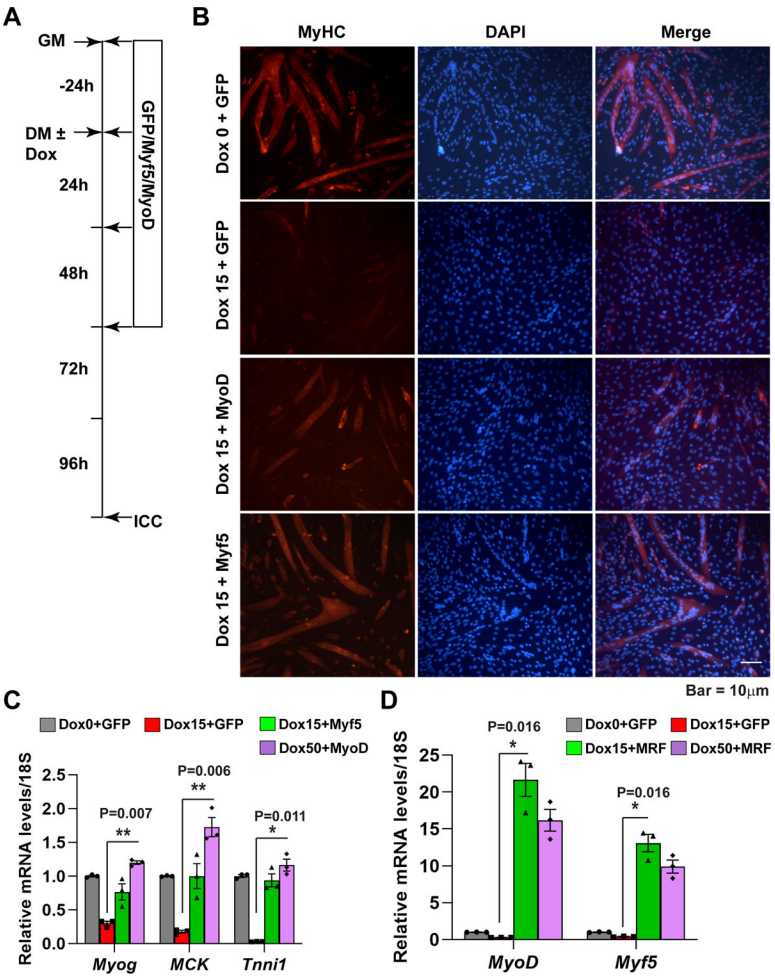

Figure 5. Ectopic expression of myogenic factors restores myogenic potential of Dox-treated iFli1-C2C12 cells.

(**A**) Schematic of experimental strategies for retrovirus-mediated ectopic expression of control (GFP) and indicated myogenic factors in untreated and Dox-treated iFli1-C2C12 myoblasts cultured in growth (GM) or differentiation (DM) medium. (**B**) ICC analysis of MyHC in Dox-treated and -untreated iFli1-C2C12 cells after 4 days in DM and transduced with retrovirus-expressing control or the indicated myogenic proteins. DAPI was used for nuclear staining (bar = 10μm). (**C, D**) qRT-PCR analyses of the indicated genes in untreated and Dox-treated iFli1-C2C12 cells transduced with control or Myf5- and MyoD-expressing retrovirus. Relative mRNA levels in control cells were set to 1. Data are depicted as mean ± SEM (n = 3), and nonparametric statistical analyses using the Kruskal-Wallis test with Dunn's multiple comparisons test were performed in both panels. Only the statistical significance (p values) for each gene vs Dox15+GFP is shown.

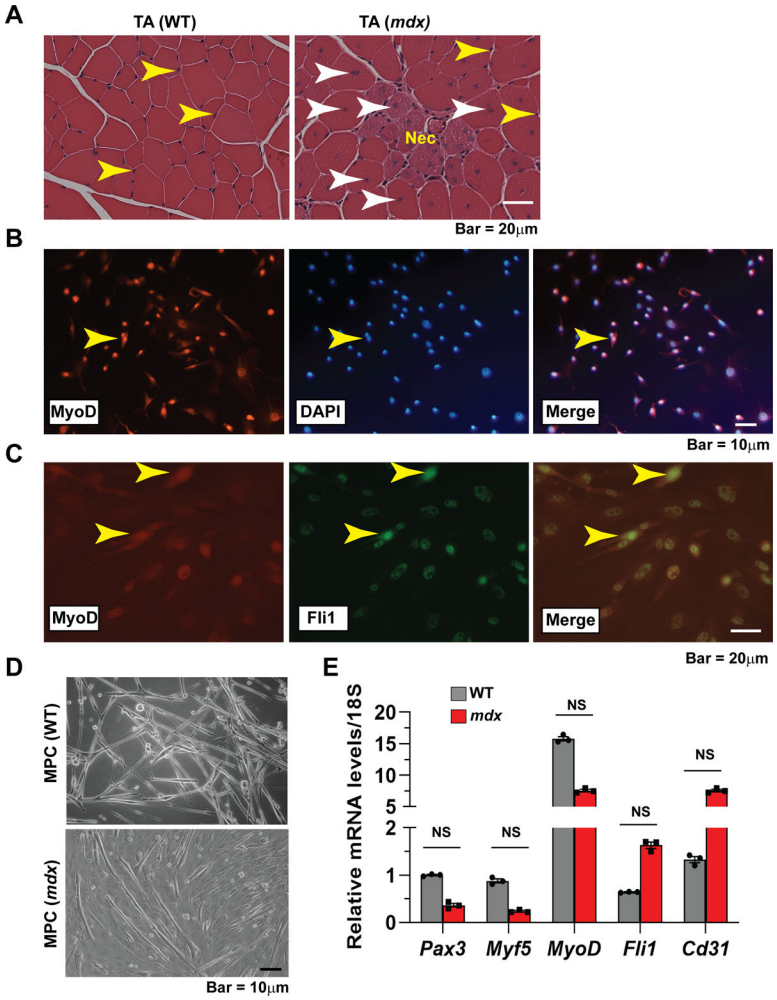

Figure 6. Elevated *Fli1* expression in MPCs from *mdx* mice coincides with attenuated myogenic gene expression and differentiation.

(**A**) Histology of hindlimb *tibialis anterior* (TA) muscle of 4-week old WT and *mdx* mice (bar = 20μm). Note that compared with the normal morphology and peripherally nucleated myofibers of WT mice, *mdx* mice manifested necrotic (Nec) and centrally nucleated myofibers, a hallmark of persistent muscle degeneration and regeneration. (**B, C**) ICC analyses and merged view of indicated proteins in MPCs/satellite cells isolated from WT mice are shown. Note the significant enrichment of MPCs and co-expression of MyoD and Fli1 in MPCs. DAPI was used for nuclear staining [bar = 10μm (B) and 20μm (C)]. (**D**) Bright field images of myotube formed by MPCs/satellite cells isolated from WT and *mdx* mice. Note that compared with WT mice, differentiation potential of *mdx* MPCs/satellite cells is diminished (bar = 10μm). (**E**) qRT-PCR analyses of the indicated genes in MPCs/satellite cells isolated from WT and *mdx* mice (n = 3). Note the inverse correlation of myogenic (*Pax3*, *Myf5* and *MyoD*) and endothelial (*Fli1* and *Cd31*) gene expression in *mdx* mice. Relative mRNA levels of *Pax3* in WT mice were set to 1 and mRNA levels of *18S* were used as control. Data are depicted as mean ± SEM, and nonparametric statistical analyses using Mann-Whitney U test were performed. NS indicates nonsignificant vs WT.

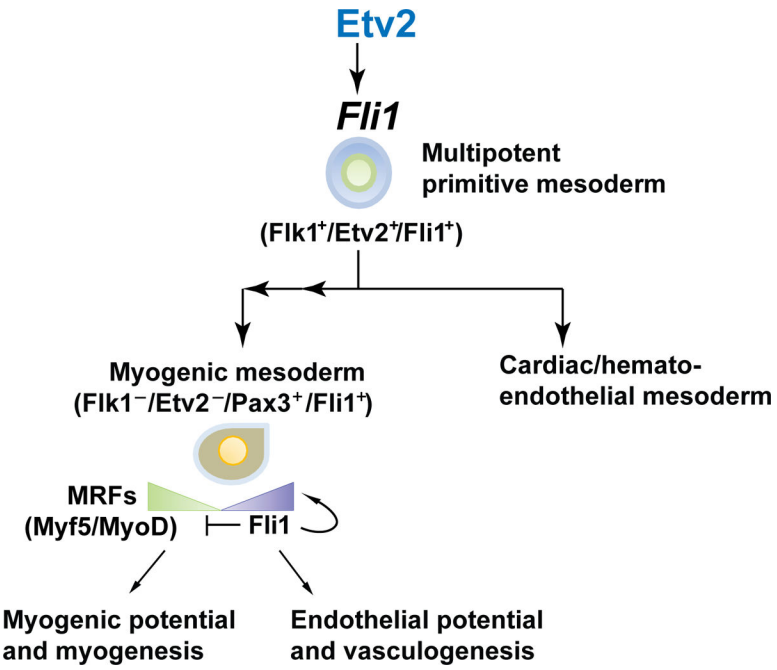

Figure 7. Working model for Fli1's role in vascular morphogenesis in the absence of its upstream regulator, Etv2.

Etv2 induces *Fli1* expression in multipotent Flk1^{+} primitive mesoderm in early embryos (~E7.5), which has been shown previously to generate mesoderm with cardiac and hemato-endothelial potential. The primitive mesoderm subsequently differentiates giving rise to Flk1^{-}/Etv2^{-} but Pax3^{+}/Fli1^{+} multipotent myogenic mesoderm in the dermomyotome (called hypaxial, paraxial and epaxial mesoderm). A gradient of myogenic regulatory factors [MRFs, (e.g. Myf5/MyoD)] in mesoderm directs myogenic fate and muscle morphogenesis. However, enriched levels of Fli1 in a subset of myogenic mesoderm governs its own expression and that of select downstream endothelial genes to regulate endothelial potential of the mesoderm at the expense of myogenic potential.