SoxF Transcription Factors Are Positive Feedback Regulators of VEGF Signaling

Kangsan Kim, Il-Kug Kim, Jee Myung Yang, Eunhyeong Lee, Bong Ihn Koh, Sukhyun Song, Junseong Park, Sungsu Lee, Chulhee Choi, Jin Woo Kim, Yoshiaki Kubota, Gou Young Koh, Injune Kim

Rationale: Vascular endothelial growth factor (VEGF) signaling is a key pathway for angiogenesis and requires highly coordinated regulation. Although the Notch pathway-mediated suppression of excessive VEGF activity via negative feedback is well known, the positive feedback control for augmenting VEGF signaling remains poorly understood. Transcription factor Sox17 is indispensable for angiogenesis, but its association with VEGF signaling is largely unknown. The contribution of other Sox members to angiogenesis also remains to be determined.

Objective: To reveal the genetic interaction of Sox7, another Sox member, with Sox17 in developmental angiogenesis and their functional relationship with VEGF signaling.

Methods and Results: Sox7 is expressed specifically in endothelial cells and its global and endothelial-specific deletion resulted in embryonic lethality with severely impaired angiogenesis in mice, substantially overlapping with Sox17 in both expression and function. Interestingly, compound heterozygosity for Sox7 and Sox17 phenocopied vascular defects of Sox7 or Sox17 homozygous knockout, indicating that the genetic cooperation of Sox7 and Sox17 is sensitive to their combined gene dosage. VEGF signaling upregulated both Sox7 and Sox17 expression in angiogenesis via mTOR pathway. Furthermore, Sox7 and Sox17 promoted VEGFR2 (VEGF receptor 2) expression in angiogenic vessels, suggesting a positive feedback loop between VEGF signaling and SoxF.

Conclusions: Our findings demonstrate that SoxF transcription factors are indispensable players in developmental angiogenesis by acting as positive feedback regulators of VEGF signaling. (Circ Res. 2016;119:839-852. DOI: 10.1161/CIRCRESAHA.116.308483.)

Key Words: endothelial cells ■ physiologic angiogenesis ■ physiological feedback ■ SoxF transcription factors ■ vascular endothelial growth factor A

Several transcription factors play key roles in determining the fate of endothelial cells during embryonic vascular development, such as emergence and differentiation. However, the main transcription factors for angiogenic remodeling and vascular maturation are poorly understood. Among E26 transformation-specific family members, Etv2 and Fli1 play essential roles in the commitment of mesodermal precursor cells to endothelial cells. The commitment of nascent endothelial cells to specific lineages is also determined by the following transcription factors: Hey1/2 and FoxC1/2 for arterial differentiation, CoupTF II for venous differentiation, and Prox1 for lymphatic differentiation. ERG, another member of the E26 transformation-specific family, was recently identified as being involved in developmental angiogenesis by promoting vascular stability. As multiple transcription factors cooperate in each process of vascular development, other unidentified transcription factors may establish a gene regulatory network for angiogenic remodeling.

The Sox transcription factor family, which consists of several subgroups based on DNA-binding domain homology, plays fundamental roles in the generation of various tissues and their organotypic morphogenesis. The repression of Sox9 is essential for acquisition of the vascular smooth muscle fate, whereas the SoxF subgroup consisting of Sox7, Sox17, and Sox18 promotes the fate specification of endothelial cells. Sox17 and Sox18 are known to be indispensable for arterial and lymphatic differentiation, respectively, in mice. Sox17 has additional roles in sprouting angiogenesis during development and artery maintenance in adulthood. However, the vascular role of Sox7 is just beginning to be understood in mammalian vascular development, although the compound silencing of Sox7 and Sox18 and the Sox7 genetic targeting

Original received February 4, 2016; revision received July 26, 2016; accepted August 12, 2016. In July 2016, the average time from submission to first decision for all original research papers submitted to Circulation Research was 13.27 days.

From the Department of Biological Sciences (K.K., J.W.K., G.Y.K., I.K.), Graduate School of Medical Science and Engineering (I.-K.K., J.M.Y., B.I.K., S.L., G.Y.K., I.K.), Biomedical Science and Engineering Interdisciplinary Program (E.L., G.Y.K., I.K.), and Department of Bio and Brain Engineering (J.P., C.C.), Korea Advanced Institute of Science and Technology, Daejeon; Center for Vascular Research, Institute for Basic Science, Daejeon, Republic of Korea (K.K., S.S., G.Y.K.); Department of Otolaryngology-Head and Neck Surgery, Chonnam National University Hospital, Gwangju, Republic of Korea (S.L.); and The Laboratory of Vascular Biology, Keio University, Tokyo, Japan (Y.K.).

The online-only Data Supplement is available with this article at http://circres.ahajournals.org/lookup/suppl;doi:10.1161/CIRCRESAHA.116.308483/-/DC1.

Correspondence to Injune Kim, PhD, Graduate School of Medical Science and Engineering, Korea Advanced Institute of Science and Technology, Daejeon 34141, Republic of Korea. E-mail injune@kaist.ac.kr

© 2016 American Heart Association, Inc.

Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/CIRCRESAHA.116.308483
Microarray Analysis
For microarray analysis, total RNA was purified from HUVECs 36 hours after Sox7-/-, Sox17-/-, or Sox7/Sox17-siRNA silencing. Two independent sets of total RNA were subjected to quality control based on concentration, OD260/230, OD260/290, ratio (28s/18s), and RIN number using an Agilent 2100 Bioanalyzer. Agilent Human GE (V2) 4 X 44K was used for microarray, and normalization and clustering were performed using the Agilent GeneSpring software. Raw data were uploaded onto the Gene Expression Omnibus database of the National Center for Biotechnology Information (GSE75380).

Statistics
Experimental values are presented as mean±SE unless otherwise indicated. Statistical difference was determined performing the Mann–Whitney U test between 2 groups or the Kruskal–Wallis test followed by Bonferroni correction for more than 3 groups. Statistical significances are represented as * (P<0.05) or ** (P<0.01).

Results

Specific and Robust Expression of Sox7 in Blood Endothelial Cells During Vascular Development
Sox17 indispensable for angiogenesis is specifically expressed in endothelial cells. To find additional Sox members that regulate angiogenesis, we fractionated VE-cadherin+CD45−Ter119− endothelial cells and other cells from embryos at embryonic day 11.5, 14.5, and 17.5 to compare the gene expression of all Sox family members. Only the SoxF subgroup consisting of Sox7, Sox17, and Sox18 was specifically expressed in endothelial cells throughout all embryonic stages (Figure 1A), suggesting that all SoxF subgroup members, including Sox7, could play key roles in blood vessel formation during embryonic development.

To investigate the role of Sox7, we generated a Sox7-targeted allele, Sox7fl/fl, by replacing the coding region of the mouse Sox7 allele with mCherry fluorescent protein cDNA (Online Figure IA and IB). Loss of Sox7 alleles was verified by the absence of Sox7 transcripts in Sox7 knockout embryos (Online Figure IC). To characterize the Sox7 expression pattern, we examined the mCherry expression of Sox7fl/fl+ heterozygous embryos. Fluorescence-activated cell sorting (FACS) analysis revealed that 2.6% of cells from Sox7fl/fl+ heterozygous embryos expressed mCherry, but wild-type littermate embryos did not express mCherry (Figure 1C). To confirm whether mCherry expression truly reflects Sox7 expression, we fractionated whole cells from Sox7fl/fl+ embryos at E10.5 based on mCherry fluorescence and evaluated Sox7 expression. Sox7 transcripts were highly enriched in mCherry-positive cells compared with mCherry-negative cells (Figure 1D), validating that mCherry expression in the Sox7fl/fl+ mouse model faithfully reflected Sox7 expression.

In accordance with the aforementioned gene expression analysis in endothelial cells, most mCherry expression in E10.5 Sox7fl/fl+ embryos exhibited a prominent vascular pattern (Figure 1B). FACS analysis of mCherry-positive cells further confirmed robust Sox7 expression in endothelial cells (Figure 1E). In whole-mount embryos, Sox7 expression was restricted to PECAM (platelet endothelial cell adhesion molecule)-expressing endothelial cells in embryos and yolk sacs (Figure 1F). In particular, Sox7 expression was stronger in angiogenic vessels than stabilized vessels, as shown by higher mCherry expression in subventricular vessels than perineural vessels in the hindbrain at E10.5 (Figure 1G). In contrast, Sox7 was not detectable in Lyve1-positive lymphatic vessels (Figure 1H).

have been reported to induce defective arterial differentiation in zebrafish.14–17 Multiple Sox factors often cooperate for tissue morphogenesis, acting redundantly, sequentially, or complementarily depending on the developmental context.18 Thus, how the SoxF members jointly regulate vascular morphogenesis remains an unexplored subject of great interest.

Although biochemical signaling triggered by angiogenic ligands has been elucidated, how extracellular angiogenic cues regulate the transcriptional basis of vascular morphogenesis still remains poorly understood. The DLL4–Notch pathway has been shown to regulate angiogenesis by converting extracellular stimuli into transcriptional regulation.19 Vascular endothelial growth factor (VEGF) promotes DLL4 expression in endothelial cells, which activates Notch signaling and suppresses VEGF receptor 2 (VEGFR2) expression. Thus, the DLL4–Notch pathway functions as a negative feedback system by stabilizing VEGF-induced angiogenic vessels.20,21 Whether transcription factors exist that are upregulated by VEGF and can promote VEGFR2 expression to reinforce VEGF signaling remains to be elucidated.

This study demonstrates the indispensable role of Sox7 in angiogenic remodeling by using loss-of-function mouse models. We found that Sox7 and Sox17 jointly regulate vascular morphogenesis with a substantial overlap in expression and function. By establishing the VEGF stimulus as an upstream regulator and VEGFR2 as one of the downstream genes of Sox7 and Sox17, we demonstrate that Sox7 and Sox17 interact with VEGF signaling to create a positive feedback loop for angiogenesis.

Methods

Mutant Mice
For Sox7 knockout, Sox7mCh/+ mice were intercrossed. For deleting Sox7 and Sox17 from embryonic endothelial cells, Tie2-Cre transgenic mice were crossed with Sox7mCh/+ and Sox17mCh/+ respectively. For excising Sox7 and Sox17 in endothelial cells after birth, Cdh5(BAC)-CreER12 driver line was bred with Sox7mCh/+ and Sox17mCh/+ mice, respectively, and their progeny were subcutaneously administered with tamoxifen as described.13 For compound haploinsufficiency of Sox7 and Sox17, Sox7mCh/+ and Sox17mCh/+ mice were crossed with Sox17mCh/+ mice.

Histology and Tissue Immunofluorescence
Mouse tissues were fixed in 2% paraformaldehyde, dehydrated in phosphate-buffered saline with 20% sucrose, and prepared for cryosection. For immunofluorescence, tissue sections were blocked in phosphate-buffered saline with 0.1% Triton X-100 containing 5% donkey or goat serum, incubated with primary and fluorescently labeled secondary antibodies, and counterstained with DAPI (4,6-diamidino-2-phenylindole). Immunofluorescent images were obtained by Zeiss LSM780 confocal fluorescence microscopes.

In Vitro 3-Dimensional Sprouting Assay and Tubule-Forming Assay
Microfluidic device for 3-dimensional (3D) sprouting assay was prepared as previously described.22 Briefly, empty space between the middle and side channels, in which cells sprout, was filled with collagen type I gels (BD Bioscience) and fibronectin (Invitrogen). Human umbilical vascular endothelial cells (HUVECs) silenced with control, Sox7-/-, or Sox7-siRNA were seeded into the device with EGM2 media lacking VEGF. VEGF gradient was applied after 1 day and culture media was daily exchanged. HUVECs in the device were fixed and processed for immunostaining 4 days after seeding. For tube-forming assay, 50,000 cells were seeded in a Matrigel matrix (BD Biosciences) 1 day after siRNA knockdown. One day later, node number and tube length were quantified.
Thus, Sox7 is specifically and robustly expressed in blood endothelial cells during vascular development in mice.

Sox7 Is Indispensable for Developmental Angiogenesis

Given its predominant expression in embryonic endothelial cells, we studied the role of Sox7 in vascular development by examining the vasculature of Sox7<sup>mCh+mCh</sup> knockout embryos. Sox7 knockout mice were embryonic lethal at E11.5 (Online Figure ID) and exhibited overall growth retardation and a pale appearance compared with Sox7 heterozygous embryos at E10.5 (Online Figure IIA and IIB). Although the primitive vasculature formed without Sox7 (Online Figure IIC), vessels in Sox7 knockout embryos remained primitive plexus structures and had less branching in the hindbrain, intersomitic region, and yolk sac, compared with the well-branched vascular morphology in Sox7 heterozygous embryos (Online Figure IID through IIF). Some Sox7 knockout embryos exhibited disoriented sprouting in intersomitic vessels (Online Figure IIG). Notably, E9.5 Sox7 knockout embryos already showed impaired angiogenesis but proper body size comparable to littermate Sox7 heterozygous embryos (Online Figure IIH through IJK). Vascular defects by Sox7 deficiency are unlikely to be secondary to growth retardation. These results indicate that Sox7 is essential for embryonic angiogenesis and early vascular morphogenesis.

To further specify the role of Sox7 in endothelial cells, we generated another Sox7-targeted allele, Sox7<sup>fl</sup>, in which the first coding exon is flanked by 2 loxP sites (Online Figure IIIA and IIIB). We deleted floxed Sox7 alleles specifically in endothelial cells using the Tie2-Cre system (Online Figure IIIC). Tie2-Cre;Sox7<sup>mCh/fl</sup> embryos exhibited growth retardation and
paleness (Figure 2A and 2B), resulting in embryonic lethality at E11.5 (Online Figure IIID). Similar to the global knockout embryos, mutant embryos lacking endothelial Sox7 had nascent vasculatures (Figure 2C), but their vessels were limited to the primary plexus and exhibited poor branching in the hindbrain, trunk, and yolk sac (Figure 2D through 2F). These findings indicate that endothelial Sox7 plays an angiogenic, rather than vasculogenic, role in a cell-autonomous manner.

To investigate whether Sox7 regulates sprouting angiogenesis, we further examined E10.5 hindbrain vessels, which serve as a representative model of vascular sprouting.23 Compared with Sox7 heterozygous embryos, the overall vascular extension was severely impaired in mutant embryos lacking endothelial Sox7 (Figure 2G). Vascular networks in the heterozygous hindbrain had a densely branched stereoscale consisting of 2 separated vascular layers in the peri-neurial and subventricular zones, and interconnecting radial vessels (Figure 2H). However, Sox7-deficient hindbrain vessels had only a single-layered plexus with a primitive shape and reduced thickness and severely less sprouting than controls (Figure 2H–2I), indicating failure of both perpendicular and horizontal sprouting (Figure 2K). In addition, Sox7-deficient hindbrain vessels often lacked a patent lumen (Figure 2L).

We further examined the postnatal retinal vessels, which is another model of sprouting angiogenesis. We conditionally excised floxed alleles in endothelial cells by injecting tamoxifen daily into P1–P3 pups also harboring the Cdh5-CreER T2 allele. Mutant mice lacking endothelial Sox7 displayed no discernable growth retardation or hemorrhage in the retina (data now shown). However, compared with controls, Sox7 deletion repressed radial extension, branching in the plexus area, and sprouting at the front line (Figure 3A through 3F). Sox7 deletion also suppressed retinal angiogenesis to almost the same magnitude as Sox7 deletion, implying a functional overlap between Sox7 and Sox17 (Figure 3A through 3F). In contrast with controls, Sox7-deficient retinal vessels frequently lacked a patent lumen (Figure 3G). Taken together, the results indicate that Sox7 is indispensable for sprouting angiogenesis in embryonic and postnatal vascular development.

**Compound Haploinsufficiency of Sox7 and Sox17 Phenocopies the Vascular Defects of Sox7 Homozygous Knockout Embryos**

The aforementioned endothelial-specific expression and angiogenic role of Sox7 are almost identical to those of Sox17.11 Therefore, we were interested in whether Sox7 and Sox17 double knockout has a more severe detrimental effect on vascular development than the single knockout of either gene. We bred Sox7mCh/+ mice with Sox17GFP/+ mice to obtain Sox7mCh/+;Sox17GFP/+ mice, which we were expecting to use as breeding founders to generate Sox7mCh/mCh;Sox17GFP/GFP double

---

**Figure 2.** Endothelial Sox7 is indispensable for embryonic angiogenesis. A and B, Gross morphology of E10.5 control (Sox7mCh/+ and Sox7mCh/+;Tie2-Cre;Sox7mCh/+ embryos). C to F, Vascular morphology by platelet endothelial cell adhesion molecule (PECAM) immunostaining. C, Vasculature of the whole embryo proper. D, Higher magnification of hindbrain vessels (yellow boxes in C) and (E) intersomitic vessels (red boxes in C). F, Yolk sac vessels. G to L, Flat-mounted PECAM immunostaining of E10.5 control and Sox7mCh/+ hindbrain tissues. G, Entire hindbrain structure. H, Magnified views of the boxed area in G, showing separated vascular layers along the radial position. I, Quantification of vascular thickness and J number of sproutings per field. Data are presented as mean±SE (n=5). **P<0.01. K, Schematic representation of the hindbrain vascular structure. Note the lack of vascular sprouting in Sox7mCh/+ embryo. L, Projection showing the vascular lumen. Scale bars, 500 μm (A through H), 20 μm (L).
knockout embryos. Unexpectedly, we failed to obtain any Sox7mCh/+;Sox17GFP/+ (Sox7 and Sox17 compound heterozygous) pups. These compound heterozygous mice exhibited embryonic lethality, growth retardation, and pale appearance, resembling Sox7mCh/mCh single knockout mice (Online Figure IV A through IVC). The gross vascular pattern of E10.5 Sox7 and Sox17 compound heterozygous embryos was comparable to that of other genotypes (Figure 4A). However, compared with other genotypes, the vasculature of compound heterozygous embryos exhibited a primitive morphology with impaired branching in the hindbrain, trunk, and yolk sac (Figure 4B).

We further examined sprouting angiogenesis in the hindbrain vessels. Compared with Sox7 single heterozygous embryos, Sox7 and Sox17 compound heterozygous embryos had remarkably reduced vascular extension (Figure 4E). Sox7 single heterozygous embryos had a 3D hindbrain vascular network consisting of 2 exquisitely branched horizontal layers and interconnecting vertical vessels. However, Sox7 and Sox17 compound heterozygous embryos had a single-layered...
Figure 4. Compound haplodeficiency of Sox7 and Sox17 impairs developmental angiogenesis. **A** to **D**, Whole-mount platelet endothelial cell adhesion molecule (PECAM) immunostaining of E10.5 wild-type, Sox7\textsuperscript{mCh/+}, Sox17\textsuperscript{GFP/+}, and Sox7\textsuperscript{mCh/+}; Sox17\textsuperscript{GFP/+} embryos. **A**, Vasculature of the whole embryo proper. **B**, Yolk sac vessels. **C**, Higher magnification of hindbrain vessels (yellow boxes in **A**) and **D** intersomitic vessels (red boxes in **A**). **E** to **I**, Flat-mounted PECAM immunostaining of hindbrain tissues. **E**, Whole vascular networks. **F**, Magnified (Continued)
vascular plexus with nascent and thin morphology and little sprouting, indicating the failure of 3D vascular sprouting (Figure 4F through 4H). Sox7 and Sox17 compound heterozygous embryos also exhibited improper lumen formation (Figure 4I). Thus, the vascular phenotypes of Sox7 and Sox17 compound heterozygous embryos substantially resembled the vascular defects in Sox7 or Sox17 homozygous knockout embryos. Loss of any of 2 of 4 copies of Sox7 and Sox17 leads to defective angiogenesis, suggesting genetic cooperation between Sox7 and Sox17 in vascular morphogenesis.

To evaluate whether the gene dosage of Sox7 and Sox17 is important for angiogenesis, we examined postnatal retinal vessels of mutant pups with different numbers of null alleles. Vessel branching was more severely decreased in mutants lacking more Sox7 and Sox17 alleles, whereas the reduction of radial extension was not further affected by deleting more than 2 copies (Figure 4J through 4M). Notably, Sox7 and Sox17 double-deficient retinal vessels failed to form tubular structure and showed a primitive morphology, indicating that the double deficiency of Sox7 and Sox17 genes had a dramatic effect on postnatal angiogenesis. These results demonstrate the dosage-dependent joint function of Sox7 and Sox17 in angiogenesis.

VEGF Upregulates Sox7 and Sox17 Expression Via the mTOR Pathway

To understand how Sox7 and Sox17 genetically cooperate during vascular morphogenesis, we investigated their vascular expression pattern during development. Angiogenic vessels coexpressed Sox7 and Sox17, as shown by strong Sox17 immunostaining in mCherry-expressing endothelial cells from E10.5 Sox7mCh; Sox17CreERT2 hindbrain vessels (Figure 5A). We further explored the potential interaction between Sox7 and Sox17 expression in angiogenic vessels. In the embryonic hindbrain, Sox7-deficient vessels retained robust Sox17 expression (Figure 5B). Similarly, Sox17-deficient hindbrain vessels retained Sox7 expression (Figure 5C). Coincident Sox7 and Sox17 expression was also found in angiogenic vessels at the vascular front of postnatal retinal vessels (Figure 5D). Sox7 and Sox17 expression in the postnatal retinal vessels did not exhibit any dependence on each other in the same single deficiency models (Figure 5E through 5H). These results suggest the absence of an epistatic relationship between Sox7 and Sox17 expression in angiogenic vessels during development.

As Sox7 and Sox17 are coexpressed in angiogenic vessels, we studied whether Sox7 and Sox17 expression can be regulated by an angiogenic stimulus, such as VEGF. VEGF treatment strikingly and persistently increased both Sox7 and Sox17 expression at the protein level, but not at the transcript level, in cultured endothelial cells (Figure 6A through 6C). VEGF did not increase Sox7 and Sox17 protein levels in cycloheximide-treated cells (Online Figure VIA and VIB), suggesting that protein synthesis may be involved in this regulation. Moreover, blockade of VEGF signaling in vivo by intraperitoneal administration of VEGF-trap (25 mg/kg) to neonatal mouse pups repressed Sox7 and Sox17 expression in postnatal retinal and hindbrain vessels, as well as vascular branching (Figure 6D through 6F). These findings confirm VEGF-induced Sox7 and Sox17 upregulation, particularly in angiogenic vessels.

The mTOR pathway downstream of several receptor tyrosine kinases, including VEGFR2, regulates the rate of protein synthesis. We explored whether mTOR signaling is involved in the regulation of endothelial Sox7 and Sox17 expression. Pharmacological inhibition of mTOR signaling by rapamycin repressed Sox7 and Sox17 proteins levels in cultured endothelial cells (Figure 6G and 6H). VEGF-induced Sox7 and Sox17 upregulation was also abolished by rapamycin (Figure 6I and 6J). Conversely, mTOR overexpression in endothelial cells increased Sox7 and Sox17 protein levels (Figure 6K and 6L). Moreover, mutant embryos lacking endothelial mTOR (Tie2-Cre; mTOR<sup>fl/fl</sup>), compared with littermate controls, had reduced Sox7 and Sox17 expression in hindbrain and yolk sac vessels (Figure 6M and 6N), indicating that endothelial mTOR regulates Sox7 and Sox17 expression in angiogenic vessels in a cell-autonomous manner. Compared with controls, mTOR-deficient vessels showed less branching in the hindbrain and intersomitic region (Online Figure VIC through VIF), implying that mTOR may play a role in embryonic angiogenesis. Taken together, VEGF promotes Sox7 and Sox17 expression coincidently in angiogenic vessels, possibly via mTOR signaling.

Sox7 and Sox17 Stimulate Endothelial Sprouting by Upregulating VEGFR2 Expression

To understand the molecular mechanisms downstream of Sox7 and Sox17, we performed microarray analysis after silencing Sox7 and Sox17 in endothelial cells. Among 25081 genes, Sox7 and Sox17 silencing downregulated 620 and 658 genes, respectively, by more than 2-fold, resulting in 432 overlapping genes. Double knockdown of Sox7 and Sox17 downregulated 679 genes by more than 2-fold, >80% of which are common with Sox7 or Sox17 downstream genes (Online Figure VIIA). We also observed a similar overlap in upregulated genes (Online Figure VIIE). Compared with individual knockdowns, double knockdown resulted in a greater change in downstream gene expression (Online Figure VIIIB and VIIIF). These results suggest that Sox7 and Sox17 substantially share downstream genes in endothelial cells and regulate them in an additive manner.

To classify the functional characteristics of Sox7 and Sox17 downstream genes, we performed gene ontology enrichment analysis on the basis of biological processes. From all of the genes sets downregulated by Sox7 and Sox17 knockdown, the predominant categories were cell surface receptor and...
Figure 5. Sox7 and Sox17 are coincidently expressed in angiogenic vessels without epistasis. A, Concurrent Sox7 and Sox17 expression. E10.5 Sox7<sup>mCh/+</sup> hindbrain vessels immunostained with Sox17 and VE-cadherin. B, Sox17 and platelet endothelial cell adhesion molecule (PECAM) immunostaining of control (Sox7<sup>mCh/fl</sup>) and Sox7<sup>i△EC</sup> (Tie2-Cre;Sox7<sup>mCh/fl</sup>) hindbrain at E10.5. C, Sox7 and PECAM immunostaining of control (Sox17<sup>GFP/fl</sup>) and Sox17<sup>i△EC</sup> (Tie2-Cre;Sox17<sup>GFP/fl</sup>) hindbrain at E10.5. D, Colocalized Sox7 and Sox17 expression at the vascular front. P5 Sox17<sup>GFP/+</sup> retina vessels immunostained with Sox7 and PECAM. See Online Figure V for Sox7 and Sox17 expression in whole retina vessels. E, Sox7 and Sox17 immunostaining of control, Sox7<sup>iEC</sup> (Cdhs-CreER<sup>T2</sup>;Sox7<sup>fl/fl</sup>), and Sox17<sup>iEC</sup> (Cdhs-CreER<sup>T2</sup>;Sox17<sup>fl/fl</sup>)) retina at P5. F, Quantification of Sox7 and Sox17 signals. Data are presented as mean±SE (n=5). Scale bars, 200 μm. **P<0.01.
Figure 6. Vascular endothelial growth factor (VEGF) upregulates Sox7 and Sox17 expression via the mTOR pathway. **A** to **C**, Human umbilical vascular endothelial cells (HUVECs) starved with 0.1% serum overnight were treated with VEGF (50 ng/mL) for the indicated duration. **A**, Immunoblotting of Sox7, Sox17, and actin. **B**, Quantification of Sox7 and Sox17 at the protein and (C) the transcript levels (n>4). **D** to **F**, Immunostaining of P5 postnatal mouse tissues after phosphate-buffered saline (PBS; top) or VEGF-trap (bottom) treatment. **D**, Platelet endothelial cell adhesion molecule (PECAM) immunostaining of entire retina. **E**, Sox7 and Sox17 immunostaining of retina and (Continued)
G-protein–coupled signaling pathway (Online Figure VIIC). Uregulated genes also exhibited the greatest enrichments in these gene ontology categories (Online Figure VIIG). This type of gene enrichment is unique to Sox7 and Sox17 targets, as the downstream genes of other endothelial transcription factors, such as ERG, or other Sox members exhibit significant enrichments in categories associated with gene regulation and biosynthesis (Online Figure VIII). These results suggest that endothelial Sox7 and Sox17 prevalently regulate the gene expression associated with transmembrane signaling.

Given the angiogenic defects induced by Sox7 or Sox17 deficiency, we selected genes in the category of angiogenesis for further analysis to identify key effectors for Sox7- or Sox17-mediated angiogenesis. Sox7 or Sox17 knockdown downregulated several genes including VEGFR2, the main receptor of VEGF signaling, by more than 2-fold (Online Figure VIID). The Ingenuity Pathway Analysis of these changes predicted that Sox7 or Sox17 knockdown would have a significant effect on signaling pathways related to various cancers. Interestingly, VEGF signaling with a high negative Z score was identified as one of the most significant pathways (Online Figure VIIJ). These results suggest that Sox7 and Sox17 silencing may inhibit VEGF signaling, in part, by repressing VEGFR2 expression. However, the analysis of genes upregulated by Sox7 or Sox17 knockdown in the category of angiogenesis showed no direct correlation with VEGF signaling (Online Figure VIIH and VIJK), suggesting that many other signaling pathways also contribute to the SoxX-mediated vascular changes.

Because VEGF upregulated Sox7 and Sox17 expression, we investigated whether VEGF can also stimulate VEGFR2 expression. VEGF treatment increased VEGFR2 expression at both the transcript and protein levels (Figure 7A through 7C). Notably, VEGFR2, Sox7, and Sox17 exhibited gradually increasing expression during a long-term period in response to VEGF stimulation (Figure 7B through 7G), implying regulation in which VEGFR2 and SoxF expression are correlated. Furthermore, blocking VEGF signaling by VEGF-trap repressed VEGFR2 expression in angiogenic postnatal brain vessels (Figure 7H and 7I). Therefore, VEGF-induced VEGFR2 upregulation suggests the possibility of a positive feedback control for reinforcing VEGF signaling.

Next, we examined whether Sox7 and Sox17 can regulate VEGFR2 expression. Sox7 or Sox17 silencing in endothelial cells decreased VEGFR2 at both the transcript and protein levels (Figure 7J through 7L). Consistently, Sox7- or Sox17-deficient postnatal brain vessels exhibited significantly repressed VEGFR2 expression, but control brain vessels robustly expressed VEGFR2 (Figure 7M and 7N). These results indicate that Sox7 and Sox17 have a stimulating effect on VEGFR2 expression in an angiogenic context.

To characterize the cellular functions regulated by Sox7 and Sox17, we analyzed endothelial cell behavior after Sox7 and Sox17 silencing. Sox7 and Sox17 knockdown did not lead to any discernable changes in proliferation and the cell cycle (Online Figure VIII through VIIIC). However, Sox7 knockdown significantly and Sox7 knockdown moderately inhibited the tubule-forming ability of endothelial cells (Online Figure VIIID through VIIF). We further assessed the angiogenic ability of endothelial cells using a 3D sprouting assay. Control endothelial cells exhibited active chemotactic sprouting toward a VEGF-secreting channel. However, both Sox7 and Sox17 silencing suppressed VEGF-stimulated sprouting (Figure 7O), suggesting that enhanced endothelial sprouting is the primary cellular mechanism for the proangiogenic functions of Sox7 and Sox17. Interestingly, Sox17 and VEGFR2 were prevalently expressed in VEGF-stimulated endothelial cells rather than in nonstimulated cells on the opposite side (Figure 7P). This result suggests that VEGF regulation of SoxF and VEGFR2 might be intimately related to sprouting angiogenesis. Collectively, these findings suggest that Sox7 and Sox17 commonly stimulate VEGF-triggered endothelial sprouting by upregulating VEGFR2 expression.

**Discussion**

Transcription factors are important for a wide range of fate determination processes in vascular development, but their contribution to angiogenic morphogenesis remains to be determined. We identified angiogenic functions for 2 SoxF transcription factors, Sox7 and Sox17, during vascular development. Both Sox7 and Sox17 are indispensable for developmental angiogenesis with high similarity in expression and function. Here, we uncovered their role in a positive feedback loop for VEGF signaling.

VEGF is one of the most powerful stimulators of angiogenesis. Thus, its activity should be carefully regulated to build a functional vascular network. In general, multiple positive and negative feedback controls exist for the balanced regulation of various signaling pathways. A negative feedback system is essential for maintaining the homeostasis of signaling activity, whereas a positive feedback control can amplify a faint input signal for a greater output. The Dll4–Notch pathway is a well-known negative feedback system for VEGF signaling. In this feedback loop, VEGF stimulates Notch activity via Dll4 upregulation, and the subsequently activated Notch signaling pathway represses VEGFR2 expression. Thus, the Dll4–Notch pathway suppresses excessive vascular sprouting and stabilizes angiogenic vessels. In contrast, a positive feedback system for amplifying the VEGF signal has not been revealed.

In this study, we revealed the existence of positive feedback regulation for VEGF signaling. We found that the VEGF stimulus can increase the expression of its main receptor VEGFR2 in angiogenic vessels, as well as in vitro endothelial culture. Furthermore, we elucidated the molecular players...
of this regulatory system. VEGF promoted the expression of Sox7 and Sox17, and then these 2 SoxF members upregulated VEGFR2 in endothelial cells, creating a positive feedback loop (Online Figure IX). This positive feedback control may enhance the sensitivity of endothelial cells to VEGF cues and generate a sufficient angiogenic output for developmental angiogenesis in response to weak VEGF stimulation. Sox7 and Sox17 are critical for VEGF-triggered chemotactic sprouting of endothelial cells and sprouting angiogenesis in vascular development. When the regulatory balance between pro- and antiangiogenesis is disturbed, this positive feedback loop may easily lead to a vicious cycle, ultimately leading to pathological angiogenesis. In line with this concern, we observed that Sox17 upregulates VEGF receptor 2 (VEGFR2) expression in tumor vessels and promotes tumor angiogenesis in ectopic tumor models. Our current study identifies SoxF transcription factors as positive feedback regulators of VEGF signaling.

We also found that the regulation of the expression and function of Sox7 and Sox17 is complicated. Although VEGF upregulated Sox7 and Sox17 for a long-period, this increasing pattern of expression was not continuous. Instead, upregulation of Sox7 and Sox17 by VEGF was halted transiently (Figure 6A) and resumed (Figure 7A), suggesting that multiple mechanisms downstream of VEGF signaling may regulate
Sox7 and Sox17 expression. Although we suggest VEGFR2 is a functional player downstream of Sox7 and Sox17, more molecular mechanisms may be involved in the SoxF-mediated vascular changes, as there were several transcripts noticed in our microarray.

Biochemical signaling pathways activated by VEGF stimulation have been elucidated, but how VEGF cues are engaged in the transcriptional regulation of angiogenesis remains poorly understood. We revealed that VEGF can upregulate SoxF transcription factors, which are essential for angiogenesis. Interestingly, VEGF promotes SoxF expression at the protein level, but not at the transcript level. Endothelial cells may utilize post-transcriptional regulation to elicit a rapid increase in SoxF expression in response to angiogenic cues by skipping transcriptional regulation. Analogously, previous studies have reported several lines of evidence for post-transcriptional regulation of gene expression in the vascular system. In this regard, mTOR, which is a key molecule downstream of various tyrosine kinase receptors including VEGFR2, is a well-characterized regulator of protein synthesis. Here, we showed that mTOR activity regulates Sox7 and Sox17 expression in endothelial cells and that Sox7 and Sox17 expression is reduced in mTOR-deficient vessels. Recently, mTOR signaling was reported to build the gene regulatory network for metabolic systems by regulating key transcription factors, such as HIF1α (hypoxia-inducible factor 1 alpha) and SREBPs (sterol regulatory element-binding proteins). The regulation of SoxF expression by mTOR suggests a potential mechanism by which mTOR regulates the gene regulatory network for angiogenesis. Additional studies should be carried out to validate this potential mechanism.

In this study, Sox7 and Sox17 promote the transcript levels of VEGFR2. Regarding the SoxF regulation of VEGFR2 expression, it has already been reported that Sox7 and Sox18 control VEGFR2 expression by modulating the gene regulatory element in zebrafish. Further investigation is needed to understand how Sox7 and Sox17 regulate VEGFR2 expression in mice.

Several transcription factors govern the acquisition and maintenance of cell identity by building transcriptional networks and inducing the expression of characteristic markers. For example, Sox2 in embryonic stem cells and ERG in endothelial cells prevalently regulate genes associated with transcriptional regulation. In contrast, Sox7 and Sox17 mainly regulate a set of genes involved in transmembrane signaling, such as cell surface receptors and G-protein–coupled receptors, rather than genes involved in transcriptional regulation and endothelial marker expression. Sox7 and Sox17 seem to be specialized transcription factors for sensing environmental cues and relaying them to the cell interior in angiogenic contexts. Sox7, Sox17, and Sox18 were originally categorized under the SoxF subfamily based on amino acid sequence homology. Now, they are regarded as Sox members specialized for vascular development. We also observed that, among the whole Sox family, only SoxF members had robust and specific expression in endothelial cells, distinguishing them as endothelial Sox members. During mouse vascular development, Sox17 is crucial for arterial specification and angiogenesis, whereas Sox18 promotes lymphatic emergence by inducing Prox1 expression. These observations indicate that SoxF members are functionally divergent depending on the vascular context. However, compound silencing of Sox7 and Sox18 in zebrafish impairs arterial differentiation, suggesting a potential functional redundancy in endothelial fate determination within the SoxF subfamily.

The phenotypic variation found in different SoxF loss-of-function animal models has prevented the establishment of a functional relationship among SoxF members in vascular morphogenesis. In addition, vascular analysis of the Sox7 knockout mouse model has been insufficient in studies determining the role of the whole SoxF subfamily in mouse vascular development.

Here, we investigated the expression, function, and regulation of SoxF in vascular development by generating new Sox7-targeted mouse models. Importantly, we found a high degree of resemblance between Sox7 and Sox17, both in their expression and function. Sox7 and Sox17 were coincidently expressed in endothelial cells from angiogenic vessels. Sox7 deletion impaired embryonic and postnatal developmental angiogenesis, phenocopying Sox17 deletion. Sox7 and Sox17 also shared upstream and downstream regulatory components, creating a positive feedback loop for VEGF signaling. This multifaceted overlap strongly suggests functional cooperation between Sox7 and Sox17. Moreover, the impaired developmental angiogenesis found in SoxF7 and Sox17 double heterozygous embryos decisively demonstrates the genetic cooperation between Sox7 and Sox17 in vascular development.

Multiple Sox members frequently cooperate during organogenesis in several different ways. Sox1, Sox2, and Sox3, which belong to the SoxB1 subfamily, function redundantly in neural stem cell maintenance, as single or double knockouts of these members induces no phenotypic change. Sox2, Sox3, and Sox11 sequentially regulate the same set of genes in consecutive differentiation processes toward the neuronal fate. Sox2 and Sox9 complementarily cooperate in lung morphogenesis by generating 2 different cell types in the pulmonary system: bronchole and accessory lobe, respectively. Our findings exclude sequential and complementary modes for Sox7 and Sox17 cooperation because Sox7 and Sox17 were coincidently expressed and lack an epistatic relationship in angiogenic vessels. Sox7 and Sox17 exhibited a substantial overlap in expression, function, and regulation, implying a redundant type of cooperation. However, Sox7 and Sox17 exert their vascular function nonredundantly, as single knockout of either Sox7 or Sox17 induces severe angiogenic defects and embryonic lethality. On the contrary, Zhou et al reported redundant roles of Sox7, Sox17, and Sox18 in postnatal retina vascular development. Differences in gene excision efficiency and developmental stage for analysis may lead to discrepant conclusion between 2 studies. Our study reveals that Sox7 and Sox17 jointly and nonredundantly play an indispensable role in developmental angiogenesis, presenting a novel mode of genetic cooperation in the Sox family.

Sox members exhibit dosage-sensitive actions under certain circumstances, as exemplified by Sox2 in embryonic stem cells, retinal progenitors, and foregut-derived tissues. Similarly, we found that the genetic cooperation between Sox7 and Sox17 is sensitive to their collective gene dosage. Loss of any 2 alleles of Sox7 and Sox17 sufficiently impairs embryonic angiogenesis. In addition, mutants lacking more alleles
of the Sox7 and Sox17 genes showed more severe vascular phenotypes in postnatal retina angiogenesis. We speculate that the positive feedback regulation of VEGF signaling may underlie the dosage-sensitive characteristics of SoxF function. Positive feedback control generally amplifies the system gain and can have great consequences, even with a small initial input. Insufficient dosage of Sox7 and Sox17, which serve as the amplifiers, may cause a significant decrease in angiogenic output. Taken together, our results indicate that the genetic cooperation of a SoxF duo promotes angiogenesis in a dosage-sensitive manner by reinforcing VEGF signaling.

Acknowledgments

We thank S.I. Chang and Y.M. Lee at the KAIST Animal Facility for maintaining mouse colonies and blastocyst injection. We thank Y.G. Lee for drawing schematic illustration.

Sources of Funding

This work was supported by the National Research Foundation of Korea (NRF-2015R1A2A100102880, NRF-2015M3A9B6046565, and NRF-2015M3A9C6030280, I. Kim), the KAIST Future Systems Healthcare Project (N01150030, I. Kim), and the KAIST Venture Research Program (N01140240, I. Kim) of the Ministry of Science, ICT and Future Planning.

Disclosures

None.

References


Kim et al

SofX Members Reinforce VEGF Signaling

This page contains references related to the study by Kim et al. on the role of SoxF members in reinforcing VEGF signaling in angiogenesis. The references cover various aspects of vascular development, including transcriptional control, endothelial cell differentiation, and the role of transcription factors such as ERG and Sox7/17/18. The study highlights the importance of positive feedback regulation in angiogenesis and the potential mechanisms by which SoxF genes influence vascular development.

Novelty and Significance

What Is Known?

- Transcription factors Sox7 and Sox17 belonging to SoxF subgroup are important for vascular development.
- Vascular endothelial growth factor (VEGF) signaling in angiogenesis is antagonized by Notch-mediated negative feedback control.

What New Information Does This Article Contribute?

- Sox7 and Sox17 jointly promote developmental angiogenesis with overlapping expression and function.
- The VEGF pathway is reinforced by SoxF-mediated positive feedback control.

Vascular signaling is a key pathway for angiogenesis and requires highly coordinated regulation. Although the Notch pathway is well known to suppress excessive VEGF activity via negative feedback, the positive feedback control for augmenting VEGF signaling remains poorly understood. Here, we identified a positive feedback control of VEGF signaling by revealing the upstream and downstream mechanisms of Sox7 and Sox17 in endothelial cells. Sox7 and Sox17 are individually indispensable for developmental angiogenesis and furthermore jointly cooperate with overlapping expression and function. The genetic cooperation of Sox7 and Sox17 is sensitive to their combined gene dosage. VEGF signaling upregulated both Sox7 and Sox17 expression in angiogenesis. In turn, Sox7 and Sox17 promoted VEGFR2 (VEGF receptor 2) expression in angiogenic vessels, suggesting a positive feedback loop between VEGF signaling and Sox17. This positive feedback control may be responsible for generating sufficient angiogenic output from weak VEGF cue. SoxF transcription factors are indispensable players in developmental angiogenesis by acting as positive feedback regulators of VEGF signaling.
SoxF Transcription Factors Are Positive Feedback Regulators of VEGF Signaling
Kangsan Kim, Il-Kug Kim, Jee Myung Yang, Eunhyeong Lee, Bong Ihn Koh, Sukhyun Song, Junseong Park, Sungsu Lee, Chulhee Choi, Jin Woo Kim, Yoshiaki Kubota, Gou Young Koh and Injune Kim

_Circ Res._ 2016;119:839-852; originally published online August 15, 2016; doi: 10.1161/CIRCRESAHA.116.308483

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2016 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/119/7/839

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2016/08/12/CIRCRESAHA.116.308483.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org//subscriptions/
SUPPLEMENTAL MATERIAL

SoxF transcription factors are positive feedback regulators of VEGF signaling

Kangsan Kim\textsuperscript{1,5}, Il-Kug Kim\textsuperscript{2}, Jee Myung Yang\textsuperscript{2}, Eunhyeong Lee\textsuperscript{3}, Bong Ihn Koh\textsuperscript{2}, Sukhyun Song\textsuperscript{5}, Junseong Park\textsuperscript{4}, Sungsu Lee\textsuperscript{6}, Chulhee Choi\textsuperscript{4}, Jin Woo Kim\textsuperscript{1}, Yoshiaki Kubota\textsuperscript{7}, Gou Young Koh\textsuperscript{1,2,3,5}, and Injune Kim\textsuperscript{1,2,3}

\textbf{Short title}: Kim, SoxF members reinforce VEGF signaling.

\textsuperscript{1}Department of Biological Sciences, \textsuperscript{2}Graduate School of Medical Science and Engineering, \textsuperscript{3}Biomedical Science and Engineering Interdisciplinary Program, \textsuperscript{4}Department of Bio and Brain Engineering, Korea Advanced Institute of Science and Technology, Daejeon 34141, Republic of Korea
\textsuperscript{5}Center for Vascular Research, Institute for Basic Science, Daejeon 34141, Republic of Korea.
\textsuperscript{6}Department of Otolaryngology-Head and Neck Surgery, Chonnam National University Hospital, Gwangju 61469, Republic of Korea
\textsuperscript{7}The Laboratory of Vascular Biology, Keio University, Tokyo 160-8582, Japan
DETAILED METHODS

Generation of Sox7 targeted mouse models

Sox7 genomic clone was isolated from mouse BAC DNA library (clone: RPCIB731A13232Q, Source Bioscience Life Sciences). Targeting constructs for Sox7mCh and Sox7fl alleles were generated using a recombineering-based method. E14 embryonic stem cells were electroporated with targeting constructs and were positively selected with G418 (A.G. Scientific). Targeted clones were confirmed by Southern blot analysis with two external probes and their karyotypes were examined. For each line, two independent targeted clones were injected into donor C57BL/6 mouse blastocyst embryo. Male chimeras were bred with C57BL/6 female mice to achieve germline transmission. Neo cassette was removed by mating germline transmitted mice with Protamine-Flpe driver line. Removal of the neo-cassette from targeted alleles was verified by Southern blot analysis. Genotypes of Sox7mCh/+ progeny were examined by PCR with the following primers: common forward, 5’-CCCGGGTGCCCAGCTGATAA-3’; reverse primer 1, 5’-CAGCCCATCCGCAGCTGC-3’; reverse primer 2, 5’-GAACGTGAGGGACAGGATGTCC-3’. 317 and 209 bp products were amplified from mutant and wild-type alleles, respectively. Genotypes of Sox7fl progeny were checked by PCR with the following primers: forward primer: 5’-CGAATAGGCTGGGTTCGCTCATC-3’; reverse primer, 5’-CCCCAATTCTCTGGCCTGTCTG-3’. 247 and 165 bp products were generated from mutant and wild-type alleles, respectively.

Mutant mice and pharmacologic inhibition

Sox7mCh, Sox7fl, Sox1fl, Tie2-Cre, Cdh5(BAC)-CreER, and mTORfl mice were housed in a pathogen-free animal facility at KAIST. mTORfl mouse model was provided by Dr. Jinwoo Kim at KAIST. All mouse lines were backcrossed onto C57BL/6 background at least ten times. For Sox7 knockout, Sox7mCh mouse were intercrossed. For deleting Sox7, Sox17, and mTOR specifically from embryonic endothelial cells, the Tie2-Cre driver line was crossed with Sox7mCh/fl, Sox1fl, and mTORfl mice, respectively. For excising Sox7 and Sox17 specifically in endothelial cells after birth, the Cdh5(BAC)-CreERT2 driver line was bred with Sox7fl/fl and Sox17fl/fl mice, respectively, and their progeny were subcutaneously administered with 25 mg/kg tamoxifen (Sigma) every day during postnatal days 1 to 3. For compound haploinsufficiency of Sox7 and Sox17, Sox7mCh mice were crossed with Sox1fl mice. To block VEGF signalling, PBS or VEGF-trap (25 mg/kg) was subcutaneously injected into experimental mice at postnatal days 3 and 4. Mice were anesthetized at postnatal day 5 and their retinas and brains were harvested for histological experiments. All animal experiments were approved by the Animal Care Committee of Korea Advanced Institute of Science and Technology.

Histology and tissue immunofluorescence
Mouse tissues were fixed in 2% paraformaldehyde on ice for 1-2 hour. For high-resolution image of embryonic vascular sprouting, mouse embryo hindbrain tissues were prepared as described. To obtain cryosections, tissues were dehydrated in phosphate buffered saline (PBS) with 20% sucrose at 4°C overnight and were embedded in OCT compound (Leica). Tissues were sectioned using a cryomicrotome (Leica). For immunofluorescence, tissue sections were blocked in PBST (0.1% Triton X-100 in PBS) containing 5% donkey or goat serum, incubated with primary antibodies overnight and fluorescently labeled secondary antibodies for 5 hours. The following primary antibodies were used: anti-PECAM (Chemicon, 1:200), anti-SMA (Sigma, 1:400), anti-Sox7 (R&D systems, 1:100), anti-Sox17 (R&D systems, 1:100), anti-Lyve1 (angiobio, 1:200), and anti-VE-cadherin (BD Biosciences, 1:200), anti-VEGFR2 (R&D systems, 1:200), and anti-actin (Sigma, 1:400). The secondary antibodies for immunofluorescent staining were used as previously described. Immunofluorescent images were obtained by Zeiss LSM780 confocal fluorescence microscopes.

**Morphometric analysis**

For quantification of embryonic vasculatures, thickness of PECAM-positive hindbrain vessels was measured by z-stack information obtained from Zeiss confocal fluorescence microscopes. Number of sprouting vessels was counted on the vascular front from at least three representative fields of embryonic hindbrain vessels. ImageJ software was used for quantifying branching point number of retina vessels, immunofluorescence intensity, and length and junction number of tubules in the tubule formation assay.

**Western blot analysis**

HUVECs and whole embryo tissues were harvested and dissolved in RIPA buffer (150mM sodium chloride, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 50mM Tris-HCl pH7.5, 2mM EDTA) with proteinase inhibitor cocktail (Roche). Protein samples were separated by SDS-PAGE and transferred to nitrocellulose membrane. Membranes were blocked in Tris-buffered saline containing 0.1% Tween-20 and 5% non-fat skim milk (or 5% bovine serum albumin) for 1 hour, incubated with primary antibody overnight at 4°C, and HRP-conjugated secondary antibody for 2 hours. The following primary antibodies were used: anti-Sox7 (R&D systems, 1:2500), anti-Sox17 (R&D systems, 1:2500), anti-actin (Sigma, 1:5000), anti-p4EBP1 (Cell Signaling, 1:1000), and anti-VEGFR2 (Cell Signaling, 1:2500).

**Flow cytometry analysis and cell sorting**

For flow-cytometry analysis, embryos were digested into single cell suspension by incubating in 1 mg/ml collagen type 4 at 37°C for 1 hour. Then cells were filtered through a 40μm-pore nylon mesh to remove cell clumps. For Sox expression profiling in endothelial cells, cells from E11.5, E14.5, and
E17.5 embryos were stained with the following antibodies on ice: PE-conjugated anti-CD144 (BD Biosciences, 1:200), APC-conjugated anti-CD45 (Biolegend, 1:400), and APC-conjugated anti-TER119 (Biolegend, 1:400). CD144+CD45-Ter119- endothelial cells and other cells were fractionated by a cell sorter. To examine mCherry expression, cells of Sox7<sup>mCh</sup>/+ embryos were stained with FITC-conjugated anti-PECAM (Biolegend, 1:400), APC-conjugated anti-CD45 (Biolegend, 1:400), and APC-conjugated anti-TER119 (Biolegend, 1:400). To discriminate dead cells, cells were resuspended in 2 μg/ml 7-amino-actinomycin D (Invitrogen) before analysis. All flow-cytometry was performed on a FACSARiaII (BD Biosciences). Data were analyzed by FlowJo software (Tree Star).

**RNA interference and DNA transfection**

Human umbilical vein endothelial cells (HUVECs) were maintained in EGM2 media (Lonza). For small RNA interference (siRNA), oligonucleotides against Sox7 (5’-GCAUAACAGUGUGCUGAAA-3’, Dharmacon), against Sox17 (5’-ACGCCAGCUCCGCGUAUA-3’, Dharmacon), or scrambled siRNA for negative control were used. For mTOR overexpression, plasmids encoding wild-type and constitutively active form of mTOR were used. HUVECs with a 40-60% confluence were transfected with siRNAs using Lipofectamine™ RNAiMAX (Invitrogen) or plasmids using Lipofectamine™ LTX (Invitrogen) in OptiMEM media. Culture media was changed 4-6 hours after transfection. siRNA-mediated silencing and overexpression were usually valid up to 48 hours post-transfection.

**Microarray analysis**

For microarray analysis, total RNA was purified from HUVECs 36 hours after Sox7-, Sox17-, or Sox7/Sox17-siRNA silencing. Two independent sets of total RNA were subjected to quality control based on concentration, OD260/230, OD260/290, ratio (28s/18s), and RIN number using an Agilent 2100 Bioanalyzer. Agilent Human GE (V2) 4 X 44K was used for DNA microarray, and normalization and clustering were performed using the Agilent GeneSpring software. Raw data was uploaded onto the Gene Expression Omnibus (GEO) database of the National Center for Biotechnology Information (GSE75380). Gene Ontology was analyzed by the DAVID method.

**Quantitative reverse transcription polymerase chain reaction**

Total RNA was extracted from HUVECs and sorted cells from whole embryos by using an RNeasy mini kit (Qiagen). cDNA was synthesized with SuperScriptII reverse transcriptase (Invitrogen). Quantitative PCR was performed on a CFX96 real-time PCR detection system (Bio-Rad) with fast SYBR green master mix (Roche). Primer pairs used are listed in Online Table I. Real-time PCR data were analyzed with the CFX manager software (Bio-Rad). GAPDH was used as an internal reference gene for normalization.
Cell cycle analysis

Equal number of HUVECs was seeded after control, Sox7-, or Sox17- siRNA silencing and cultured cell number was counted at indicated time points. For cell cycle analysis, HUVECs were incubated with 10 μM BrdU, washed three times with PBS to remove unincorporated BrdU, and examined with the FITC BrdU flow kit (BD Bioscience). The cells were analyzed with a FACS AriaII (BD Biosciences).

In vitro 3D sprouting assay and tubule forming assay

Microfluidic device for three-dimensional sprouting assay was prepared as previously described. Briefly, three channels in the device were made with poly-. The two empty spaces between the middle and side channels, in which cells sprout, were filled with collagen type I gels (BD Bioscience) and fibronectins (Invitrogen). HUVECs silenced with control, Sox7-, or Sox17-siRNA were seeded into the device with EGM2 media lacking VEGF. VEGF gradient was applied after one day and culture media was exchanged every day. HUVECs in the device were fixed and processed for immunostaining four days after seeding. For tubule forming assay, 50,000 cells were seeded in a Matrigel matrix (BD Biosciences) one day after siRNA knockdown. One day later, node number and tube length were quantified.

Statistics

Experimental values are presented as the mean ± standard error unless otherwise indicated. Statistical difference was determined performing the Mann-Whitney U test between two groups or the Kruskal-Wallis test followed by Bonferroni correction for more than three groups. Statistical significances are represented as * (P < 0.05) or ** (P < 0.01).
Online Figure I. Generation of the Sox7 mCherry knock-in mouse model.

(A) Schematics showing Sox7 targeting in the mouse genome. The genomic locus spanning protein-coding sequences scattered in two neighboring exons (black boxes) and an intercalated intron of murine Sox7 gene is replaced by the mCherry coding sequence and neo cassette by homologous recombination. Later, the neo cassette flanked by FRT sequences is removed by Flp recombinase. (B) Southern blot analysis of heterozygous F1 (before neo excision) and F2 (after neo excision) mice. (C) We confirmed no Sox7 transcript in Sox7^mCh/mCh embryos. ND: not detectable. Data are presented as mean ± standard error (n = 4). * P < 0.05. (D) Genotypes of progeny derived from Sox7^mCh/+ intercrosses. Lethality appears around E11.5.
Online Figure II. Loss of Sox7 impairs developmental angiogenesis.

(A-B) Gross appearance of E10.5 Sox7^mCh/+ and Sox7^mCh/mCh embryos (A) and yolk sacs (B).

(C-G) Whole-mount PECAM immunostaining of Sox7^mCh/+ and Sox7^mCh/mCh embryos. (C) Whole embryo images. (D) Higher magnification of hindbrain vessels (yellow boxes in C) and (E) intersomitic vessels (red boxes in C). (F) Yolk sac vessels. (G) Three-dimensional representation of intersomitic vessels. Intersomitic vessels migrate dorsally along the dorsal roof of somites on each side and pair-up at the top midline along the trunk (arrow) in Sox7^mCh/+ embryos. However, Sox7-deficient vessels are displaced from the dorsal roof and fail to make a connection with vessels from the opposite side (arrowheads).

(H) Gross appearance and (I-K) whole-mount PECAM immunostaining of E9.5 Sox7^mCh/+ and Sox7^mCh/mCh embryos. (I) Whole embryo images. (J) Higher magnification of hindbrain vessels (yellow boxes in I) and (K) intersomitic vessels (red boxes in I). Note arrest of angiogenesis in E9.5 Sox7^mCh/mCh embryos. Scale bars: 500μm.
Online Figure III. Endothelial-specific targeting of the floxed Sox7 mouse model.

(A-C) Generation of the floxed Sox7 mouse model. (A) Targeting strategy to generate a floxed Sox7 allele. LoxP elements (white arrowheads) were inserted at intronic regions on both sides of the first exon of murine Sox7 gene by homologous recombination. The neo-resistant cassette flanked by two FRT sequences (black arrowheads) was removed by Flp recombinase. (B) The targeted allele of Sox7 gene was verified by Southern blot. (C) Remarkable decrease in Sox7 transcripts by Tie2-Cre-mediated deletion of floxed Sox7 alleles in endothelial cells. Data are presented as mean ± standard error (n = 4). * P < 0.05. (D) Genotypes of progeny derived from mating Tie2-Cre;Sox7^{mCh/fl} males with Sox7^{fl/fl} females. Lethality appears around E11.5.
Online Figure IV. Compound haploinsufficiency of Sox7 and Sox17 leads to embryonic lethality. Sox7^{mCh/+};Sox17^{GFP/+} double heterozygous embryos presented with severe growth retardation and a pale appearance of the embryo proper (A) and yolk sac (B). Scale bars: 500\,\mu m. (C) Genotype of progeny derived from mating between Sox17^{GFP/+} male and Sox7^{mCh/+} female mice.
Online Figure V. Arterial expression of Sox7 and Sox17 in retina vessels.

(A) PECAM immunostaining of Sox7\textsuperscript{mCh/+} and (B) Sox17\textsuperscript{GFP/+} retina at P5. Arrows indicate arteries. Scale bars: 500 µm.
Online Figure VI. *mTOR* deletion delays embryonic vascular development.

(A) Immunoblotting and (B) quantification of Sox7 and Sox17 at 24 hours after VEGF and cycloheximide treatment. NC: negative control treated with vehicles, VE: VEGF (50 ng/ml), CHX: cycloheximide (20 µg/ml). Data are presented as mean ± standard deviation ($n = 4$). * $P < 0.05$.

(C) Gross morphology and (D) whole-mount PECAM immunostaining of E11.5 control (*mTOR* $^{fl/fl}$) and *mTOR* $^{i-EC}$ (*Tie2-Cre; mTOR* $^{fl/fl}$) embryos. (E) Higher magnification of hindbrain vessels (yellow boxes in D) and (F) intersomitic vessels (red boxes in D). Scale bars: 500 µm.
Online Figure VII. Genes and signaling pathways regulated by Sox7 and Sox17 silencing.
Microarray analysis using total RNA from HUVECs after Sox7, Sox17, or Sox7/Sox17 double siRNA knockdown (KD). (A) Venn diagrams showing the number of genes down-regulated or (E) up-regulated by more than 2-fold. (B) Heat map of down-regulated or (F) up-regulated genes emphasizing the additive effect of double knockdown compared with single knockdown. (C) Top 10 gene ontology (GO) terms in biological processes using the list of down-regulated or (G) up-regulated genes ($P < 0.05$). (D) Heat map of down-regulated or (H) up-regulated genes in the category of angiogenesis by more than 2-fold. See Online Table 2 for raw data of the entire genes in the category.
of angiogenesis. (I) Comparison of GO terms enriched in target genes of ERG, Sox2, Sox9, Sox7, and Sox17. (J) Twenty most significant pathways of down-regulated or (K) up-regulated genes associated with angiogenesis by IPA analysis ($P < 0.0001$). Bars and dots depict Z-scores and $P$ values, respectively.
Online Figure VIII. *Sox7* and *Sox17* silencing reduces the tubule-forming capacity of endothelial cells.

(A-C) Cell proliferation assessment of HUVECs after *Sox7* or *Sox17* knockdown (KD). (A) Cell number (*n* = 6). (B) FACS plots showing BrdU incorporation. Boxes represent each phase of the cell cycle. (C) Quantification of the analysis in (B) (*n* = 3). (D) Tubule formation of HUVECs after *Sox7* or *Sox17* knockdown. Scale bars: 100μm. (E) Quantification of tubule length and (F) tube nodes (*n* = 8). Data are presented as mean ± standard error. *P* < 0.05, **P** < 0.01, N.S.: not significant.
Online Figure IX. Feedback controls of VEGF signaling.

SoxF transcription factors Sox7 and Sox17 are key players in the positive feedback control of VEGF signaling. This positive feedback control can generate significant angiogenic output by amplifying VEGF cues. On the other hand, excessive VEGF signaling can be suppressed by negative feedback control. VEGF stimulus up-regulates Dll4, a Notch ligand, thereby increasing Notch activity in endothelial cells. Notch activity represses VEGFR2 expression, stabilizing vessels.
### SUPPLEMENTAL TABLE

**Online Table I. Primers used in qRT-PCR**

<table>
<thead>
<tr>
<th>Species</th>
<th>Gene</th>
<th>Direction</th>
<th>Sequence (5' → 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>Sox7</td>
<td>forward</td>
<td>GAGTGCCGCCTCTATCCCTTCC</td>
</tr>
<tr>
<td>Human</td>
<td>Sox7</td>
<td>reverse</td>
<td>CACTGGGAAGACGAACCTCAGGG</td>
</tr>
<tr>
<td>Human</td>
<td>Sox17</td>
<td>forward</td>
<td>CAGACTCCTGGGGTTTTTGTGCTG</td>
</tr>
<tr>
<td>Human</td>
<td>Sox17</td>
<td>reverse</td>
<td>GAAATGGAGGAAGCTGTGGGACAC</td>
</tr>
<tr>
<td>Human</td>
<td>Vegfr2</td>
<td>forward</td>
<td>ATGACATTCTGATCATGGAGC</td>
</tr>
<tr>
<td>Human</td>
<td>Vegfr2</td>
<td>reverse</td>
<td>CCCAGATGCGCTGATGAG</td>
</tr>
<tr>
<td>Human</td>
<td>Gapdh</td>
<td>forward</td>
<td>CTTCGCTCTCTGCTCTCTCT</td>
</tr>
<tr>
<td>Human</td>
<td>Gapdh</td>
<td>reverse</td>
<td>GTTAAAGCAGCCTGGTG</td>
</tr>
<tr>
<td>Mouse</td>
<td>Sox1</td>
<td>forward</td>
<td>AGTCTCTCTGGGCTGATCTCCT</td>
</tr>
<tr>
<td>Mouse</td>
<td>Sox1</td>
<td>reverse</td>
<td>CAGGTCGGTCTCCATCTCA</td>
</tr>
<tr>
<td>Mouse</td>
<td>Sox2</td>
<td>forward</td>
<td>CTGGACTGCGAAGTGGAAAG</td>
</tr>
<tr>
<td>Mouse</td>
<td>Sox2</td>
<td>reverse</td>
<td>GCAATATCAAGATTCTACTCTC</td>
</tr>
<tr>
<td>Mouse</td>
<td>Sox3</td>
<td>forward</td>
<td>AGATCGACAATCCCGAGATCAG</td>
</tr>
<tr>
<td>Mouse</td>
<td>Sox3</td>
<td>reverse</td>
<td>ACTTGATGCTCCGGTAGTCTCTCC</td>
</tr>
<tr>
<td>Mouse</td>
<td>Sox4</td>
<td>forward</td>
<td>CTGCTACAAGGTCGGACTC</td>
</tr>
<tr>
<td>Mouse</td>
<td>Sox4</td>
<td>reverse</td>
<td>CAGGCTTTGCAACAGGTAG</td>
</tr>
<tr>
<td>Mouse</td>
<td>Sox5</td>
<td>forward</td>
<td>GAAGATGGGAGAAGCTGATCGAAG</td>
</tr>
<tr>
<td>Mouse</td>
<td>Sox5</td>
<td>reverse</td>
<td>TCGAGATCCCTTTATCTTCTCC</td>
</tr>
<tr>
<td>Mouse</td>
<td>Sox6</td>
<td>forward</td>
<td>AGCTGCTCTACGCTCTAGGAG</td>
</tr>
<tr>
<td>Mouse</td>
<td>Sox6</td>
<td>reverse</td>
<td>TCACTCCACATAGGCTCTTCTG</td>
</tr>
<tr>
<td>Mouse</td>
<td>Sox7</td>
<td>forward</td>
<td>ATACCGCAGAGCTAGCAAGAC</td>
</tr>
<tr>
<td>Mouse</td>
<td>Sox7</td>
<td>reverse</td>
<td>ATACGATGGGAGGATGCTG</td>
</tr>
<tr>
<td>Mouse</td>
<td>Sox8</td>
<td>forward</td>
<td>TGCTATTTGTAATCTGGGCTG</td>
</tr>
<tr>
<td>Mouse</td>
<td>Sox8</td>
<td>reverse</td>
<td>CTGCTTTCTCTGCTCTGAC</td>
</tr>
<tr>
<td>Mouse</td>
<td>Sox9</td>
<td>forward</td>
<td>CTAGCAAGACTCTGCGCAAG</td>
</tr>
<tr>
<td>Mouse</td>
<td>Sox9</td>
<td>reverse</td>
<td>GTAATTGGAATCTGAGCTTTT</td>
</tr>
<tr>
<td>Mouse</td>
<td>Sox10</td>
<td>forward</td>
<td>ATCCGGACTAACAGTACCAACCTT</td>
</tr>
<tr>
<td>Mouse</td>
<td>Sox10</td>
<td>reverse</td>
<td>GCACTTCTGTAGTCTAGCTG</td>
</tr>
<tr>
<td>Mouse</td>
<td>Sox11</td>
<td>forward</td>
<td>ACCTGGGTGTTACGTTAGAG</td>
</tr>
<tr>
<td>Mouse</td>
<td>Sox11</td>
<td>reverse</td>
<td>CCACTTTGAGCATCAGGAG</td>
</tr>
<tr>
<td>Mouse</td>
<td>Sox13</td>
<td>forward</td>
<td>ATGTTGGAAGCTAAGGAGTCAAA</td>
</tr>
<tr>
<td>Mouse</td>
<td>Sox13</td>
<td>reverse</td>
<td>CCACTTCTCTCTCTACAGTG</td>
</tr>
<tr>
<td>Mouse</td>
<td>Sox14</td>
<td>forward</td>
<td>GAAGGAGCCACCCTGACTCAAGT</td>
</tr>
<tr>
<td>Mouse</td>
<td>Sox14</td>
<td>reverse</td>
<td>CACTGCAACCACCTACTGAG</td>
</tr>
<tr>
<td>Mouse</td>
<td>Sox15</td>
<td>forward</td>
<td>CCACTTTGAGGACTACCT</td>
</tr>
<tr>
<td>Mouse</td>
<td>Sox15</td>
<td>reverse</td>
<td>GCTACTGGTAAGGGAGAAGAGG</td>
</tr>
<tr>
<td>Mouse</td>
<td>Sox17</td>
<td>forward</td>
<td>GAGGGCCAGAAAGCAGTGTTACAC</td>
</tr>
<tr>
<td>Mouse</td>
<td>Sox17</td>
<td>reverse</td>
<td>CTGGCTAAAACCTGGGAGTATTG</td>
</tr>
<tr>
<td>Mouse</td>
<td>Sox18</td>
<td>forward</td>
<td>ACAACGCAGTATCGAGCAAAGT</td>
</tr>
<tr>
<td>Mouse</td>
<td>Sox18</td>
<td>reverse</td>
<td>CGTACTTTGATTTGGAAGTGC</td>
</tr>
<tr>
<td>Mouse</td>
<td>Sox21</td>
<td>forward</td>
<td>CCTATCCCTCCTCCTCCT</td>
</tr>
<tr>
<td>Mouse</td>
<td>Sox21</td>
<td>reverse</td>
<td>CTCTCCCTCTCCTCCTGCT</td>
</tr>
<tr>
<td>Mouse</td>
<td>Sox30</td>
<td>forward</td>
<td>ATCCACCTACCTCCACTGGA</td>
</tr>
<tr>
<td>Mouse</td>
<td>Sox30</td>
<td>reverse</td>
<td>GCCTATGTTTTTGGTGATCTC</td>
</tr>
</tbody>
</table>
SUPPLEMENTAL REFERENCES