FOXF1 Transcription Factor Is Required for Formation of Embryonic Vasculature by Regulating VEGF Signaling in Endothelial Cells

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ABSTRACT

**Rationale:** Inactivating mutations in the FOXF1 gene locus are frequently found in patients with Alveolar Capillary Dysplasia with Misalignment of Pulmonary Veins (ACD/MPV), a lethal congenital disorder, which is characterized by severe abnormalities in the respiratory, cardio-vascular and gastro-intestinal systems. In mice, haploinsufficiency of the Foxf1 gene causes alveolar capillary dysplasia and developmental defects in lung, intestinal and gall bladder morphogenesis.

**Objective:** While FOXF1 is expressed in multiple mesenchyme-derived cell types, cellular origins and molecular mechanisms of developmental abnormalities in FOXF1-deficient mice and ACD/MPV patients remain uncharacterized due to lack of mouse models with cell-restricted inactivation of the Foxf1 gene. In the present study, the role of FOXF1 in endothelial cells was examined using a conditional knockout approach.

**Methods and Results:** A novel mouse line harboring Foxf1-floxed alleles was generated by homologous recombination. Tie2-Cre and Pdgfb-CreER transgenes were used to delete Foxf1 from endothelial cells. FOXF1-deficient embryos exhibited embryonic lethality, growth retardation, polyhydramnios, cardiac ventricular hypoplasia and vascular abnormalities in the lung, placenta, yolk sac and retina. Deletion of FOXF1 from endothelial cells reduced endothelial proliferation, increased apoptosis, inhibited VEGF signaling and decreased expression of endothelial genes critical for vascular development, including VEGF receptors Flt1 and Flk1, Pdgfb, Pecam1, CD34, integrin β3, ephrin B2, Tie2 and the non-coding RNA Fendrr. ChIP assay demonstrated that Flt1, Flk1, Pdgfb, Pecam1 and Tie2 genes are direct transcriptional targets of FOXF1.

**Conclusions:** FOXF1 is required for formation of embryonic vasculature by regulating endothelial genes critical for vascular development and VEGF signaling.

**Keywords:** FOXF1, endothelial cells, VEGF, Flk1, vascular development, angiogenesis, pulmonary circulation, developmental biology, gene regulation, genetically altered mice

**Nonstandard Abbreviations and Acronyms:**

- Cre: Cre recombinase
- E: Embryonic day
- FOXF1: Forkhead Box transcription factor F1
- VEGF: Vascular Endothelial Growth Factor
- ACD/MPV: Alveolar Capillary Dysplasia with Misalignment of Pulmonary Veins
- HSV-TK: Herpes simplex virus-thymidine kinase
- EC: Endothelial cells

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INTRODUCTION

Development of the embryonic vasculature depends on vasculogenesis (de novo formation of blood vessels) and angiogenesis (branching of preexisting blood vessels) in a process requiring appropriate levels of Vascular Endothelial Growth Factor (VEGF). Targeted disruption of the Vegf gene produces an embryonic lethal phenotype displaying impaired blood-island formation and delayed endothelial cell differentiation, leading to abnormal blood vessel development. VEGF is the ligand for tyrosine kinase receptors Flk1 and Flt1, both of which are expressed in endothelial cells and their mesenchymal precursors. Flk1-/- mice die in utero due to inhibition of vasculogenesis and formation of angioblast cells in the blood islands, whereas Flt1-/- embryos fail to form mature blood vessels. Other signaling pathways involved in formation of embryonic vasculature include Angiopoietin/Tie2, PDGF, PI3K/AKT, TGF-β, Shh, Wnt and Notch, as well as transcription factors Etv2, Hand1, MEF2c, Prox1, Hey1/2, COUP-TFI, Tbx4, Snail, FOXC2, GATA, Sox and KLF (reviewed in 5-7). Identification of additional proteins that regulate embryonic vascular development will provide information regarding pathogenesis of human vascular disorders.

Alveolar Capillary Dysplasia with Misalignment of Pulmonary Veins (ACD/MPV) is a congenital disorder of neonates and infants, which is characterized by severe defects in development of pulmonary capillaries, hypoxemia, pulmonary hypertension and thickening of small pulmonary arteries, malposition of pulmonary veins, lung edema and impaired lobular development. Structural abnormalities of the genitourinary, gastrointestinal and cardiovascular systems are also common. Due to the severity of developmental defects and progressive respiratory insufficiency in ACD/MPV infants, the survival after the first month of birth is rare. While genetic factors associated with ACD/MPV are not fully characterized, heterozygous deletions and point mutations in the Forkhead Box F1 (FOXF1) gene locus account for approximately 40% of ACD/MPV cases. In addition, genomic deletions in FOXF1 gene were recently found in prenatal cystic hygroma, a congenital vascular defect which can result in fetal hydrops (tissue edema) and embryonic death. These clinical data illustrate a critical role of FOXF1 in vascular development.

FOXF1 protein (previously known as HFH-8 or Freac-1) is a member of the Forkhead Box (Fox) family of transcription factors that share homology in the Winged helix/Forkhead DNA binding domain. FOXF1 is expressed in extraembryonic mesoderm, allantois, splanchnic mesoderm and septum transversum mesenchyme. Foxf1-/- mice die by E8.5 due to severe abnormalities in development of the yolk sac and allantois. While FOXF1 haploinsufficiency causes alveolar capillary dysplasia, fusion of the lung lobes and various developmental defects in mesenchyme of the gallbladder, esophagus and trachea, Foxf1+/ mice do not recapitulate all histopathological features of human ACD/MPV. Approximately half of Foxf1+/ mice survived past birth but these mice exhibited severe pulmonary hemorrhage in response to lung injury and abnormal liver regeneration after liver injury.

FOXF1 is activated by the Shh signaling pathway through a direct binding of Gli transcription factors to the Foxf1 promoter region. Deletions of Gli-binding sites were found in the FOXF1 gene locus of ACD/MPV patients, implicating Shh/Gli signaling in regulation of Foxf1 gene expression. FOXF1 induces migration of mesenchymal cells through direct transcriptional activation of Integrin β3 and Notch-2 genes. While FOXF1 is expressed in multiple mesenchyme-derived cell types, including fibroblasts, peribronchial smooth muscle, endothelial and hepatic stellate cells, cellular origins and molecular mechanisms of developmental abnormalities in FOXF1-deficient mice and ACD/MPV patients remain uncharacterized due to the lack of mouse models with cell-restricted inactivation of the Foxf1 gene. In the present study, we generated mice harboring Foxf1-floxed alleles and used Tie2-Cre and Pdgfb-CreER transgenes to investigate the role of FOXF1 in endothelial cells. We demonstrated that FOXF1 is critical for formation of embryonic vasculature by...
stimulating endothelial proliferation and promoting the VEGF, PDGF and Angpt/Tie2 signaling pathways in endothelial cells through direct transcriptional activation of Flk1, Flt1, Pdgfb and Tie2 genes.

METHODS

Generation of Foxf1-floxed mice and deletion of FOXF1 from endothelial cells.

Foxf1-targeting vector contained a LoxP site inserted into the Foxf1 promoter and PGK-gb2 LoxP/FRT-flanked Neomycin (neo) cassette placed into the first intron (Fig. 2A). The PGK promoter-driven herpes simplex virus-thymidine kinase (HSV-TK) gene was placed outside of the Foxf1 gene homology region for negative selection of non-homologous recombination in ES cells. The Foxf1β-targeting vector was used for electroporation of mouse ES cells (C57Bl/6 x 129/SVEV), which were selected for neo (G418) and HSV-TK resistance (ganciclovir). ES cells with the appropriate Foxf1β-targeted locus were used to generate chimeric mice by injecting Foxf1β ES cells into mouse blastocysts. Mice containing the Foxf1β-targeted allele were determined by PCR amplification with primers flanking the LoxP sequence located in the Foxf1 promoter (P1 and P2) and primers located in the 3’ region of the Foxf1β allele (P3 and P4) (Fig. 2A-B and Online Table I). To produce Foxf1β/β mice, chimeric mice were bred with C57Bl/6 mice in the animal facility of Cincinnati Children’s Hospital Medical Center. The Neo cassette was deleted by breeding of Foxf1β/β mice with ACT-FLP1 mice (Jackson Lab.) (Fig. 2A). The loss of Neo in Foxf1β/β mice was confirmed by PCR using P5 and P6 primers (Fig. 2A and Online Table I). Foxf1β/β mice were backcrossed to generate viable Foxf1β/β mice that were bred into the C57Bl/6 background for ten generations. Deletion of the Foxf1β alleles from endothelial cell lineage was accomplished through breeding with Tie2-Cre (C57Bl/6, Jackson Lab.) and Pdgfb-CreER (C57Bl/6, 22) transgenic mice. To activate Pdgfb-CreER, tamoxifen was given in food (200mg of tamoxifen citrate with 24.8g of sucrose per kg of diet, Harlan Lab.) at E9.5. For postnatal activation of Cre, tamoxifen was injected i.p. (20μg/ day) at postnatal days P0, P1 and P2. Deletion of FOXF1 was confirmed by breeding FOXF1-deficient mice with LoxP-stop-LoxP-β-gal (R26R) and LoxP-tldTomato-LoxP-GFP (mT/mG) reporter mice (both from Jackson Lab.). Flk1-null mutant mice were previously described 23. Animal studies were approved by the Animal Care and Use Committee of Cincinnati Children’s Hospital Research Foundation.

RNA preparation and quantitative real-time RT-PCR (qRT-PCR).

Total RNA was prepared from MFLM-91U cells, mouse tissue and flow-sorted endothelial cells using RNeasy micro kit (Qiagen). qRT-PCR analysis was performed using a StepOnePlus Real-Time PCR system (Applied Biosystems) as described 24. Samples were amplified using inventoried TaqMan primers (Online Table II). Reactions were analyzed in triplicates and expression levels were normalized to β-actin mRNA.

siRNA transfection, Western blot and matrigel angiogenesis assay.

MFLM-91U cells 20 were cultured in serum-free UltraCULTURE medium (Lonza, Walkersville, MD). To inhibit FOXF1, we transfected either non-targeting siRNA or siRNA specific to mouse Foxf1 (Dharmacon) using Lipofectamine™ 2000 reagent (Invitrogen) as described 20, 25. Cells were harvested 48 hours after transfection and used for matrigel angiogenesis assay (BD Biosciences). VEGF 165 (20 ng/ ml, Millipore) was added to matrigel for 14 hr. Cells in matrigel were stained with calcein AM fluorescent viability dye which is transported through the cellular membrane into live cells. Confocal 3D images were quantitated using IMARIS software (Bitplane, CT). Western Blot analysis was performed using antibodies described in Supplemental Material. Detection of the immune complex was accomplished by using secondary antibodies directly conjugated with HRP followed by the Supersignal chemiluminescence substrate (Pierce, Rockford, IL).
Immunohistochemical staining and Flow cytometry.
Paraffin sections were stained with hematoxylin and eosin (H&E) or used for immunohistochemical staining as described 26, 27. Primary antibodies and detection systems are listed in Supplemental Material. For co-localization experiments, secondary antibodies conjugated with Alexa Fluor 488 or Alexa Fluor 594 (Invitrogen) were used as previously described 28, 29. Slides were counterstained with DAPI (Vector Lab). Fluorescent images were obtained using a Zeiss Axiosplan2 microscope equipped with an AxioCam MRm digital camera and AxioVision 4.3 Software (Carl Zeiss Microimaging, Thornwood, NY). Flow cytometry was performed using cells isolated from yolk sacs and lungs as described 27, 30. Antibodies used for flow cytometry are listed in Supplemental Materials. BrdU was injected i.p. into pregnant females 2 hr prior to embryo harvest. Annexin V kit was from eBioscience. Stained cells were separated using cell sorting (Five-laser FACS Aria II, BD Biosciences). Purified cells were used for RNA preparation and qRT-PCR analysis.

Chromatin Immunoprecipitation (ChIP) assay.
ChIP assay was performed using in situ cross-linked MFLM-91U cells as described 24, 27. Antibodies used for ChIP were: FOXF1 20 and control rabbit IgG (Vector Lab). Sense (S) and antisense (AS) PCR primers that were used to amplify mouse promoter DNA fragments in ChIP assay are provided in Online Table III.

Statistical analysis.
ANOVA and Student’s T-test was used to determine statistical significance. P values less then 0.05 were considered significant. Values for all measurements were expressed as the mean ± standard deviation (SD).

RESULTS
FOXF1 is expressed in mesenchyme and endothelial cells during embryogenesis.

Immunostaining with FOXF1 antibodies was used to visualize FOXF1-expressing cells in E13.5 mouse embryos. FOXF1 protein was found in mesenchyme of the lung, trachea, esophagus, stomach, intestine, oral cavity, tongue and cartilage (Fig. 1A-I), which is consistent with previous in situ hybridization studies 11, 13. Additional site of FOXF1 expression was found in the embryonic heart where FOXF1 was present in mesenchyme of cardiac cushion but absent from myocardium and endocardial cells (Fig. 1E and data not shown). FOXF1 was detected in hemangioblasts of the yolk sac but was absent from hematopoietic cells at E8.5 - E12.5 (Fig. 1O and data not shown). FOXF1 was also detected in nuclei of endothelial cells of the lung, yolk sac and embryonic regions of the placenta (Fig. 1J-N). FOXF1 protein co-localized with endothelial marker proteins Flk1, Isolectin B4 and vWF in lung tissue (Fig. 1P-R). Thus, FOXF1 is expressed in endothelial cells during embryogenesis.

Generation of Foxf1-floxed mice and deletion of Foxf1 from endothelial cells.

Given the importance of FOXF1 in pathogenesis of ACD/MPV in humans, we determined FOXF1 requirements in endothelial cells using a conditional knockout approach. A triple-LoxP Foxf1-floxed (fl) -targeting vector containing the Neo cassette and two Frt sites was constructed (Fig. 2A) and used for electroporation of mouse ES cells. After mouse blastocyst injection of the Neo-resistant Foxf1fl/+ ES cells, chimeric mice with germ line transmission were obtained and bred to generate a stable Foxf1fl/+ mouse line. PCR amplification of mouse tail genomic DNA was used to distinguish between Foxf1-floxed and Foxf1-wild type (wt) alleles (Fig. 2B). The Foxf1fl/+ mice were bred with Tie2-Cre mice to delete the first exon of the Foxf1 gene encoding the DNA-binding domain and part of the transcriptional activation domain of the FOXF1 protein (Fig. 2A and Online Fig. 1), both of which are required for FOXF1 transcriptional activity.
Cre-mediated recombination and the loss of FOXF1 protein in Tie2-Cre Foxf1\textsuperscript{fl/fl} endothelial cells were confirmed by Rosa26-LacZ-reporter (Fig. 2C) and immunostaining with FOXF1 antibodies (Fig. 2D).

Embryonic lethality and cardiovascular defects in Tie2-Cre Foxf1\textsuperscript{fl/fl} embryos.

Tie2-Cre Foxf1\textsuperscript{fl/fl} embryos were present in Mendelian ratio prior to E13.5 (Table 1). The number of Tie2-Cre Foxf1\textsuperscript{fl/fl} embryos progressively decreased from E13.5 to E16.5, consistent with embryonic lethality during this period (Table 1). Histological examination of the Tie2-Cre Foxf1\textsuperscript{fl/fl} embryos revealed severe growth retardation as demonstrated by decreased embryo size and body weight (Fig. 3A). Liver size was also decreased in FOXF1 mutants (Fig. 3B). Deletion of FOXF1 caused ventricular hypoplasia and an interventricular septal defect in the embryonic heart (Online Fig. IIA-B). Furthermore, FOXF1 mutants exhibited accumulation of fluid in the amniotic cavity (polyhydramnios) and pericardial cavity (pericardial efflux) (Fig. 3A), a common finding in embryos with various cardiovascular abnormalities \textsuperscript{5, 6}. Thus, deletion of FOXF1 resulted in embryonic lethality due to severe growth retardation and cardiovascular defects.

FOXF1 deletion impairs the formation of embryonic vasculature in the yolk sac and placenta.

At E13.5, vascular branching was reduced in the yolk sac and placenta of Tie2-Cre Foxf1\textsuperscript{fl/fl} embryos when compared to control Foxf1\textsuperscript{fl/fl} embryos (Fig. 3C and Online Fig. IIIA). Reduced vascular branching was confirmed by whole mount immunostaining of yolk sacs for endothelial-specific endomucin (Fig. 3D). Decreased vascular branching and polyhydramnios were also found in the yolk sac of Foxf1\textsuperscript{+/c} embryos (Online Fig. IIIB-C), findings consistent with published studies \textsuperscript{14, 15}. Vascular phenotypes in Tie2-Cre Foxf1\textsuperscript{fl/fl} and Foxf1\textsuperscript{+/c} embryos were less severe compared to Flk1-null mutant mice that exhibited growth retardation, a near complete loss of yolk sac vasculature and embryonic lethality at E10.5 (Online Fig. IIID).

Vascular defects in Tie2-Cre Foxf1\textsuperscript{fl/fl} yolk sacs were associated with reduced Foxf1 mRNA (Fig. 4A) and decreased expression of Flt1, Flk1 and angiopoietin-1 (Angpt1) (Fig. 4B), all of which are critical for angiogenesis and VEGF signaling in endothelial cells \textsuperscript{3, 4, 23, 31}. Angpt2 and Nrp1 mRNAs were increased in FOXF1 mutants (Fig. 4B). Expression of Ephrin B2 was reduced in arteries of Tie2-Cre Foxf1\textsuperscript{fl/fl} embryos (Online Fig. IVA) as well as in isolated Pecam1\textsuperscript{+} endothelial cells (Online Fig. IVB). There were no differences in the number of lymphatic vessels stained for LYVE1 (Online Fig. IVA). Ephrin b4, Sox-18, Foxc1 and Foxc2 mRNAs were unaltered (Online Fig. IVB-C). Interestingly, when the Tie2-Cre Foxf1\textsuperscript{fl/fl} embryos were examined at E10.5, diminished branching of blood vessels was still evident in the yolk sac and placenta (Fig. 3C and data not shown) but cardiac abnormalities and polyhydramnios were absent (Fig. 3C and Online Fig. IIC). The number of Pecam1\textsuperscript{+} Tie2\textsuperscript{+} CD45\textsuperscript{−} endothelial cells was reduced in Tie2-Cre Foxf1\textsuperscript{fl/fl} E10.5 yolk sacs (Online Fig. VB), whereas the number of Pecam1\textsuperscript{+} CD45\textsuperscript{−} and Pecam \/ CD41\textsuperscript{+} hematopoietic cells was normal (Online Fig. VA). Thus, vascular insufficiency in the yolk sac and placenta occurs earlier than other embryonic defects and is likely to be a primary cause of growth retardation and embryonic lethality in Tie2-Cre Foxf1\textsuperscript{fl/fl} embryos. Altogether, FOXF1 deletion from endothelial cells impairs heart development and decreases vascular branching in the yolk sac and placenta.

FOXF1 deletion impairs the formation of pulmonary vascular plexus.

Since FOXF1 deficiency is associated with reduced numbers of pulmonary capillaries in ACD/MPV infants and Foxf1\textsuperscript{+/c} embryos \textsuperscript{8, 14}, we examined vasculature in Tie2-Cre Foxf1\textsuperscript{fl/fl} lungs. Similar to the yolk sac and placenta, diminished number of blood vessels was observed in the lung of Tie2-Cre Foxf1\textsuperscript{fl/fl} embryos, as demonstrated by reduced staining for endothelial-specific markers PECAM-1 and SOX-17 (Fig. 4C). Despite impaired vasculature, epithelial tubules were still present in Tie2-Cre Foxf1\textsuperscript{fl/fl} lungs (Fig. 4C). Flk1 staining and Flk1 mRNA were reduced after deletion of FOXF1 (Fig. 4C-D). Fltl,
Pecam-1, Sox-17, CD34 and Pdgfb mRNAs were decreased in whole lung RNA from Tie2-Cre Foxf1fl/fl embryos (Fig. 4D), confirming the loss of pulmonary vascular plexuses.

Reduced vascular branching in Pdgfb-CreER Foxf1fl/fl embryos.

Pdgfb-CreER Foxf1fl/fl mouse line was generated to achieve an inducible deletion of Foxf1 from endothelial cells without targeting hematopoietic cells. To activate Cre, tamoxifen was given to pregnant females at E9.5 and embryos were harvested at E12.5. The Foxf1-null allele was detected in tamoxifen-treated Pdgfb-CreER Foxf1fl/fl mice by PCR (Fig. 5A), a finding consistent with activation of Cre by tamoxifen. Cre-mediated recombination in lung tissue was confirmed by β-gal reporter and diminished FOXF1 immunostaining (Fig. 5B). Flow cytometry showed that approximately 60% of PECAM-1+ endothelial cells and only 2% of CD45+ hematopoietic cells in the yolk sac were positive for Cre (Fig. 5A). Pdgfb-CreER Foxf1fl/fl embryos exhibited polyhydramnios (Fig. 5A), reduced vascular branching in the placenta and yolk sac (Fig. 5C) and decreased numbers of endothelial cells in the lung (Fig. 5D). Deletion of FOXF1 during the postnatal period (P0-P2) impaired retinal angiogenesis in Pdgfb-CreER Foxf1fl/fl mice (Online Fig. VI). Thus, FOXF1 stimulates angiogenesis in the developing lung, eye, placenta and yolk sac.

FOXF1 stimulates angiogenesis in vitro.

The ability of FOXF1 to stimulate angiogenesis in endothelial cells was directly tested in vitro. siRNA transfection was used to deplete FOXF1 mRNA and protein in endothelial MFLM-91U cells (Fig. 6B-C). Depletion of FOXF1 reduced the ability of MFLM-91U cells to form vessel-like sprouts in matrigel (Fig. 6A), a common model of angiogenesis in vitro. Diminished angiogenesis in FOXF1-depleted cells was associated with reduced proteins and mRNAs of Flk1, Flt1, Pecam-1 and Pdgfb, whereas expression of Angpt2 was increased (Fig. 6B-C). After depletion of FOXF1, phosphorylation of ERK and AKT was decreased (Fig. 6D), indicating reduced VEGF signaling. Interestingly, VEGF-A ligand did not rescue the FOXF1-mediated decrease in angiogenesis (Fig. 6A and 6D), a finding consistent with reduced expression of VEGF receptors in FOXF1-depleted cells. Thus, FOXF1 promotes VEGF signaling and increases angiogenesis in vitro.

Decreased proliferation and increased apoptosis in endothelial cells of Tie2-Cre Foxf1fl/fl embryos.

VEGF induces cellular proliferation and inhibits apoptosis by activating the tyrosine kinase receptor Flk1, which is present on the surface of endothelial cells. Given that Flk1 mRNA and protein were decreased in FOXF1-deficient mice (Fig. 4B-D) and cultured MFLM-91U cells (Fig. 6B-C), we next determined whether FOXF1 affects endothelial proliferation and apoptosis in vivo. Yolk sacs from E12.5 embryos were enzymatically digested and cells were stained for the endothelial marker Pecam-1 and the hematopoietic marker CD45 followed by flow cytometry. Consistent with reduced VEGF/Flk1 signaling, DNA replication was decreased in Pecam-1+/CD45- endothelial cells of Tie2-Cre Foxf1fl/fl yolk sacs as demonstrated by reduced BrdU incorporation into DNA (Fig. 7A). In addition, apoptosis of FOXF1-deficient endothelial cells was increased as shown by annexin V staining (Fig. 7A). Thus, reduced proliferation and increased apoptosis of endothelial cells can contribute to reduced angiogenesis and embryonic lethality in Tie2-Cre Foxf1fl/fl mice.

FOXF1 directly regulates expression of endothelial genes critical for angiogenesis and VEGF signaling.

To label endothelial cells that underwent Cre-mediated recombination, Tie2-Cre Foxf1fl/+ mice were crossed with mT/mG reporter mice that contain the LoxP-tdTomato-LoxP-GFP cassette knocked into the Rosa26 locus. The use of mTmG reporter enabled us to distinguish between Cre-targeted (GFP+) and non-targeted (tdTomato) endothelial cells in Tie2-Cre Foxf1fl/fl mTmG embryos. In control Foxf1fl/fl/mTmG embryos, both endothelial (Pecam-1+/CD45+) and hematopoietic cells (Pecam-1-/CD45+)
were negative for GFP but positive for tdTomato reporter, indicating the lack of Cre-mediated recombination (Fig. 7B). In contrast, 84.1 ± 0.5% of endothelial cells and 71.6 ± 4.6% of hematopoietic from Tie2-Cre Foxf1fl/fl mTmG embryos were positive for GFP (Fig. 7B). We next used flow cytometry-based cell sorting to isolate GFP+ and tdTomato+ endothelial cells and use these cells for qRT-PCR. Loss of Foxf1 mRNA and decreased expression of the FOXF1 target gene, integrin β3 20, were specifically found in GFP+ endothelial cells compared to control tdTomato+ endothelial cells (Fig. 7C). Deletion of FOXF1 efficiently reduced mRNAs of Flk1, Flt1, Pdgfb, Pecam-1, CD34, Tie2 and the non-coding RNA Fendrr, all of which are critical for embryonic vascular development 5-7, 32, 33. In contrast, Angpt2, Nrp1, Dll4, Notch2 and VEGFb mRNAs were increased (Fig. 7C). There was no difference in expression levels of Notch1 and Notch target genes Hey2 and Hes1 (Fig. 7C). Finally, Chromatin Immunoprecipitation assay (ChIP) demonstrated that FOXF1 protein directly bound to promoter DNAs of Flk1, Flt1, Pdgfb, Pecam-1 and Tie2 (Fig. 7D), a finding consistent with a direct transcriptional regulation of these genes by FOXF1. FOXF1 protein did not bind to Nrp1 and Angpt2 promoters (Fig. 7D). Thus, FOXF1 directly regulates expression of endothelial genes critical for angiogenesis and VEGF signaling.

DISCUSSION

Although various vascular abnormalities were previously reported for Foxf1−/− and Foxf1+/- mouse embryos as well as for ACD/MPV infants with FOXF1 mutations 9, 13, 14, cellular origins and molecular mechanisms of these developmental defects remain uncharacterized. In the present study, we showed that FOXF1 transcription factor is expressed in endothelial cells and that endothelial deletion of FOXF1 causes a variety of developmental defects, including impaired vasculature in the yolk sac, placenta, lung and retina. These data demonstrate that FOXF1 functions in a cell-autonomous manner to induce formation of embryonic vasculature. This hypothesis is consistent with diminished angiogenesis in FOXF1-deficient endothelial MFLM-91U cells in vitro. Interestingly, during embryogenesis FOXF1 is abundantly expressed in endothelial cells of capillaries and small blood vessels of the yolk sac, placenta and lung, but found only in a subset of endothelial cells of vena cava and pulmonary vein. In adult mice, FOXF1 was absent from endothelium of large pulmonary vessels but present in pulmonary capillaries 34. Therefore, FOXF1 is not a marker of endothelial cells. It is possible that FOXF1 expression in endothelial cells depends on proliferation or differentiation status, or reflects endothelial responses to various stimuli.

While Tie2-Cre Foxf1fl/fl mutant mice exhibited a complex developmental phenotype, we believe that vascular insufficiency in the yolk sac and placenta were primary causes of growth retardation and embryonic lethality in Tie2-Cre Foxf1fl/fl embryos. This conclusion is based on the fact that diminished branching of blood vessels was found at E10.5, whereas as other developmental abnormalities, such as cardiac defects and polyhydramnios, occurred after E13.5. Vascular insufficiency in the yolk sac and placenta may alter embryonic circulation in FOXF1 mutants, causing secondary heart defects and polyhydramnios and contributing to embryonic death. Interestingly, FOXF1 is expressed in the cardiac cushion and important for mesenchyme migration (20 and this manuscript). Therefore, interventricular septal defect in Tie2-Cre Foxf1fl/fl embryos can be a direct consequence of FOXF1 deletion from mesenchymal cells of cardiac cushion, which is critical for formation of interventricular septum and cardiac valves. FOXF1 was not detected in hematopoietic cells that are targeted by the Tie2-Cre transgene in our mouse model and therefore, it is unlikely that FOXF1 deletion in hematopoietic cell lineages contributed to the vascular phenotype in FOXF1 mutant mice. However, we can not exclude the possibility that FOXF1 is expressed in rare population(s) of hematopoietic progenitors and targeting these cells contributed to the phenotype in Tie2-Cre Foxf1fl/fl embryos. Interestingly, the Pdgfb-CreER transgene, which is more specific to endothelial cells compared to the Tie2-Cre 22, caused similar defects in vascular development, suggesting that endothelial cells are the main cellular targets of FOXF1. It is also possible that FOXF1 deletion reduces the number of circulating endothelial progenitors or mesenchymal stem cells, contributing to vascular
insufficiency in FOXF1-deficient embryos. Previous studies demonstrated that Foxc2 and Foxc1 genes are critical for vascular development in zebrafish and mice. We found no significant differences in expression of FOXC2 and FOXC1 after deletion of FOXF1. Since FOXC proteins and FOXF1 have high homology in their DNA binding domains, it is possible that FOXC1/2 can compensate for the loss of FOXF1 from endothelial cells by regulating similar target genes.

Published studies reported a direct correlation between FOXF1 levels and the number of pulmonary capillaries during lung development and lung injury. In the present study, we used in vivo and in vitro models to demonstrate that FOXF1 induces angiogenesis, endothelial proliferation and VEGF signaling through transcriptional activation of VEGF receptors Flk1 and Flt1 (Fig. 7). Reduction of Flk1 and Flt1 in FOXF1 mutants is consistent with the role of these genes in stimulating proliferation and differentiation of endothelial cells, leading to formation and maturation of blood vessels. In addition to VEGF signaling, FOXF1 may influence other signaling pathways critical for endothelial development, such as PDGF and Angpt/Tie2 pathways. Reduced expression of Pdgfb and Tie2 may account for decreased proliferation and increased apoptosis of endothelial cells in FOXF1-deficient embryos. Our data also suggest that FOXF1 stimulates angiogenesis through transcriptional activation of integrin-β3, pecam-1 and lncRNA Fendrr, all of which are critical for vascular development. Altogether, disruption of several key EC regulators can contribute to the FOXF1 phenotype. Since FOXF1 is a downstream target of the Shh signaling pathway, it is possible that FOXF1 mediates cross-talk between the Shh and VEGF pathways during development of embryonic vasculature. Our results suggest that inability of FOXF1-deficient endothelial cells to respond to VEGF, PDGF and Angpt/Tie2 signaling is a key mechanism in development of alveolar capillary dysplasia in ACD/MPV fetuses and infants harboring inactivating mutations in the FOXF1 gene locus.

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DISCLOSURES
No relationships to disclose

REFERENCES


Table 1. Genotype frequency of offspring from the breeding of Tie2-Cre<sup>+/−</sup>Foxf1<sup>fl/wt</sup> males and Foxf1<sup>fl/fl</sup> females.

<table>
<thead>
<tr>
<th>Theoretical Ratio</th>
<th>Total embryos</th>
<th>Foxf1&lt;sup&gt;fl/wt&lt;/sup&gt;</th>
<th>Foxf1&lt;sup&gt;fl/fl&lt;/sup&gt;</th>
<th>Tie2-Cre&lt;sup&gt;+/−&lt;/sup&gt; Foxf1&lt;sup&gt;fl/wt&lt;/sup&gt;</th>
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<td>8 (21%)</td>
<td>7 (18%)</td>
<td>10 (26%)</td>
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<td>9 (15%)</td>
<td>12 (20%)</td>
<td>19 (32%)</td>
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<tr>
<td>E13.5</td>
<td>74</td>
<td>21 (28%)</td>
<td>20 (27%)</td>
<td>20 (27%)</td>
<td>13 (18%)*</td>
</tr>
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<td>E14.5</td>
<td>21</td>
<td>6 (29%)</td>
<td>7 (33%)</td>
<td>6 (29%)</td>
<td>2 (10%)*</td>
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<td>E15.5-16.5</td>
<td>17</td>
<td>2 (12%)</td>
<td>7 (41%)</td>
<td>7 (41%)</td>
<td>1 (6%)*</td>
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* shows significant differences between experimental and theoretical ratios.
FIGURE LEGENDS

Figure 1. FOXF1 is expressed in mesenchymal and endothelial cells of the developing embryo. (A-J) Immunostaining shows that FOXF1 is expressed in mesenchyme-derived cells in E13.5 wild type mouse embryos (A). FOXF1 is detected in cartilage primordiums of vertebral bodies (CPVB in B), oral cavity (Ca in C), tongue (To in D), atrioventricular cardiac cushions (CC in E), trachea (Tr) and esophagus (Es) (F), lung (Lu in G), small intestine (Si in H), stomach (St in I). (J-O) FOXF1 is expressed in endothelial cells. Low levels of FOXF1 were detected in a subset of endothelial cells (shown with arrows) located in the inferior vena cava (J) and pulmonary vein (K). FOXF1 is detected in blood vessels of the yolk sac (L and O), lung (N) and the fetal part of the placenta (M). A composite of four different images is shown in O. FOXF1 protein was not found in hematopoietic cells. (P-R) FOXF1 co-localizes with endothelial markers Flk1 (P), Isolectin B4 (Q) and vWF (R) in E12.5 lungs. Location of FOXF1 in nuclei of endothelial cells is shown with white arrows. Autofluorescence of red blood cells is denoted with *. Abbreviations: ep, epithelial cells; me, mesenchyme; Ve, blood vessel; AG, adrenal gland; He, heart; Ki, kidney; Li, liver; LJ, lower jaw; UJ, upper jaw; Sp, spleen; Vb, vertebrae. Scale bars: A, 100μm; B-M, 50μm; N-O, 20μm; P-R, 10μm.

Figure 2. Generation of Foxf1fl/fl mice and conditional deletion of FOXF1 from endothelial cells. (A) Schematic diagram of Foxf1 gene-targeting construct with two Frt sites (white arrows) and three LoxP sites (black arrowheads) that surround the Neomycin (neo) gene and exon 1 (E1), encoding the DNA-binding domain of the FOXF1 protein. The Neo cassette was removed after breeding of Foxf1-floxed mice with β-actin (ACT)-FLP1 mice. Endothelial deletion of FOXF1 was achieved by breeding with Tie2-Cre mice. (B) PCR of mouse tail DNA using primers (P1-P7). Locations of primers are indicated in panel A. (C) β-gal activity (blue staining) is detected in both endothelial and hematopoietic cells in the yolk sac of Tie2-Cre Foxf1fl/fl/R26R E8.5 embryos. Slides were counterstained with nuclear fast red (red nuclei). Inserts show blood vessels in the yolk sac (top insert) and embryo proper (bottom insert). (D) Immunostaining shows the presence of FOXF1 protein in endothelial cells (black arrowheads) of control Foxf1fl/fl embryos. FOXF1 staining is absent from majority of endothelial cells of Tie2-Cre Foxf1fl/fl embryos (white arrowheads). Abbreviations: Br, bronchiole; Ve, blood vessel. Scale bars are 50μm.

Figure 3. Embryonic abnormalities in Tie2-Cre Foxf1fl/fl embryos. (A) Tie2-Cre Foxf1fl/fl embryos (KO) exhibit growth retardation and severe polyhydramnios (white arrowheads) at E13.5-14.5. Foxf1fl/fl littermates (fl/fl) are shown for comparison. Boundary of the liver is indicated by blue line. Pericardial efflux is shown with a black arrow on H&E-stained section of Tie2-Cre Foxf1fl/fl embryos. Body weight is decreased in Tie2-Cre Foxf1fl/fl E13.5 embryos (n=10) compared to control Foxf1fl/fl littermates (n=43). (B) Liver size was decreased in Tie2-Cre Foxf1fl/fl embryos. Occipital frontal diameter (OFD) and the ratio between OFD and the Crown rump length (CRL) were significantly reduced after deletion of FOXF1 (n=5). (C-D) Diminished vascular branching in the yolk sac of FOXF1 deficient embryos. The whole mount immunostaining was performed using endomucin Abs. Confocal microscopy was used to quantitate the vessel density as a ratio between endothelial (endomucin) and epithelial (E-cadherin) staining (bottom panel in D). p<0.05 is *, p<0.01 is **. Abbreviations: Ht, heart; Li, liver; Lu, lung. Scale bars: A, 50μm; B, 1mm; C, 50μm, D, 100μm.

Figure 4. Altered expression of endothelial genes in the yolk sac and lung of Tie2-Cre Foxf1fl/fl embryos. (A-B) Total RNA was prepared from yolk sacs of Tie2-Cre Foxf1fl/fl (KO) and control Tie2-Cre Foxf1fl/+ E12.5 embryos and analyzed by qRT-PCR. Decreased mRNAs of Fox1, Flt-1, Flk-1 and Angpt 1 were found in Tie2-Cre Foxf1fl/fl yolk sacs. Angpt2 and Npr1 mRNAs were increased (n=5). Expression levels were normalized to β-actin mRNA. (C) Immunohistochemical staining shows reduced PECAM1 and SOX-17 in lungs of FOXF1 KO embryos. The intensity of Flk1 staining was also reduced in FOXF1 KO lungs. (D) Decreased mRNAs of Pecam1, Sox-17, CD34, Flt1, Flk1 and Pdgfb were found in whole lung RNA from E12.5 KO embryos (n=6). p<0.05 is *, p<0.01 is **. Scale bars are 50μm.
Figure 5. Reduced vascular branching in Pdgfb-CreER Foxf1fl/fl embryos. (A) Pdgfb-CreER Foxf1fl/fl embryos were treated with tamoxifen at E9.5 and harvested at E12.5. Polyhydramnios in FOXF1-deficient embryos is indicated with white arrowhead. PCR of genomic tail DNA shows Cre-mediated recombination. Flow cytometry shows GFP fluorescence, which is associated with the Pdgfb-CreER transgene, in 60% of Pecam1+ endothelial cells (EC) isolated from the yolk sac. GFP is not detected in a majority of CD45+ hematopoietic cells (HC). (B) Increased β-gal activity and decreased FOXF1 staining is observed in lungs of Pdgfb-CreER Foxf1fl/fl/R26R E17.5 embryos. (C) Immunostaining for Pecam1 and endomucin shows reduced vessel (Ve) density in the placenta and yolk sac of FOXF1-deficient embryos. Quantification is shown in bottom panel. (D) Decreased numbers of Pecam1+ endothelial cells are found in FOXF1-deficient lungs by flow cytometry (n=5). p<0.05 is *, p<0.01 is **. Scale bars are 50μm.

Figure 6. siRNA-mediated depletion of FOXF1 impairs angiogenesis in matrigel and inhibits VEGF signaling in cultured MFLM-91U cells. (A) MFLM-91U cells were transfected with either FOXF1 siRNA or control non-targeting siRNA. Forty-eight hours after siRNA transfection, matrigel angiogenesis assay was performed in the presence and absence of VEGF-A(165). FOXF1 knockdown reduced the number and total length of endothelial sprouts (n=5). (B) qRT-PCR shows reduced Foxf1, Flk1 and Pdgfb mRNAs at 24 hrs after siRNA transfection. Angp2 mRNA was increased after depletion of FOXF1. (C) Altered protein levels of FOXF1, Flk1, Flt1, PECAM-1, PDGFb and Angpt2 at 48 hrs after siRNA transfection are shown by Western blots. (D) FOXF1-depleted MFLM-91U cells are resistant to VEGF-A(165) stimulation as indicated by decreases in phosphorylated Erk1/2 (pErk1/2) and phosphorylated Akt (pAkt). Quantification of Western blots is shown in right panels in C and D. p<0.05 is *, p<0.01 is **. Scale bars are 500μm.

Figure 7. FOXF1 directly regulates expression of endothelial genes critical for angiogenesis and VEGF signaling. (A) Reduced BrdU incorporation and increased annexin V staining is detected by flow cytometry in endothelial cells of Tie2-Cre Foxf1fl/fl E12.5 embryos. The dotted line represents non-specific staining with BrdU Abs. (B) GFP and tdTomato fluorescence were measured in endothelial (CD45+ PECAM-1+) and hematopoietic cells (CD45+ PECAM-1-) isolated from Tie2-Cre Foxf1fl/fl mT/mG yolk sacs. GFP is not detected in cells from control Foxf1fl/fl mT/mG yolk sacs. Efficiency of Cre-mediated recombination is shown as a percentage of GFP+ cells among total cells isolated from the yolk sac (n=5) (right panel). BrdU incorporation is decreased in GFP+ endothelial cells at E10.5 (right middle panels). (C) Endothelial cells were flow sorted from E12.5 yolk sacs. qRT-PCR was used to examine mRNAs. (D) ChIP assay shows that FOXF1 binds to promoter regions of Pecam-1, Pdgfb, Tie-2, Flt1 and Flk1 genes. FOXF1 does not bind to Nrp1 and Angpt2 promoters. Fetal endothelial MFLM-91U cells were used for ChIP. p<0.05 is *, p<0.01 is **.
Novelty and Significance

What Is Known?

- Inactivating mutations in the FOXF1 gene are found in 40% of patients with Alveolar Capillary Dysplasia with Misalignment of Pulmonary Veins (ACD/MPV).
- Haploinsufficiency of the Foxf1 gene causes alveolar capillary dysplasia and developmental defects in lung, intestinal and gall bladder morphogenesis in mice.
- FOXF1 is a transcription factor, which is found in multiple cell types, including endothelial cells.

What New Information Does This Article Contribute?

- Disruption of Foxf1 gene in endothelial cells caused embryonic lethality, growth retardation and cardiovascular abnormalities.
- Disruption of Foxf1 reduced cell proliferation, increased apoptosis and inhibited the Vascular Endothelial Growth Factor (VEGF) signaling pathway in endothelial cells.
- FOXF1 induces transcription of VEGF receptor genes Flk1 and Flt1.

Inactivating mutations in the FOXF1 gene were recently found in 40% of human patients with ACD/MPV. The molecular mechanisms by which these mutations cause vascular defects remain unknown. In the present study, we used transgenic mice with endothelial-specific inactivation of the Foxf1 gene to demonstrate that FOXF1 is critical for formation of embryonic vasculature. FOXF1 stimulates endothelial proliferation and promotes the VEGF signaling pathway in embryonic endothelial cells through direct transcriptional activation of VEGF receptor genes. Our results suggest that inability of FOXF1-deficient endothelial cells to respond to VEGF signaling is a key mechanism in development of alveolar capillary dysplasia in ACD/MPV fetuses and infants harboring inactivating mutations in the FOXF1 gene. Pharmacological agents that stimulate or stabilize FOXF1 protein might serve as promising therapeutic agents in patients with ACD/MPV caused by heterozygous loss-of-function mutations in FOXF1 gene.
**Ren et al., Figure 6**

**A.**

Control siRNA | siFoxf1

- VEGF

+ VEGF

**B.**

qRT-PCR

**C.**

Mock Cont siRNA siFoxf1

Fold change

Foxf1 Flt-1 PECAM-1 Flk-1 PDGFB Angpt2 

β-Actin

**D.**

VEGF: - +

Cont siRNA siFoxf1 Cont siRNA

p-Erk1/2 Erk1/2 p-Akt pan-Akt 

β-Actin

Graphs showing relative fold change and protein levels.
Ren et al., Figure 7

A. 

B. 

C. qRT-PCR from sorted endothelial cells

D. 

- PECAM-1 (268 bp)
- PDGFb (176 bp)
- Flk-1 (362 bp)
- Fth-1 (528 bp)
- Tie-2 (326 bp)
- Nrp-1 (309 bp)
- Angpt 2 (243 bp)
FOXF1 Transcription Factor Is Required for Formation of Embryonic Vasculature by Regulating VEGF Signaling in Endothelial Cells

Xiaomeng Ren, Vladimir Ustiyen, Arun Pradhan, Yuqi Cai, Jamie A Havrilak, Craig S Bolte, John M Shannon, Tanya V Kalin and Vladimir V Kalinichenko

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SUPPLEMENTAL MATERIAL

Detailed Methods

**Mice.** Tie2-Cre transgenic mice were purchased from Jackson Lab., whereas Pdgfb-CreER transgenic mice were obtained from Pierre Chambon \(^1\). Flk1-null mutant mice were previously described \(^2\). The LoxP-stop-LoxP-flgal (R26R) and LoxP-tdTomato-LoxP-GFP (mT/mG) reporter mouse lines were from Jackson Lab. The Foxf1\(^{fl/fl}\) mouse line was generated in the Kalinichenko lab (Cincinnati Children’s Hospital Medical Center). Foxf1-targeting vector contained a LoxP site inserted into the Foxf1 promoter and PGK-gb2 LoxP/FRT-flanked Neomycin (neo) cassette placed into the first intron. The PGK promoter-driven herpes simplex virus-thymidine kinase (HSV-TK) gene was placed outside of the Foxf1 gene homology region for negative selection of non-homologous recombination in ES cells. The Foxf1\(^{fl}\)-targeting vector was used for electroporation of mouse ES cells (C57Bl/6 x 129/SVEV mouse background), which were selected for neo (G418) and HSV-TK resistance (ganciclovir) at the inGenious Targeting Laboratory (Stony Brook, NY). ES cells with the appropriate Foxf1\(^{fl}\)-targeted locus were identified by PCR and Southern blot analysis and were used to generate chimeric mice by injecting Foxf1\(^{fl}\) ES cells into mouse blastocysts. Mice containing the Foxf1\(^{fl}\)-targeted allele were determined by PCR amplification with primers flanking the LoxP sequence located in the Foxf1 promoter and primers located in the 3’ region of the Foxf1\(^{fl}\) allele. Primer sequences are provided in Suppl. Table. S1. To produce Foxf1\(^{fl/fl}\) mice, chimeric mice were bred with C57Bl/6 mice in the animal facility of Cincinnati Children’s Hospital Medical Center. The Neo cassette was deleted by breeding of Foxf1\(^{fl/fl}\) mice with ACT-FLP1 mice (Jackson Lab.). The loss of Neo in Foxf1\(^{fl/fl}\) mice was confirmed by PCR using primers described in Suppl. Table. S1. Foxf1\(^{fl/fl}\) mice were backcrossed to generate viable Foxf1\(^{fl/fl}\) mice that were bred into the C57Bl/6 background for ten generations. Deletion of the Foxf1\(^{fl}\) alleles from endothelial and hematopoietic cell lineages was accomplished through breeding with Tie2-Cre and Pdgfb-CreER transgenic mice. Deletion of FOXF1 was confirmed by breeding FOXF1-deficient mice with LoxP-stop-LoxP-flgal (R26R) and LoxP-tdTomato-LoxP-GFP (mT/mG) reporter mice. Animal studies were approved by the Animal Care and Use Committee of Cincinnati Children’s Hospital Research Foundation.

**RNA preparation and quantitative real-time RT-PCR (qRT-PCR).** Total RNA was prepared from cultured MFLM-91U cells, mouse tissue and flow-sorted endothelial cells using RNeasy micro kit from Qiagen (Germantown, MD). qRT-PCR analysis was performed using a StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA) as described \(^3\). Samples were amplified using inventoried TaqMan primers for the gene of interest as indicated in Online Table II. Reactions were analyzed in triplicates and expression levels were normalized to \(-\)actin mRNA. Five embryos were used in each group.

**siRNA transfection, Western blot and matrigel angiogenesis assay.** MFLM-91U cells were cultured in serum-free UltraCULTURE medium (Lonza, Walkersville, MD). To inhibit FOXF1, we transfected siRNA specific to mouse Foxf1 (siFoxf1, 5’-GAA AGG AGU UUG UCU UCU C-3’, Dharmacon) using Lipofectamine\(\text{TM} \ 2000\) reagent (Invitrogen) as described \(^4, 5\). Controls included mock-transfected cells and cells transfected with control non-targeting siRNA (Dharmacon). Cells were harvested 48 hours after transfection and used for matrigel angiogenesis assay (BD Biosciences). VEGF 165 (20 ng/ml, Millipore) was added to matrigel for 14 hr. Cells in matrigel were stained with calcein AM fluorescent viability dye which is transported through the cellular membrane into live cells. 3D images were acquired using a Nikon Eclipse Ti confocal microscope in conjunction with NIS-Elements AR software. Vessel filament total length and circularity were measured using IMARIS software (Bitplane, CT).

Western Blot analysis was performed using following antibodies: Foxf1 \(^4\), Pecam-1 (ABCAM, Cat# ab28364-100), Fli1 (Santa Cruz, Cat# SC-6251), Fli1 (R&D, Cat# AF471), Pdgfb, (Aviva Systems Biology, Cat# ARP58509), Angpt2 (Rockland, Cat# 100-401-402), p-ERK 1/2 (Cell signaling, clone D13.14.4E), total ERK 1/2 (Cell signaling, clone 137F5), p-Akt (Cell signaling, clone 736E11), total Akt
Detection of the immune complex was accomplished by using secondary antibodies directly conjugated with HRP followed by the Supersignal chemiluminescence substrate (Pierce, Rockford, IL).

**Immunohistochemical staining and Flow cytometry.** Paraffin sections were stained with hematoxylin and eosin (H&E) or used for immunohistochemical staining as described. The following antibodies were used for immunohistochemistry: FOXF1, Pecam-1 (ABCAM, Cat# ab28364-100), Sox-17 (generated in Dr. Whitsett lab), Flk1 (Cell signaling, Cat# 55B11), LYVE-1 (Novas biologicals, Cat# NB100-725), Ephrin B2 (R&D systems, Cat# AF496) and endomucin (R&D systems, Cat#AF4666). Antibody-antigen complexes were detected using biotinylated secondary antibody followed by avidin-horseradish peroxidase (HRP) complex and DAB substrate (Vector Labs, Burlingame, CA). Sections were counterstained with nuclear fast red. To stain endothelial cells, Alexa Fluor 488-conjugated Isolecitn B4 (Invitrogen) was used according to manufacturer recommendations and previous studies. For co-localization experiments, secondary antibodies conjugated with Alexa Fluor 488 or Alexa Fluor 594 (Invitrogen) were used. Slides were counterstained with DAPI (Vector Lab). Fluorescent images were obtained using a Zeiss Axioplan2 microscope equipped with an AxioCam MRm digital camera and AxioVision 4.3 Software (Carl Zeiss Microimaging, Thomwood, NY).

Flow cytometry was performed using cells isolated from yolk sacs and lungs as described. Cells were stained with fluorescently-labeled antibodies against CD45 (eBioscience, clone 30-F11), CD41 (eBioscience, clone eBioMWRReg30), Tie2 (eBioscience, clone TEK4), endomucin (eBioscience, clone eBioV.7C7), Pecam-1 (eBioscience, clone 390) or BrdU (eBioscience, clone BU20A). BrdU-labeling reagent (Invitrogen) was injected i.p. into pregnant females 2 hr prior to embryo harvest. Apoptosis was measured using the Annexin V kit (eBioscience). Cells stained with fixed viability dye, CD45 and Pecam-1 Abs were separated using cell sorting (Five-laser FACSria II, BD Biosciences). Purified cells were used for RNA preparation and qRT-PCR analysis.

**Chromatin Immunoprecipitation (ChIP) assay.** ChIP assay was performed with in situ cross-linked MFLM-91U cells as described. Nuclear extracts from MFLM-91U cells were cross-linked by addition of formaldehyde, sonicated and used for the immunoprecipitation with Abs against FOXF1 or control rabbit IgG (Vector Lab). DNA fragments were 500-700 bp. Reverse cross-linked ChIP DNA samples were subjected to PCR using the oligonucleotides specific to promoter regions of potential FOXF-target genes. Sense (S) and antisense (AS) PCR primers that were used to amplify mouse promoter DNA fragments in ChIP assay are provided in Online Table III.

**Statistical analysis.** ANOVA and Student’s T-test was used to determine statistical significance. P values less then 0.05 were considered significant. Values for all measurements were expressed as the mean ± standard deviation (SD).
Online Figure Legends

Online Fig. I. Genomic sequences of Foxf1-floxed and Foxf1 wild type alleles. The first LoxP site was inserted into the promoter region of Foxf1 gene. Primer P1 and P2 were used for genotyping. The second LoxP site is located in the intron of the Foxf1 gene. The Neo sequence located in Foxf1-floxed allele was excised through Flp1-Frt recombination. Open box shows sequences that were deleted in Tie2-Cre Foxf1 fl/fl endothelial cells. Deleted sequences include part of the Foxf1 promoter (977 bp), the first exon (white letters on gray background), Frt sequence and 62 bp of the intron. Identity of DNA sequence is denoted with *.

Online Fig. II. Ventricular hypoplasia and interventricular septum defect in Tie2-Cre Foxf1 fl/fl embryos. (A-B) Tie2-Cre Foxf1 fl/fl embryos were harvested at E12.5, E13.5 and E14.5. Transverse paraffin sections of thoracic region were stained with H&E. Tie2-Cre Foxf1 fl/fl embryos exhibited ventricular hypoplasia and the interventricular septum defect (asterisk in A). Decreased thickness of the left ventricular wall is observed in Tie2-Cre Foxf1 fl/fl embryos (black arrowheads in B). (C) H&E staining shows normal heart structure in Tie2-Cre Foxf1 fl/fl E10.5 embryos. Abbreviations: RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle; ES, esophagus; DAo, dorsal aorta. Scale bars are 50 μm.

Online Fig. III. Vascular abnormalities in Tie2-Cre Foxf1 fl/fl, Foxf1 +/- and Flk1-null embryos. (A) Diminished branching of blood vessels is observed in placenta of Tie2-Cre Foxf1 fl/fl E13.5 embryos (KO) compared to control Foxf1 fl/fl littermates (fl/fl). (B-C) Vascular abnormalities in Foxf1 +/- embryos. Foxf1 +/- E12.5 embryos exhibit polyhydramnios (white arrowheads in left B panel). Wild type littermates (WT) are shown for comparison. PCR of genomic tail DNA identifies the Foxf1-null and Foxf1 WT alleles (middle panel in B). Western blot shows reduced FOXF1 protein in lung tissue obtained from Foxf1 +/- embryos (right panel in B). Diminished vascular branching in the yolk sac of Foxf1 +/- E12.5 embryos (C). The whole mount immunostaining of the yolk sac was performed using endomucin Abs (upper panel in C). Confocal microscopy was used to quantitate vessel density by calculating a ratio between endomucin + endothelial area and E-cadherin + epithelial area (bottom panel in C). p<0.05 is *. (D) Flk1-null embryos show growth retardation at E10.5. Endomucin staining shows the lack of blood vessels in Flk1-null yolk sacs. Scale bars are 200 μm.

Online Fig. IV. Expression of arterial, venous and lymphatic markers in Tie2-Cre Foxf1 fl/fl embryos. (A) Paraffin sections from Tie2-Cre Foxf1 fl/fl (KO) and control Foxf1 fl/fl E12.5 embryos were stained for LYVE1 and Ephrin B2. Slides were counterstained with nuclear fast red (red nuclei). Ephrin B2 staining was decreased in endothelial cells of aorta. Lymphatic vessels (LYVE1+) were not altered after FOXF1 deletion. LYVE1 staining (dark brown) is shown with arrowheads. The images were taken from transverse sections of thoracic region. (B) Total RNA was prepared from either yolk sacs (left panel) or purified yolk sac endothelial cells (right panel) of Tie2-Cre Foxf1 fl/fl (KO) and control Foxf1 fl/fl E12.5 embryos and analyzed by qRT-PCR. Decreased ephrin B2 mRNA was found in Tie2-Cre Foxf1 fl/fl yolk sacs (p<0.05 is *). Ephrin B4, Sox-18, Foxc1 and Foxc2 mRNAs were not significantly altered (n=5). Expression levels were normalized to β-actin mRNA. Abbreviations: DA, dorsal aorta; Es, esophagus; Br, bronchus; Sk, skin. Scale bars are 20 μm.

Online Fig. V. Flow cytometry for hematopoietic and endothelial markers in Tie2-Cre Foxf1 fl/fl E10.5 yolk sacs. (A-B) Cells purified from E10.5 yolk sacs were used for flow cytometry. Hematopoietic cells were subdivided on CD45+ (mature) and CD45-CD41+ (immature) subsets. There was no difference in the number of either CD45+ or CD45-CD41+ cells in Tie2-Cre Foxf1 fl/fl E10.5 yolk sacs (A). Endothelial cells were identified as CD45+PECAM-1+ cells that also expressed Tie2 and endomucin (EDCN). The number of endothelial cells was significantly decreased in Tie2-Cre Foxf1 fl/fl E10.5 yolk
sacs compared to controls (B). p<0.05 is *, p<0.01 is **. (C) Immunostaining shows that FOXF1 is expressed in atrioventricular cardiac cushion (CC) and endothelial cells of placenta and yolk sac. FOXF1 is not expressed in hematopoietic cells located within blood vessels. Scale bars are 20μm.

**Online Fig. VI. Reduced angiogenesis in retina of Pdgfb-CreER Foxf1^{fl/fl} mice.** (A) Pdgfb-CreER Foxf1^{fl/fl} newborns were treated with tamoxifen at P0, P1 and P2. Mice were harvested at P6.5. (B-C) Retinal blood vessels were visualized using whole mount staining for isolectin B4. Red arrow shows retinal vascular outgrowth from the optic stalk. White arrowheads indicate endothelial sprouts at angiogenic front. Decreased retinal vasculature outgrowth is found in tamoxifen-treated Pdgfb-CreER Foxf1^{fl/fl} mice (n=5) compared to Foxf1^{fl/fl} controls (n=6). p<0.05 is indicated with * (C). Scale bars: B, upper panels, 300μm; B, lower panels, 100μm.
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(A) Paraffin sections from Tie2-Cre Foxf1<sup>fl/fl</sup> (KO) and control Foxf1<sup>fl/fl</sup> E12.5 embryos were stained for LYVE1 and Ephrin B2. Slides were counterstained with nuclear fast red (red nuclei). Ephrin B2 staining was decreased in endothelial cells of aorta. Lymphatic vessels (LYVE1<sup>+</sup>) were not altered after FOXF1 deletion. LYVE1 staining (dark brown) is shown with arrowheads. The images were taken from transverse sections of thoracic region. (B) Total RNA was prepared from either yolk sacs (left panel) or purified yolk sac endothelial cells (right panel) of Tie2-Cre Foxf1<sup>fl/fl</sup> (KO) and control Foxf1<sup>fl/fl</sup> E12.5 embryos and analyzed by qRT-PCR. Decreased ephrin B2 mRNA was found in Tie2-Cre Foxf1<sup>fl/fl</sup> yolk sacs (p<0.05 is *). Ephrin B4, Sox-18, Foxc1 and Foxc2 mRNAs were not significantly altered (n=5).

Expression levels were normalized to β-actin mRNA. Abbreviations: DA, dorsal aorta; Es, esophagus; Br, bronchus; Sk, skin. Scale bars are 20 μm.
Online Fig. V. Flow cytometry for hematopoietic and endothelial markers in Tie2-Cre Foxf1<sup>fl/fl</sup> E10.5 yolk sacs. (A-B) Cells purified from E10.5 yolk sacs were used for flow cytometry. Hematopoietic cells were subdivided on CD45<sup>+</sup> (mature) and CD45<sup>+</sup>CD41<sup>+</sup> (immature) subsets. There was no difference in the number of either CD45<sup>+</sup> or CD45<sup>+</sup>CD41<sup>+</sup> cells in Tie2-Cre Foxf1<sup>fl/fl</sup> E10.5 yolk sacs (A). Endothelial cells were identified as CD45<sup>+</sup>PECAM-1<sup>+</sup> cells that also expressed Tie2 and endomucin (EDCN). The number of endothelial cells was significantly decreased in Tie2-Cre Foxf1<sup>fl/fl</sup> E10.5 yolk sacs compared to controls (B). p<0.05 is *, p<0.01 is **. (C) Immunostaining shows that FOXF1 is expressed in atrioventricular cardiac cushion (CC) and endothelial cells of placenta and yolk sac. FOXF1 is not expressed in hematopoietic cells located within blood vessels. Scale bars are 20μm.
Online Fig. VI. Reduced angiogenesis in retina of Pdgfb-CreER Foxf1<sup>fl/fl</sup> mice. (A) Pdgfb-CreER Foxf1<sup>fl/fl</sup> newborns were treated with tamoxifen at P0, P1 and P2. Mice were harvested at P6.5. (B-C) Retinal blood vessels were visualized using whole mount staining for isoelectin B4. Red arrow shows retinal vascular outgrowth from the optic stalk. White arrowheads indicate endothelial sprouts at angiogenic front. Decreased retinal vasculature outgrowth is found in tamoxifen-treated Pdgfb-CreER Foxf1<sup>fl/fl</sup> mice (n=5) compared to Foxf1<sup>fl/fl</sup> controls (n=6). p<0.05 is indicated with * (C). Scale bars: B, upper panels, 300μm; B, lower panels, 100μm.
Online Tables

**Online Table I. Primers for genotyping**

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## Online Table II. TaqMan primers for qRT-PCR reactions

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Supplemental References


