Notch Pathway Targets Proangiogenic Regulator Sox17 to Restrict Angiogenesis

Seung-Hun Lee,* Sungsu Lee,* Hanseul Yang,* Sukhyun Song, Kangsan Kim, Thomas L. Saunders, Jeong K. Yoon, Gou Young Koh, Injune Kim

Rationale: The Notch pathway stabilizes sprouting angiogenesis by favoring stalk cells over tip cells at the vascular front. Because tip and stalk cells have different properties in morphology and function, their transcriptional regulation remains to be distinguished. Transcription factor Sox17 is specifically expressed in endothelial cells, but its expression and role at the vascular front remain largely unknown.

Objective: To specify the role of Sox17 and its relationship with the Notch pathway in sprouting angiogenesis.

Methods and Results: Endothelial-specific Sox17 deletion reduces sprouting angiogenesis in mouse embryonic and postnatal vascular development, whereas Sox17 overexpression increases it. Sox17 promotes endothelial migration by destabilizing endothelial junctions and rearranging cytoskeletal structure and upregulates expression of several genes preferentially expressed in tip cells. Interestingly, Sox17 expression is suppressed in stalk cells in which Notch signaling is relatively high. Notch activation by overexpressing Notch intracellular domain reduces Sox17 expression both in primary endothelial cells and in retinal angiogenesis, whereas Notch inhibition by delta-like ligand 4 (Dll4) blockade increases it. The Notch pathway regulates Sox17 expression mainly at the post-transcriptional level. Furthermore, endothelial Sox17 ablation rescues vascular network from excessive tip cell formation and hyperbranching under Notch inhibition in developmental and tumor angiogenesis.

Conclusions: Our findings demonstrate that the Notch pathway restricts sprouting angiogenesis by reducing the expression of proangiogenic regulator Sox17. (Circ Res. 2014;115:215-226.)

Key Words: delta-like ligand 4 • Notch receptors • physiologic angiogenesis • Sox17 transcription factor

The Notch pathway plays a pleiotropic role in vascular development such as angiogenic regulation and arteriovenous specification. Notch activation stabilizes growing vascular network by suppressing excessive tip cell formation and maintaining stalk cell property at the vascular front.1–6 In line with this, Notch inhibition by delta-like ligand 4 (Dll4) blockade induces excessive but nonfunctional sprouting in tumor angiogenesis,7,8 becoming a potential therapeutic option for tumor vessels. Several genetic studies demonstrated that the Notch pathway is required for the formation of dorsal aorta and arterial specification of endothelial cells across vertebrate species.1,10–14 After activation by Notch ligands, the Notch intracellular domain released from Notch receptors translocates into nucleus and interacts with the transcription factor CBF1/RBP-J/Suppressor of Hairless/LAG-1.15 By using this signaling pathway, Notch system plays a role mainly by regulating expression of its downstream target genes such as Hes and Hey, which regulate the expression of further downstream genes as transcription factors. In fact, Notch signaling regulates the transcription of arterial marker genes including ephrinB216 and a key player in sprouting angiogenesis, VEGFR2.17 Although the study of Hey1/Hey2 double-null embryo showing no arterial endothelial marker expression identified Hey1 and Hey2 as transcription factors downstream of the Notch pathway in arterial fate determination,13 transcription factors involved in Notch-mediated vascular stabilization have not been revealed yet. Although differential gene expression between tip and stalk cells has been fairly elucidated,18–20 how the Notch pathway governs transcriptional regulation in tip–stalk specification remains incompletely understood.

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Transcription factor Sox17 belonging to Sox F subgroup of Sox family is crucial for many developmental processes,
such as endoderm organogenesis, fetal hematopoiesis, and vascular formation.\textsuperscript{21–23} Interestingly, Sox7 and Sox18, other members of Sox F subgroup, are necessary for arterial specification in zebra fish\textsuperscript{24–26} and lymphatic emergence in mouse,\textsuperscript{27} implicating Sox F members in cell fate determination during vascular development. Recently, it has been revealed that Sox F members can regulate arterial Dil4 expression by binding to enhancer region of Dil4,\textsuperscript{28} and Sox17 is indispensable for arterial specification in mouse vascular development by regulating the expression of Dil1, Dil4, and Notch4, key elements of the Notch pathway.\textsuperscript{29} These studies identified Sox17 as an upstream regulator of the Notch pathway in arterial specification. However, the role of Sox17 in sprouting angiogenesis during vascular development has not been well studied, although the compound heterozygosity of Sox18 and Sox17 during vascular development has not been well studied, although the compound heterozygosity of Sox17 and Sox18 results in abnormal heart and vascular development.\textsuperscript{30} A recent study elucidating the proangiogenic role of Sox17 in tumor angiogenesis\textsuperscript{31} suggests that Sox17 might play a proangiogenic role in vascular development. Furthermore, how Sox17 interacts with Notch signaling in sprouting angiogenesis remains unclear.

In this study, we investigated that Sox17 is expressed in endothelial cells at the vascular front and promotes sprouting angiogenesis in vascular development using Sox17 genetic mouse models. We found that Sox17 expression is reduced in the stalk cells, whereas it is robust in the tip cells. By gain and depletion of Notch activity, we unveiled that the Notch pathway inversely regulates Sox17 expression in sprouting angiogenesis. Taken together, our findings establish the reduced Sox17 expression as a key mechanism for Notch-mediated vascular stabilization.

### Methods

An expanded Methods section can be found in the Online Supplement.

#### Mutant Mice

For inducible Sox17 deletion and N1CID overexpression in endothelial cells, Sox17\textsuperscript{fl/fl} and Gt(Rosa)26Sox\textsuperscript{tm1(Notch1)Dam/J} mice were crossed with Cdh5(PAC)-Cre\textsuperscript{ERT2} transgenic mice, respectively. For endothelial-specific Sox17 deletion in embryonic development, Sox17\textsuperscript{fl/fl} mice were crossed with Tie2-Cre transgenic mice. For inducible Sox17 overexpression in endothelial cells, Tet-O–Sox17 mice were interbred VE-cadherin–tTA mice. The administrations of tamoxifen and doxycycline were performed as described.\textsuperscript{31–33} All animal experiments were approved by the Animal Care Committee of Korea Advanced Institute of Science and Technology.

#### Histology and Tissue Immunofluorescence

Cells and embryonic tissues (in 2% paraformaldehyde for 60 minutes), tumor tissues (in 1% paraformaldehyde for 60 minutes), hindbrain tissues (in 4% paraformaldehyde for 40 minutes), and extracted eye-balls (in 4% paraformaldehyde for 20 minutes) were fixed at 4°C and prepared for either whole mount or cryosection. For immunofluorescence, all samples were washed in PBS, blocked in PBS containing 0.1% Triton X-100 with 5% donkey serum, incubated with primary and fluorescently labeled secondary antibodies, counterstained with DAPI (4′-diamidino-2-phenylindole) or Hoechst33258, and finally mounted in Vectashield (Vector Laboratories). Immunofluorescent images were acquired using Zeiss LSM510 confocal fluorescence microscope.

### Embryonic Stem Cell Culture and Endothelial Differentiation

Mouse embryonic stem cell (ESC) lines were derived from Sox17\textsuperscript{fl/fl} blastocysts in a standard manner as described.\textsuperscript{34} Sox17-null ESC line was obtained by adenoviral Cre delivery to the Sox17\textsuperscript{fl/fl} ESC line. ESC lines were maintained on mouse embryonic fibroblast feeders in DMEM containing 15% fetal bovine serum and leukemia inhibitory factor. Endothelial cells were induced as described.\textsuperscript{35} Briefly, ESCs were cultured in the absence of leukemia inhibitory factor. After 4.5 to 5.0 days, fetal liver kinase 1-positive mesodermal precursor cells were purified using AutoMACS Pro Separator (Miltenyi) and plated onto 0.1% gelatin-coated dish in α-minimum essential medium with 10% fetal bovine serum in the presence of 50 ng/mL vascular endothelial growth factor-A165 (R&D) for 4 days.

### In Vitro Modulation of Notch Signaling

For in vitro activation of Notch signaling, human umbilical vein endothelial cells were transfected with EFhICN1.CMV.GFP vector (17623, Addgene) containing human Notch1 intracellular domain or EFhCMV.RFP vector (17619, Addgene) as a negative control. Twenty-four hours after transfection, cells were collected for immunoblotting or transcript analysis. Alternatively, human umbilical vein endothelial cells were plated on culture dishes coated with Dil4 (R&D) or BSA as a negative control for timed Notch activation. Cells were collected at the indicated time points for biochemical and transcriptional analyses. For in vitro inhibition of Notch signaling, human umbilical vein endothelial cells were treated with 10 μmol/L DAPT (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butylester) dissolved in dimethylsulfoxide or equal amount of dimethylsulfoxide as a negative control. After 12 hours, cells were harvested for analysis.

### Flow Cytometry Analysis and Cell Sorting

Differentiated cells were dissociated, incubated with antibodies, and analyzed by fluorescence-activated cell sorter AriAl (Beckton Dickinson). Dead cells were stained using 7-aminoactinomycin D (Invitrogen) and excluded for analysis. Tumor endothelial cells were purified as described.\textsuperscript{31} Briefly, tumor tissues were dissociated and incubated with antibodies. After enrichment using AutoMACS Pro Separator (Miltenyi), tumor endothelial cells (CD31^+CD45^-Ter19^-) were purified using fluorescence-activated cell sorter AriAl, and cell purity was checked to be >95%.

### Statistics

Values are reported as mean±SD unless otherwise indicated. Significant differences between means were determined by ANOVA followed by 2-tailed Student–Newman–Keuls test. Statistical significance was set at P<0.01 or P<0.05.

### Results

#### Sox17 Promotes Sprouting Angiogenesis in Embryonic Vascular Development

We examined Sox17 expression in sprouting angiogenesis by immunostaining embryonic vessels. We detected robust Sox17 staining only in the nuclei of endothelial cells of angiogenic capillary vessels (Figure 1A) as well as in arterial endothelial cells (Online Figure IA), suggesting the role of Sox17 in sprouting angiogenesis. To characterize the role of endothelial Sox17 in vascular development, we analyzed vascular phenotypes in mutant embryos (Tie2-Cre Sox17\textsuperscript{fl/fl}), of which Sox17 alleles had been excised from endothelial cells by Tie2-Cre. Mutant embryos died around embryonic day 11.5 with vascular defects and growth retardation.

### Nonstandard Abbreviations and Acronyms

<table>
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<tr>
<td>DAPT</td>
<td>(N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butylester)</td>
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<td>ESC</td>
<td>embryonic stem cell</td>
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(\textsuperscript{21–23})
Figure 1. Sox17 promotes sprouting angiogenesis during embryonic development. A. Angiogenic capillary vessels of embryonic day (E) 10.5 hindbrain stained with Sox17 (red), isolectin-B4 (IB4; green), and an endothelial nuclear marker, ETS-related gene (ERG; blue). Platelet endothelial cell adhesion molecule (PECAM) immunostaining of control (Sox17^{GFP/−}) and Tie2-Cre Sox17^{GFP/−} embryos at E10.5. B, Whole embryo images (left column). Higher magnification of hindbrain vessels (middle column) and intersomitic vessels (ISV, right column). C, Flat-mounted hindbrain tissues. Layered vasculatures are separately displayed depending on the radial position. D, Depth-coded 3-dimensional reconstructions for transverse views of layered hindbrain vascular network. Note no prominent radial vessel in Sox17-deficient mutant (bottom). The arrow indicates abnormality resembling vascular turf. E, Schematic drawings depicting hindbrain vascularization. F, Yolk sac vasculatures. PECAM immunostaining of control and Sox17^{GOF} embryos at E10.5. G, Hindbrain (left column) and yolk sac (right column). Note abundant vascular sprouting in Sox17^{GOF} mutants compared with controls. H, Higher magnification of yolk sac vessels, showing defective lumen formation in Sox17^{GOF} mutants. Scale bars, 100 μm (except B, 500 μm). PNP indicates perineural vascular plexus; RV, radial vessel; and SVP, subventricular vascular plexus.
(Figure 1B). Mature dorsal aorta successfully formed in control embryos, but mature vessels were rare in Sox17-deficient embryos (Online Figure 1B). In contrast to a well-branched patterning of control vessels, Sox17-deficient vessels were less branched in the head and trunk (Figure 1B) and frequently remained as primary plexuses (Online Figure 1C). To analyze sprouting angiogenesis more precisely, we examined blood vessels in embryonic day 10.5 hindbrain as a standard angiogenesis model during embryonic development.36 Control vessels sprouted perpendicularly from perineural vascular plexus toward the ventricular zone and then formed capillary plexus by horizontal sprouting at subventricular layer. However, Sox17-deficient vessels failed to sprout from perineural vascular plexus (Figure 1C–1E). Sprouting angiogenesis was also reduced in Sox17-deficient yolk sac vessels compared with controls (Figure 1F). Conversely, Sox17 overexpression in endothelial cells induced excessive angiogenesis as shown by increased vascular branching and more vascular sprouts in hindbrain and yolk sac relative to controls (Figure 1G). In addition, it led to the endothelial cells failing to form functional vessels as shown by defective lumen formation (Figure 1H) and remarkably less erythrocytes within yolk sac vessels in contrast to controls (Online Figure ID). These findings indicate that endothelial Sox17 is indispensable for proper angiogenesis in embryonic vascular development and suggest that proper Sox17 expression is crucial for stabilized vessel formation.

**Sox17 Promotes Sprouting Angiogenesis in Postnatal Vascular Development**

Because retinal angiogenesis is an important angiogenic event after birth, we further studied the expression and role of Sox17 in postnatal retinal vessels. Sox17 was specifically detected in endothelial cells but not in pericytes of retinal vessels (Online Figure IIA). Deletion of endothelial Sox17 in Cad5-CreER2 Sox17floxed/pup pups resulted in reduced radial extensions and vascular defects with a high variation, ranging from reduced vascular branching in moderate cases to an improperly organized vascular network with remarkably few tip cells and defective hierarchy formation in severe cases (Online Figure IIB and IIC). To exclude a potential long-term effect of Sox17 haploinsufficiency, we analyzed vascular changes in retinal angiogenesis by treating Cad5-CreER2 Sox17+/– pups with tamoxifen. Significant declines in radial extension, vascular branching, and tip cell number were found in Sox17-deficient vessels (Figure 2A–2E). Delayed Sox17 deletion from postnatal day 6 also reduced vascular branching on both superficial and deep layers of developing retinal vessels (Online Figure IID). Oppositely, Sox17 overexpression in endothelial cells significantly increased vascular branching and sprouting of postnatal retinal vessels (Figure 2F–2H). It also induced excessive angiogenesis after wounding compared with controls (Figure 2L–2M), confirming the proangiogenic function of Sox17 in pathological angiogenesis as well as developmental angiogenesis. Collectively, these results indicate that Sox17 promotes angiogenesis persistently in postnatal vascular development.

In line with its supportive role in tip cell formation at the vascular front, nuclear Sox17 was robustly detected in tip cells, but its expression was markedly reduced in stalk cells (Figure 2I–2K). This result suggests that Sox17 expression is regulated in tip–stalk specification.

**Sox17 Destabilizes Endothelial Cells and Upregulates Tip Cell Preferential Genes**

To understand the cellular mechanism by which Sox17 regulates the angiogenic process, we recapitulated vascular development by differentiating Sox17-null ESCs toward the endothelial lineage. Control and Sox17-null ESCs showed no appreciable difference in loss of pluripotency, emergence of mesodermal precursor cells, and endothelial generation (Online Figure IIIA–IID). These results suggest that Sox17 is dispensable for the emergence of endothelial population during early vascular development. In contrast, Sox17 deletion changed the behaviors of differentiated endothelial cells. Control endothelial cells had obvious destabilized junctional distribution of vascular endothelial cadherin and β-catenin, stellate stress fibers with cortical actin disassembly, and abundant lamellipodia and microspikes at the periphery; however, Sox17-null endothelial cells had linear junctional colocalization of vascular endothelial cadherin and β-catenin, considerable cortical bundles at the periphery, and few cellular protrusions (Figure 3A). Of note, ≈40% of cells within control endothelial colonies compared with only 17% of cells within Sox17-null endothelial colonies migrated freely between the center and peripheral regions, indicating reduced positional exchanging in Sox17-deficient endothelial cells (Figure 3B and 3C). In addition, Sox17-null endothelial cells migrated ≈25% more slowly compared with controls (Figure 3D). These results indicate that Sox17 deletion reduces cell migratory behavior by stabilizing endothelial junctions and cytoskeletal structures. Interestingly, Sox17-deficient hyaloid vessels showed a substantially delayed regression, suggesting increased vascular stability by Sox17 deletion in postnatal vascular remodeling (Online Figure IVA). Conversely, Sox17 overexpression in endothelial cells induced vascular destabilization as is shown by reduced vascular endothelial cadherin at endothelial junctions and impaired structural integrity in yolk sac vessels (Online Figure IVB and IVC). These findings suggest that Sox17 might have a vascular destabilizing role during vascular remodeling and that Sox17 expression should be properly regulated during vascular development.

To gain insight into the molecular events underlying Sox17-mediated vascular changes, we analyzed the expression of several angiogenic regulators in Sox17-silenced endothelial cells. Among several genes upregulated in tip cells relative to stalk cells,18–20 the expression of Ang2, PDGFB, Apelin, ESM1, Dll4, and VEGFR2 was significantly decreased in Sox17-silenced endothelial cells at the transcript level, whereas Sox17 knockdown did not change the expression of VEGFR1 and Jag1, which are preferentially expressed in stalk cells21,22 (Figure 3E). We confirmed decreased Ang2 expression at the vascular front of Sox17-deficient retinal vessels at the protein level compared with control vessels (Figure 3F). This finding suggests that Sox17 might promote angiogenesis by increasing...
Figure 2. Sox17 promotes postnatal angiogenesis and its expression is suppressed in stalk cells. A to E, Reduced retinal angiogenesis in Sox17\textsuperscript{i∆EC} mutants. A, PECAM-stained control and Sox17\textsuperscript{i∆EC} P6 retina (left column). Higher magnification of boxed area (right column). B, Higher magnification of vascular front in A. Quantification of radial length (C), branching (D), and sprouting (E) of P6 retinal vessels (n\geq12). F, Platelet endothelial cell adhesion molecule (PECAM)-stained control and Sox17\textsuperscript{iGOF} P6 retina (left column). Higher magnification of the boxed areas is shown on the right. Quantification of branching (G) and sprouting (H) of P6 retinal vessels (n\geq4). I to K, Reduced Sox17 expression in stalk cells. I, Angiogenic front of P6 retina stained with Sox17 (red), isolectin-B4 (IB4; green), and ETS-related gene (ERG; blue). Arrows indicate nuclei of stalk cells at vascular sprouts. J, Quantification of Sox17 expression in tip and stalk cells (n=18). K, Comparison of Sox17 expression in adjacent tip and stalk cells as tip/stalk ratio (n=95). Ratio >1.0 and ratio \leq1.0 denote the adjacent tip–stalk alignments with higher Sox17 expression and equivalent or less Sox17 expression, respectively, in tip cell compared with stalk cell. L, Dermal vasculature of control and Sox17\textsuperscript{iGOF} mice 7 days after wounding. Dashed lines indicate the margin of punching holes in ears. M, Quantification of vascular density in L (n=3). Data are presented as mean±SEM; *P<0.01; **P<0.05. Scale bars, 100 \mu m. Ctrl indicates control; i∆EC, Sox17\textsuperscript{i∆EC} mutant; and iGOF, Sox17\textsuperscript{iGOF} mutant.
gene expression involved in tip cell formation rather than by decreasing gene expression implicated in stalk cell property.

**Notch Pathway Inhibits Angiogenesis by Reducing Sox17 Expression**

Given the predominant role of the Notch pathway in tip–stalk determination and differential Sox17 expression between tip and stalk cells (Figure 2I–2K), we explored whether the Notch pathway might regulate Sox17 expression in endothelial cells. Nuclear Sox17 declined specifically in endothelial cells successfully transfected with exogenous Notch1 intracellular domain (N1CD), whereas mock-transfected or nontransfected endothelial cells robustly expressed Sox17 (Figure 4A and 4B). This result indicates that the Notch pathway can...
regulate Sox17 expression in a cell-autonomous manner. Furthermore, activated Notch signaling by gain of N1ICD in endothelial cells substantially repressed Sox17 expression at the vascular front, as well as tip cell number and branching in postnatal retinal vessels (Figure 4C). Conversely, inhibition of endogenous Notch activity by blocking Dll4, a vascular-specific Notch ligand, increased Sox17-expressing endothelial cells together with hyperbranching (Figure 4D). This result indicates that the Notch pathway suppresses Sox17 expression in endothelial cells at the vascular front during postnatal retinal angiogenesis. We also observed an inhibitory effect of Notch activity on Sox17 expression in tumor angiogenesis as shown by stronger Sox17 staining in Lewis lung carcinoma tumor vessels on Dll4 blockade compared with control IgG treatment (Figure 5I).

To characterize the functional involvement of Sox17 in Notch signaling, we studied the vascular effect of the Notch pathway in retinal angiogenesis of mutant pups lacking endothelial Sox17. Anti-Dll4 antibody induced hypopersprouting and hyperdense vascular network in controls but did not result in increased filopodia number and vascular density in Sox17-deficient vessels (Figure 5A–5C). The angiogenic effect of Dll4 blocking on tumor angiogenesis was also significantly reduced in Sox17-deficient tumor vessels (Figure 5D–5F). These findings indicate that the angiogenic effect of Notch inhibition significantly depends on Sox17.

### Notch Pathway Downregulates Sox17 Expression Mainly at the Post-Transcriptional Level

We then studied the mechanism for reduced Sox17 expression by Notch signaling in primary endothelial cells. Notch activation by N1ICD overexpression reduced Sox17 by 80% (Figure 6A), whereas Notch inactivation by the γ-secretase inhibitor DAPT increased Sox17 by 3.2-fold at the protein level (Figure 6E). In contrast, Notch pathway did not change Sox17 expression at the transcript level, although some known Notch downstream genes were transcriptionally regulated (Figure 6B and 6F). In addition, the change in Sox17 expression by Notch modulation was found at the protein level but not at the mRNA level in endothelial cells from both postnatal lung (Figure 6C, 6D, 6G, and 6H) and tumors (Figure 6I and 6J). These results indicate that Notch signaling inversely regulates Sox17 expression mainly at the post-transcriptional level.

To further specify how Notch activation decreases Sox17 protein levels, we established Notch activation in endothelial cells by Dll4 coating, which successfully increased the expression of several well-known Notch downstream genes (Figure 7A). Sox17 protein gradually decreased over 36 hours almost to a nondetectable level under Notch activation (Figure 7B), whereas Sox17 mRNA level was moderately reduced (Figure 7C), further indicating that Notch activity mainly affects Sox17 protein levels.
Expression of a certain protein can be decreased at the post-transcriptional level mainly by reducing protein synthesis and increasing protein degradation. Because of the emerging significance of protein turnover in angiogenic regulation, we examined whether Notch activation can destabilize Sox17. On blocking translation by cycloheximide treatment, Sox17 degradation was modestly reduced in Notch-activated endothelial cells but was not statistically significant (Figure 7D and 7E), implying that reduced Sox17 protein level by Notch activity might not be achieved simply by increasing Sox17 protein degradation. However, we could not exclude the possibility of the requirement of a new protein synthesis for the Notch-mediated Sox17 degradation. Whether the Notch pathway can interfere with Sox17 protein synthesis requires further investigation.

**Discussion**

Transcription factors play an important role in endothelial emergence and arterial–venous specification during vascular development; however, their roles and significance in sprouting angiogenesis remain elusive. Here, using Sox17 loss- and gain-of-function mouse models, we demonstrate that transcription factor Sox17 promotes sprouting angiogenesis in vascular development. Sox17 is dispensable for the generation of endothelial cells at the initial stage of vascular development as shown by the existence of primitive capillary plexus in Sox17-deficient embryos and successful endothelial differentiation from Sox17-null ESCs. In contrast, Sox17-deficient vessels show reduced sprouting angiogenesis in embryo hindbrain and postnatal retina. Consistent with this vascular defect, Sox17 is robustly expressed in endothelial cells at the vascular front where sprouting angiogenesis is active. In addition, we
such as Norrin/Fz4/Lrp-mediated retinal angiogenesis and the proangiogenic role of Sox17 was reported in other vascular contexts. Consistent with these findings, the proangiogenic function of Sox17 in vascular development. Furthermore, Sox17 overexpression induced vascular sprouting in both embryonic and postnatal angiogenesis, indicating the proangiogenic function of Sox17 in vascular development.

Taken together, Sox17 contributes to several processes of vascular development in a context-dependent manner after the onset of endothelial differentiation.

Although Corada et al. reported the indispensable role of Sox17 in mouse vascular development that is largely similar to our study here, the increased vascular sprouting of Sox17-deficient retinal vessels is functionally required for excessive sprouting angiogenesis.4,5

Figure 6. Notch pathway regulates Sox17 expression mainly at the post-transcriptional level. Immunoblotting and quantification of Sox17 signal (A, C, E, G, I) and transcript level of Sox17 (B, D, F, H, J). A to D, Reduced Sox17 protein level on Notch activation. A and B, Human umbilical vein endothelial cells (HUVECs) transfected with control or Notch1 intracellular domain (N1ICD) vector (n=4). C and D, P5 lungs from control and N1ICD-iGOF pups (n=3). E to J, Increased Sox17 protein level after Notch inactivation. E and F, HUVECs treated with vehicle (control) or N-[3,5-difluorophenacetyl]-L-alanyl-S-phenylglycine t-butylerster (DAPT; n=4). G and H, P5 lungs treated with IgG or α-delta-like ligand 4 (Dll4) antibody (Ab; n=3). I, Images showing Sox17 (red) and platelet endothelial cell adhesion molecule (PECAM; blue) of Lewis lung carcinoma tumors treated with IgG or α-Dll4 Ab. Quantification of Sox17 intensity in tumor vessels (n=20; right). J, Transcript level of Sox17 in tumor endothelial cells (TECs) of tumors treated with IgG or α-Dll4 Ab (n=3). Data are presented as mean±SD; *P<0.01; **P<0.05. Scale bars, 100 μm.
circulation. Interestingly, the Dll-Notch system is a fundamental signaling pathway conserved in various kinds of cell type, but how this common system induces different biological phenomena remains elusive. Our findings suggest that the Notch pathway might use Sox17 as a specific mediator in endothelial cells to regulate specialized downstream genes for vascular stabilization.

Although the article by Corada et al.29 is not supportive of the possibility that the Notch pathway is upstream of Sox17 expression, their data do not contradict with our observations such as the little change of Sox17 transcript level by the modulation of Notch activity. We observed that Notch activity can change Sox17 protein level at vascular front as well as in cultured endothelial cells, whereas Corada et al.29 found little change in Sox17 protein level mainly in arteries by Notch activity. However, they elucidated that Sox17 plays a regulatory role as an upstream transcription factor of some Notch ligands and Notch receptors essential for arterial determination. They also found that Sox17 is highly expressed in arterial endothelial cells and is required for the acquisition and maintenance of arterial identity in mouse vascular development. Together with our findings, these studies suggest that Sox17 can interact with the Notch pathway in different ways depending on the vascular context.

In general, transcriptional regulation is a cardinal mechanism of Notch signaling. In contrast, we show that Notch signaling regulates endothelial Sox17 expression mainly at the post-transcriptional level by gain and depletion of Notch activity. In line with post-transcriptional control of vascular endothelial growth factor receptor 3 levels by the Notch pathway in postnatal retinal angiogenesis,46 our findings describe that the Notch pathway can control angiogenic regulators at the post-transcriptional level. However, we do not exclude the possibility that the Notch pathway can regulate the transcription

Figure 7. Notch activation suppresses Sox17 protein level without acceleration of Sox17 degradation. A, Quantitative polymerase chain reaction analysis of Notch downstream genes in human umbilical vein endothelial cells (HUVECs) plated on dishes coated with BSA or extracellular domain of delta-like ligand 4 (Dll4) at several different time points. Transcript levels in HUVECs on Dll4 coating were normalized based on GAPDH mRNA and are represented as fold differences compared with HUVECs on BSA coating at the same time point. Data are presented as mean±SD; n=3. *P<0.01 and **P<0.05 compared with values at the initial time point (0 hours). Sox17 protein level (B) and Sox17 mRNA level (C) in HUVECs on BSA or Dll4 coating at indicated time points after plating. D, Immunoblotting and quantitative analysis of Sox17 protein in HUVECs on BSA or Dll4 coating at indicated time points after blocking protein synthesis by 20 μg/mL cycloheximide (CHX). Curves were fitted using first-order exponential decay model to determine the half-life of Sox17. E, Comparison of the half-life of Sox17 protein between control (BSA coating) and Notch-activated (Dll4 coating) conditions. Data are presented as mean±SEM; n=8.
of Sox17 in some contexts. Further investigation will reveal underlying molecular mechanisms.

The Notch pathway regulates tumor angiogenesis as well as developmental angiogenesis.\(^7\)\(^9\) We previously found that Sox17 promotes tumor angiogenesis.\(^11\)\(^2\) In this report, in addition to postnatal retinal angiogenesis, we found that Notch inhibition by Dll4 blockade induces excessive tumor angiogenesis at least partly by increasing Sox17 expression. This result suggests that the Notch pathway can use Sox17 to control angiogenesis in both developmental and tumor contexts and implicates Sox17 in recent therapeutic approaches using Dll4 blocking antibodies.\(^47\)\(^48\)

Collectively, we elucidate that Sox17 plays a role as a proangiogenic regulator in vascular development, and the downregulation of Sox17 expression in stalk cells is important for proper vascular patterning. Here, we establish the Notch pathway as an upstream regulatory mechanism of Sox17 expression in sprouting angiogenesis.

Acknowledgments

We are indebted to R.H. Adams and J.M. Wells for sharing Cdh5(PAC)-CreERT2 and Tert-O;Sox17 mouse lines, respectively. We are grateful to S.J. Morrison for Sox17-null embryonic stem cell line. We thank S. Seo, E.S. Lee, and T.C. Yang for technical assistance.

Sources of Funding

This work was supported by the National Research Foundation of Korea (NRF-2009-0006850, NRF-2010-0011402, and NRF-2013M3A9B6046565, I. Kim), the KAIST High Risk High Return Project (N10110088, I. Kim) of the Ministry of Education, Science and Technology, the National R&D Program for Cancer Control (1120030, I.Kim), the KAIST High Risk High Return Project (A110076, G.Y. Koh) of the Ministry of Health & Welfare, Republic of Korea.

Disclosures

None.

References


What Is Known?

- The Notch pathway regulates vascular development by promoting arterial differentiation and preventing excessive angiogenesis.
- Endothelial-specific transcription factor Sox17 promotes arterial differentiation by increasing Dll4 expression, which is critical for Notch activation in endothelial cells.

What New Information Does This Article Contribute?

- Sox17 promotes angiogenesis by increasing vascular sprouting at the leading edge of the growing vascular network.
- The Notch pathway stabilizes growing vascular network in part by downregulating Sox17 expression at the vascular front.
- The interplay between the Notch pathway and Sox17 differs for arterial differentiation and angiogenic regulation.

Functional vascular network is established by the orchestration of multiple processes including endothelial specialization and balanced angiogenesis. The Notch pathway is central to vascular development by supporting both arterial differentiation and vascular stabilization. Nevertheless, how this signaling pathway regulates endothelial-specific changes in vascular development is yet to be elucidated. It has been recently shown that arterial differentiation is supported by the transcription factor Sox17, which upregulates the expression of Dll4, a vascular-specific Notch ligand. Here, we report that Sox17 is also expressed at vascular front and promotes angiogenesis by increasing vascular sprouting. We also found that the Notch pathway suppresses Sox17 expression at the vascular front. Sox17 promotes arterial differentiation as an upstream regulator of the Notch pathway, whereas the Notch pathway prevents excessive angiogenesis by targeting Sox17 expression. This contextual interplay between the Notch pathway and Sox17 may explain how the conserved Notch signaling system is responsible for both arterial differentiation and balanced angiogenesis specialized in vascular morphogenesis.
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Circ Res. 2014;115:215-226; originally published online April 22, 2014; doi: 10.1161/CIRCRESAHA.115.303142

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The Notch pathway targets proangiogenic regulator Sox17 to restrict angiogenesis

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\textit{Running title:} Lee \textit{et al.}, The Notch pathway suppresses Sox17 expression.

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SUPPLEMENTAL METHODS

Mutant mice, tumor model, and pharmacological inhibition

Sox17\textsuperscript{GFP+}, Sox17\textsuperscript{fl/+}, Cdh5(PAC)-Cre\textsuperscript{ERT2}, VE-cadherin–tTA and Tet-O–Sox17 mice were housed as previously reported. Tie2-Cre mice and Gt(ROSA)26Sor\textsuperscript{tm1(Notch1)Dam} mice can express constantly active Notch1 intracellular domain (N1ICD) upon Cre activation were obtained from Jackson laboratory. All mice were backcrossed into the C57BL/6 strain at least more than ten times and bred in a pathogen-free animal facility. For Sox17 deletion during embryonic period, Tie2-Cre mice were crossed with Sox17\textsuperscript{gfp/fl} mice. For Sox17 deletion in tumor and retinal angiogenesis, Cdh5(PAC)-Cre\textsuperscript{ERT2} mice were interbred with Sox17\textsuperscript{fl/fl} mice. To activate Cre inducibly in retinal angiogenesis, tamoxifen (Sigma) was given intraperitoneally every day 25mg/kg from postnatal day 1 through day 3 and 50 mg/kg from postnatal day 4 and onwards (at days 1-5 for early Sox17 deletion, at days 6-8 for late Sox17 deletion, and at days 2-4 for N1ICD overexpression). Sox17 deletion in tumor angiogenesis was performed as described. VE-cadherin–tTA mice were bred with Tet-O-Sox17 mice for Sox17 overexpression in endothelial cells. Because the Sox17 transgene is turned on in the absence of doxycycline in this system, doxycycline was removed from feeding water from embryonic day 5 for developmental angiogenesis or 2 days before wounding for wound-induced angiogenesis.

To generate tumor models, suspensions of Lewis lung carcinoma (American Type Culture Collection) cells (1 × 10\textsuperscript{6} cells in 100 μl) were implanted subcutaneously in the flanks of mice. For the analysis of tumour vasculatures, mice were anesthetized by intramuscular injection of 80 mg/kg ketamine and 12 mg/kg xylazine and tumours were harvested for further analysis.

For pharmacological inhibition of Notch signalling, 5 mg/kg human IgG antibody (Jackson ImmunoResearch) or anti-Dll4 antibody (clone 26.82, US 7803377 B2) was administered subcutaneously for pups at postnatal days 3 and 4 or intraperitoneally at days 8 and 11 after LLC tumour implantation. Retina samples were collected at postnatal day 5. Tumors were isolated at 12 days after implantation for further analysis. All animal experiments were approved by the Animal Care Committee of Korea Advanced Institute of Science and Technology.

Histology and tissue immunofluorescence

Cells and embryonic tissues were fixed in 2% paraformaldehyde (PFA) at 4°C for 0.5 - 1 h. Tumor tissues were fixed in 1% PFA at 4°C for 1 h (for Sox17 staining) or overnight (for PECAM staining). Then samples were prepared for either whole mount or cryosection. For cryosections, tissue was dehydrated in phosphate buffered saline (PBS) with 30% sucrose at 4°C for 2 days and was embedded in OCT compound (Leica). Five to twenty-micrometer-thick sections for embryonic tissues
and 10 (for Sox17 staining) or 60 (for PECAM staining) μm thick sections for tumor tissues were processed using cryomicrotome (Leica). To analyze sprouting angiogenesis in developing hindbrain, hindbrain tissues were dissected, fixed in 4% PFA for 40 min, and flat-mounted as described. For retina whole-mount samples, extracted eyeballs were fixed in 4% PFA for 20 min and the retina was dissected and incised into four quadrants. Retina was then further fixed in cold methanol for 15 min. To analyze hyaloid vessel regression, hyaloid vessels were gently collected from the remaining fixed eyeballs. For immunofluorescence, all samples were washed in PBS, blocked in PBST (PBS-0.1% Triton X-100) with 5% donkey serum, incubated with primary and fluorescently labeled secondary antibodies, counterstained with DAPI or Hoechst33258 and finally mounted in Vectashield (Vector Laboratories). Immunofluorescent images were acquired using Zeiss LSM510 or LSM780 confocal fluorescence microscopes. Antibodies used in this study are listed in Online Table I.

**Morphometric analysis**

All vascular images are representative of at least three independent experimental settings. In retinal angiogenesis, radial extension was assessed by the length between optic disk and the most vascular front margin. All four quadrants in at least 12 retinas were calculated in each group. Filopodia and sprout numbers were counted in 1000 μm vascular length at the vascular front in all four quadrants. Branching number was counted at the area 100 μm proximal to the peripheral margin to exclude irregular vascular network at the most distal portion. Branching number was also quantified in 1000 μm vascular length each.

For tip-stalk ratio of Sox17 expression, endothelial nuclei were stained with ERG which showed an equal level of expression among tip and stalk cells. To exclude any obscure position of tip and stalk cells, only prominent sprouts with straight alignment of tip and stalk cell were quantified. Image J software (http://rsb.info.nih.gov/ij) was used to measure Sox17 fluorescence intensity in tip and stalk cells.

Time-lapse imaging of ESC-derived endothelial cells was performed at 20-min interval using an Axiovert 2000M inverted microscope (Zeiss) with stage-top incubator. The position of cells was obtained from time-lapse images by tracking the nuclei of individual cells using Manual Tracking plugin of an Image J software.

**RNA interference**

Human umbilical vein endothelial cells (HUVECs) were maintained in EGM2 medium (Lonza) and used for experiments before seven times passages. For small RNA interference (siRNA), oligonucleotides against Sox17 (5’-ACGCCAGCUCCGCGGUUA-3’, Dharmacon) or scrambled
siRNAs for a negative control was transfected into HUVECs using Lipofectamin 2000 (Invitrogen). After 24 h, cells were replated on culture plate at 10-20% confluency. After 12 h, cells were incubated in 0.5% FBS in EBM-2 for overnight. Then cells were stimulated by 50 ng/ml VEGF-A 165 (R&D) for 2 h and harvested for transcript analysis.

**In vitro modulation of Notch signaling**
For *in vitro* activation of Notch signaling, HUVECs were transfected with EF.hICN1.CMV.GFP vector (17623, Addgene) containing human Notch1 intracellular domain or EF.CMV.RFP vector (17619, Addgene) as a negative control using Lipofectamine LTX (Invitrogen) in Opti-MEM (Invitrogen) for 2 h. Then, cells were incubated in 0.5% FBS in EBM-2. Twenty four hours after transfection, cells were collected for immunoblotting or transcript analysis. Alternatively, HUVECs were plated on culture dishes coated with Dll4 (R&D) or BSA as a negative control for timed Notch activation. Cells were collected at the indicated time points for biochemical and transcriptional analyses. For *in vitro* inhibition of Notch signaling, HUVECs were treated with 10 μM DAPT (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butylerster) dissolved in dimethylsulphoxide (DMSO) or equal amount of DMSO as a negative control. After 12 h, cells were harvested for analysis.

**Western blotting**
Lung tissues from mouse pups and HUVECs were homogenized and lysated in PRO-PREP protein extraction solution (Intron). Proteins were loaded and separated by SDS-PAGE and transferred to a PVDF membrane. After blocking, membranes were incubated with primary and secondary antibodies serially. To verify equal amount loading, membranes were stripped and probed for actin.

Antibodies were used: rabbit anti-actin (A2066, Sigma); goat anti-VE-cadherin (sc-6458, Santa Cruz); goat anti-Sox17 (AF1924, R&D); donkey horseradish peroxidase (HRP)-linked anti-goat IgG (Santa Cruz); goat HRP-linked anti-rabbit IgG (Santa Cruz). Signals were developed by enhanced chemiluminescence HRP substrate (Millipore) and detected using LAS-3000 mini (Fuji).

**Transcript analysis by quantitative real time-PCR**
To analyze mRNA expression level, total RNA was extracted from HUVECs or lung tissues using RNeasy plus mini kit (Qiagen). Each 1 μg of RNA was used to synthesize cDNA by GoScript reverse transcriptase (Promega). Quantitative real-time PCR (qRT-PCR) was performed by using fast SYBR green master mix (Roche) and CFX96 real-time PCR detection system (Bio-Rad). The qRT-PCR data were analyzed with CFX manager software (Bio-Rad). Primer sequences used in quantitative RT-PCR are listed in Online Table II.
Embryonic stem cell culture and endothelial differentiation

Mouse embryonic stem cell (ESC) lines were derived from Sox17<sup>fl/fl</sup> blastocysts in a standard manner as described<sup>8</sup>. Sox17-null ESC line was obtained by adenoviral Cre delivery to the Sox17<sup>fl/fl</sup> ESC line. Pluripotency of these ESC lines was confirmed by checking the expression of pluripotency markers Oct4 (eBioscience), SSEA-1 (Chemicon) and successful teratoma formation on NOD/SCID mice. ESC lines were maintained on mouse embryonic fibroblast feeders in DMEM containing 15% FBS and leukemia inhibitory factor (LIF). Endothelial cells were induced as described<sup>9</sup>. Briefly, ESCs were cultured on 0.1% gelatin-coated plates at a density of 1 to 2 x 10<sup>4</sup> cells/cm<sup>2</sup> in α-MEM (Invitrogen) with 10% FBS (Welgene) in the absence of LIF. After 4.5-5.0 days, cells were incubated with allophycocyanin (APC)-conjugated anti-Flk-1 antibody (clone AVAS12a1, eBioscience) and anti-APC microbeads (Miltenyi) and then Flk-1<sup>+</sup> mesodermal precursor cells were purified using AutoMACS Pro Separator (Miltenyi) and plated onto 0.1% gelatin coated dish at a density of 4 x 10<sup>4</sup> cells/cm<sup>2</sup> in α-MEM with 10% FBS in the presence of 50 ng/ml VEGF-A 165 (R&D) for 4 days.

Flow cytometry analysis and cell sorting

Differentiated cells were dissociated with 0.25% trypsin-EDTA (Invitrogen), resuspended in HBSS containing 2% FBS, incubated with antibodies for 10 min, washed twice and resuspended. Analyses and sorting were performed by FACS AriaII (Beckton Dickinson). Antibodies were used: mouse anti-SSEA-1(clone MC-480, Chemicon); rat APC-conjugated anti-Flk-1 (clone AVAS12a1, eBioscience); rat phycocerythrin (PE)-conjugated anti-CD31 (clone MEC13.3, eBioscience); rat Alexa Fluor 647-conjugated anti-CD144 (clone BV13, eBioscience); goat Alexa Fluor 488-linked anti-IgG or -IgM (Invitrogen). Dead cells were stained using 7-aminoactinomycin D (Invitrogen) and excluded for analysis.

Tumor endothelial cells were purified as described<sup>5</sup>. Briefly, tumor tissues were dissociated with 0.1% collagenase type 4 (Worthington) and 3U/ml DNase 1 (Worthington), incubated with rat APC-conjugated anti-CD45 (clone 30-F11, BioLegend), rat APC-conjugated anti-Ter119 (clone TER119, BioLegend) and rat PE-conjugated anti-CD31 (clone MEC 13.3, BioLegend). After enrichment using anti-PE microbeads and AutoMACS Pro Separator (Miltenyi), tumor endothelial cells (CD31<sup>+</sup>CD45<sup>-</sup>Ter119<sup>-</sup>) were purified using FACS AriaII and cell purity was checked to be more than 95%. Data were analyzed using FlowJo software (Tree Star).

Statistics

Values are reported as mean ± standard deviation unless otherwise indicated. Significant differences between means were determined by analysis of variance followed by two-tailed Student-Newman-
Keuls test. Statistical significance was set at $p < 0.01$ or $p < 0.05$. 
**Online Figure I. Endothelial Sox17 is indispensable for embryonic vascular development.**

(A) Immunofluorescence image for Sox17 (red), von Willebrand factor (vWF, endothelial marker; blue), and α-smooth muscle actin (green) of dorsal aorta at E11.5. (B-C) Images of Sox17<sup>GFP/fl</sup> and Tie2-Cre Sox17<sup>GFP/fl</sup> embryos at E10.5. (B) Cross-sectional immunofluorescence images showing vasculature (vWF; red) and Sox17-expressing tissues (GFP; green). Sox17 expression is strong in the vasculature as well as gut endoderm (indicated as arrow heads) during early development. The endothelial-specific ablation of the Sox17 locus by Tie2-Cre did not affect gut formation. Arrow indicates successful formation of dorsal aorta in Sox17<sup>GFP/fl</sup> embryo. (C) Superficial view of hindbrain vasculature reflected by GFP fluorescence. (D) Immunofluorescence images of control and Sox17<sup>GOF</sup> yolk sac at E10.5, showing vessels (PECAM, red) and erythrocytes (Ter119, white). Scale bars, 100 μm.
Online Figure II. Endothelial Sox17 is indispensable for proper postnatal vascular development.

(A) Whole-mount immunofluorescence for Sox17 (red), isolectin-IB4 (IB4; white), and Nerve/Glial antigen 2 (NG2; green) of P5 retinal vessels. (B) IB4-stained Cad5(PAC)-CreER\textsuperscript{T2} Sox17\textsuperscript{fl/+} (control) and Cad5(PAC)-CreER\textsuperscript{T2} Sox17\textsuperscript{GFP/fl} (Sox17\textsuperscript{iΔEC}) P5 retina (daily tamoxifen administration at postnatal days 3 and 4). Representative image showing moderate defects with reduced vascular branching.
(middle panel). Representative image showing severe defects with branching deficits and limited sprouting at the vascular front (right panel). (C) Quantification of radial length of retinal vessels in B. (D) PECAM-stained control and \textit{Sox17}\textsuperscript{\Delta EC} P9 retina (tamoxifen treatment from postnatal day 6). Superficial layers (left column) and deep layers (right column) of retinal vessels. Data are presented as mean ± s.d.; n = 4. * \( P < 0.01 \) relative to control. Scale bars, 100 \( \mu \text{m} \) (A), 200 \( \mu \text{m} \) (B), and 500 \( \mu \text{m} \) (D).
Online Figure III. Sox17 is dispensable for endothelial emergence in vascular differentiation.

(A) Schematic diagram of endothelial differentiation from embryonic stem cells (ESCs). Around 5 days after spontaneous differentiation in the absence of leukemic inhibitory factor (LIF), Flk1-positive mesodermal precursor cells were fractionated for further differentiation during 4 days in the presence of VEGF. (B) The percentages of SSEA-1 (a marker for pluripotent cells)- or Flk1-expressing cells during differentiation of Sox17+/+ (control) and Sox17null/null (null) ESCs. (C) FACS plots showing the endothelial population (CD144+CD31+) derived from control and Sox17-null ESCs. The percentages of endothelial cells are shown inside plots. (D) Immunofluorescence images for Sox17 (red), VE-cadherin (green), and DAPI (blue) of ESC-derived endothelial colonies. Scale bars, 100 μm. (E) Immunoblot analysis confirming siRNA-mediated Sox17-knockdown in HUVECs for gene expression studies. HUVECs were transfected with scrambled- (control) or Sox17-targeting (KD) siRNA.
Online Figure IV. Sox17 plays a vascular destabilizing role in vascular development.

(A) Whole-mount immunofluorescence images for PECAM (green) and F4/80 (red) showing delayed regression of Sox17\textsuperscript{\text{IAEC}} hyaloid vessels at P10. (B) Images for PECAM (green) and VE-cadherin (red) of control and Sox17\textsuperscript{\text{IGOF}} E12.5 yolk sac. (C) Immunofluorescence images for PECAM (red) and α-smooth muscle actin (green) of control and Sox17\textsuperscript{\text{IGOF}} yolk sac at E12.5. Higher magnification images of the boxed area are shown below. Scale bars, 500 μm (A) and 100 μm (B-C).
### SUPPLEMENTAL TABLES

**Online Table I. Antibodies used for immunoflorescent analysis.**

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Online Table II. Primers used for quantitative RT-PCR.

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SUPPLEMENTAL REFERENCES


