The Transcription Factor Complex LMO2/TAL1 Regulates Branching and Endothelial Cell Migration in Sprouting Angiogenesis

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The transcription factor complex LMO2/TAL1 regulates branching and endothelial cell migration in sprouting angiogenesis

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Abstract

The transcription factor complex, consisting of LMO2, TAL1/LYL1, and GATA2, plays an important role in capillary sprouting by regulating VEGFR2, DLL4, and angiopoietin 2 in tip cells. Overexpression of the basic helix-loop-helix transcription factor LYL1 in transgenic mice results in shortened tails. This phenotype is associated with vessel hyperbranching and a relative paucity of straight vessels due to DLL4 downregulation in tip cells by forming aberrant complex consisting of LMO2 and LYL1. Knockdown of LMO2 or TAL1 inhibits capillary sprouting in spheroid-based angiogenesis assays, which is associated with decreased angiopoietin 2 secretion. In the same assay using mixed TAL1- and LYL1-expressing endothelial cells, TAL1 was found to be primarily located in tip cells, while LYL1-expressing cells tended to occupy the stalk position in sprouts by upregulating VEGFR1 than TAL1. Thus, the interaction between LMO2 and TAL1 in tip cells plays a key role in angiogenic switch of sprouting angiogenesis.

Key Words: LMO2 transcription factor complex, sprouting angiogenesis, angiogenic switch, angiopoietin 2, VEGF receptors.

Introduction

In embryogenesis, the mammalian vascular system develops via two distinctive processes, vasculogenesis, and angiogenesis. Vasculogenesis is the process in which *de novo* specification of the common precursors of blood and endothelial cells (i.e., hemangioblasts) occurs from unspecified mesoderm to form blood islands. While inner cells of the blood islands differentiate into red blood cells, outer cells become endothelial cells and form a capillary network. During development of the mature vascular system, angiogenesis remodels the capillary network into branching vascular trees. Sprouting angiogenesis is the main form of angiogenesis in which tip cells migrate into avascular areas, along with proliferation of stalk cells, to form new tubes [1], and is stimulated by soluble chemoattractants and mitogens produced in areas with insufficient oxygen and nutrients. Sprouting angiogenesis is a two-step process initiated in quiescent endothelial cells: firstly, they have to be liberated through mural cell detachment and basement membrane degradation, and secondly, the cells are activated and give rise to tip cells, which inhibit the process in neighboring endothelial cells. This specification of endothelial cells into tip vs. stalk cells occurs via lateral inhibition by Delta-like ligand 4 (DLL4)/Notch signaling [2,3]. During development, DLL4 regulates vessel branching by inhibiting endothelial tip cell formation. Heterozygous deletion of DLL4 in mice results in a hyperbranching blood vessel phenotype. Vascular endothelial growth factor (VEGF)-A is a major proangiogenic chemoattractant and mitogen that is secreted from hypoxic sites and that binds to VEGF receptor 2 (VEGFR2) on the surface of tip cells. VEGF-

A/VEGFR2 signaling induces formation of filopodia and enhances DLL4 expression in tip cells. DLL4-mediated activation of Notch in neighboring endothelial cells inhibits the tip cell phenotype and promotes differentiation into stalk cells with higher proliferative activity and fewer filopodia. In stalk cells, the expression of VEGFR2 is downregulated while that of VEGFR1 (a scavenger receptor of VEGF) is activated. Following anastomosis of new blood vessels and an increase in oxygen, activated endothelia revert to a quiescent state. During this process, tighter inter-endothelial and endothelium-extracellular matrix attachments are formed. Platelet-derived growth factor (PDGF)-B is released by endothelial tip cells and stabilizes new tubes by recruitment of pericytes. This multistep process of sprouting angiogenesis is tightly regulated and only achieved via finely tuned transcriptional activation and inactivation [4].

Several transcription factors that play important roles in angiogenesis have been identified. Among these, the LIM-only protein LMO2 is necessary for embryonic angiogenesis. A highly disorganized vascular system was observed in LMO2−/− chimeric mice, which indicates an essential regulatory role for LMO2 in angiogenesis [5]. When LMO2−/− embryonic stem cells were subcutaneously implanted into nude mice, a complete defect in vascular sprouting and vascular tree formation was observed during development of teratocarcinoma [6], which indicates a key role for LMO2 in sprouting angiogenesis and vascular tree formation. Similarly, both TAL1 and GATA2 are needed for embryonic angiogenesis [7-10] and tube formation [11,12]. LYL1 is a basic helix-loop-helix domain containing a transcription factor that is

structurally related to TAL1 [13]. LYL1 can bind to LMO2 and is therefore an alternative member of the LMO2 transcription factor complex. While TAL1 is exclusively expressed in angiogenic (activated) endothelial cells, LYL1 is also expressed in quiescent endothelium [14]. In LYL1−/− mice, which exhibit relatively minor vascular system anomalies compared with TAL1 knockout mice, vascular sprouting is enhanced, resulting in increased neovasculature in tumor xenografts. Reduced vascular stabilization via LYL1 transcriptional activation results in increased vascular permeability and a larger vascular lumen [14,15]. In contrast, TAL1 plays a variety of roles in embryonic cardiovascular development and its expression is upregulated during angiogenesis [16-18]. Considering the vascular stabilizing effect of LYL1 in the quiescent endothelium, and the positive roles of TAL1 in the angiogenic endothelium, changing the LMO2 binding partner from LYL1 to TAL1 in the endothelium could trigger initiation of vascular sprouting.

LMO2 can directly bind to TAL1/LYL1 and GATA2 to form a multimeric protein complex via its LIM domain zinc-finger-like structure [19-26]. This protein complex functions as a transcription factor (hereafter referred to as the LMO2 transcription factor complex) and regulates the expression of hundreds of hematopoiesis- and angiogenesis-related downstream genes [27]. Among angiogenesis-related genes, VE-cadherin, angiopoietin 2, VEGFR2, the VEGF co-receptor neuropilin 2, and DLL4 have been identified as direct targets of the LMO2 transcription factor complex [11,28-30]. VE-cadherin is a key component of endothelial adherens junctions and plays an important role in regulating vascular integrity and permeability. VEGF promotes rapid

endocytosis of VE-cadherin, and Notch/VEGFR-regulated differential dynamics of VE-cadherin junctions drive competitive endothelial tip cell rearrangement during sprouting angiogenesis [31,32]. VEGFR2-DLL4-Notch signaling constitutes a negative feedback loop that is essential for tip and stalk cell selection. Angiopoietin 2 secreted from tip cells is thought to be another soluble factor important for endothelial cell activation [33]. Angiopoietin 2 is an antagonist of the vascular stabilizing protein angiopoietin 1 and plays a key role in pericyte detachment by opposing angiopoietin1/Tie2 signaling. It also binds to integrin, which activates Rac1 signaling and promotes endothelial cell migration [34].

Neovascularization is associated with many physiological and pathological conditions such as the menstrual cycle in the endometrium, inflammation, tissue repair, and tumor growth. In the adult body, in which blood and lymphatic circulation is at a steady state, blood vessel endothelia are mainly quiescent and neovascularization is largely inhibited. In hypoxia, tissue damage, inflammation, and tumor growth, chemical signals produced from those sites activate endothelial cells in existing blood vessels. To investigate these dynamic transcriptional processes during sprouting angiogenesis, with a focus on the quiescent/angiogenic switch in the endothelium and competition between tip cells in sprouts mediated by the LMO2 transcription factor complex, we analyzed LYL1-overexpressing transgenic mice and discovered that their short and deformed tail phenotype correlates with fewer straight arteries in their tails. In addition, using a 3D spheroid-based angiogenesis assay [35] combined with downregulation/upregulation of

components of the LMO2 complex, we provide evidence that it plays a pivotal role in endothelial cell migration, tube formation, and continuous tip cell competition during sprouting angiogenesis by transcriptionally regulating downstream target genes such as angiopoietin 2 and VEGF receptors.

Methods

All methods were performed in accordance with the guidelines for Recombinant DNA Experiments (MEXT, Japan). The biomedical experiment committee of Aino University approved these experiments.

Generation of transgenic mice

LYL1 transgenic mice were produced in our laboratory according to the procedure described by [36]. For the present study, this strain of mouse, B6.Cg-Tg(BOS-mLYL1)/Rbrc (RBRC01839), was provided by RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. Homozygous mice were obtained through heterozygote intercrossing. Genotyping of each mouse was done using PCR with transgene-specific primers set using tail DNA. The animal experiment committee of the Graduate School of Medicine, Kyoto University and Aino University approved these experiments. This study is reported in accordance with ARRIVE guideline.

CD31 immunohistochemistry and histological analysis

A cryostat (Leica) was used to produce 10 μm-thick sections of fresh-frozen tissue. After fixation in −30 °C methanol for 10 min, hematoxylin-eosin was used to stain the sections. Immunohistochemistry was then performed on the sections by treatment with 10 μg/ml of anti-CD31 goat polyclonal antibody (R & D systems, AF3628) for 16 hours at 4 °C. After washing, staining was done using Goat IgG VisUCyte HRP Polymer Detection Reagent (R & D systems) according to the manufacturer's protocol.

Cell culture

Human umbilical vein endothelial cells (HUVECs) were obtained and cultured in endothelial cell growth medium 2 (EGM2 medium) with supplements (PromoCell, Heidelberg, Germany). Cells were used for nucleic acid transfection immediately following a single 1:3 passage.

siRNA transfection

Small interfering RNAs (siRNA) were transfected in HUVECs at 90% confluence by using Lipofectamine RNAiMAX Reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. The sequences of duplex RNAs were described by [12,29]. eGFP siRNA was used as the transfection control.

DNA transfection

The whole cDNA sequences of TAL1, LYL1, and β-galactosidase were cloned into the Xba I site in the mammalian expression vector pEF-BOS [37] to prepare BOSTAL1, BOSLYL1, and BOSβgal, respectively. The empty expression vector pEF-BOS (BOSXba) was used as a transfection control. Each of the DNA sequences was transiently transfected to the cultured HUVECs at 90% confluence using the Lipofectamine 3000 Reagent or jetPEI-HUVEC transfection reagent (Polyplus-transfection S. A., France) for higher transfection efficiency according to the manufacturer's protocol.

Real-time qPCR

For the relative expression study, cell lysates were prepared 48 hours after siRNA transfection and 72 hours after DNA transfection. Following this, cDNA synthesis and quantitative real-time PCR were performed using the CellAmp Direct SYBR RT-qPCR Kit (TaKaRa, Shiga, Japan). Reactions were run on an Eco Real-Time PCR System (Illumina, CA, USA). The signal of each RNA was normalized to that of GAPDH or HPRT1. Oligo. Statistical analysis of the relative expression of each target gene was done after three separate transfections and duplicate PCR reactions (n = 3 × 2)

ELISA

Angiopoietin 2 protein levels in cell culture media were quantified by enzyme-linked immunosorbent assay (ELISA), using the Quantikine ELISA human angiopoietin 2 immunoassay (R&D Systems, MN, USA) according to the manufacturer's protocol. HUVECs were transfected with RNAs or DNAs. Then, 24 hours after transfection, the medium was changed and angiopoietin 2 secretion into the culture medium was measured for 24 hours. In cases where DNA transfection was performed, the medium was changed 48 hours after transfection.

3D spheroid-based angiogenesis assay

A 3D spheroid-based angiogenesis assay was performed according to the procedure described by Heiss [35] with a slight modification. After one passage, HUVECs were plated for siRNA or DNA transfection. Then, 24 hours after transfection, cells were suspended in sterile 0.24% w/v methylcellulose

solution in EGM2 medium with 10% FBS and aggregated overnight in hanging drops (25 µL) to form cellular spheroids (500 cells/spheroid). Spheroids were embedded into 1 mg/mL bovine acid-treated collagen gels (Nippi, Tokyo, Japan). Vessel sprouting was stimulated with 20 ng/mL (final concentration) VEGF165 (Sigma-Aldrich, MO, USA) 30 min after embedding the spheroids. After a 24 hr incubation at 37 °C, spheroids in collagen gels were fixed with 10% paraformaldehyde and *in vitro* angiogenesis was quantified using cellSens Standard2 software (Olympus, Japan) to measure the cumulative sprout length (CSL) of capillaries, defined by the presence of lumens, that grew from the spheroids. In case of HUVEC transfection with BOS β gal, whole-mount X-gal staining of spheroids was performed according to the protocol described in [5].

Endothelial cell invasion assay

After one passage, HUVECs were plated for DNA transfection. Then, 48 hours after transfection, cells were suspended in EGM2 medium with 10% FCS, 1 ng/ml VEGF, and 20 ng/ml bFGF. 5×10^4 cells were plated on BioCoat Matrigel Invasion Chambers (Corning, MA, USA) and the plates were incubated for 24 hours in a humidified CO_2 incubator. After removing non-migrated cells on the upper side of the membrane, migrated cells were fixed with cold methanol (4℃) for 20 minutes and stained with 0.1% crystal violet (Sigma-Aldrich, MO, USA).

Mouse aorta ring assay

Thoracic aortas from 6-week-old mice were dissected. After removing all surrounding connective tissue in the medium, they were cut into segments of 1 mm length and placed in Matrigel (Corning, MA, USA). They were incubated in EC growth medium-2 (Promocell, Heidelberg, Germany) containing 10% FBS for 9 days. Aortic explants were then imaged daily by using an inverted microscope (Olympus, Japan). Bifurcation of the sprouting vessels in the explants was quantified by using cellSens Standard2 imaging software (Olympus, Japan).

Results

Mice overexpressing LYL1 possess deformed tails

A tail shortening phenotype was observed in transgenic mice that overexpressed the basic helix-loop-helix transcription factor LYL1 driven by the promoter of the human elongation factor gene (Figure 1A,B). The foundation and maintenance of these transgenic mice was described in our previous report [36]. These mice possessed short and deformed tails at birth (Figure 1C), organs with a round shape that were about 10% lower in weight and length than their wild type littermates at 6 weeks of age. Later in life these mice exhibited marked alopecia (Figure 1D) and developed malignant lymphoma as described in [36]. Histologically, they were characterized by hypervascularity (increase in vascular density) in every organ. Spleens of 6-week-old TG/+ mice have a round shape and are smaller than those of their wild type littermates that usually exhibit dumbbell-shaped spleens (Figure 1E,F). They also exhibited increased blood vessel density (Figure 1G,H). The average size of blood vessels in the red pulp of spleens was smaller in TG/+ mice than in their wild type littermates. Especially in the lung, which is organized by two closely related branched anatomical systems (tracheal tubes and blood vessels), TG/+ mice were observed to have smaller alveolar spaces and capillaries than their wild type littermates at 6 weeks of age (Figure 1I,J). Immunohistochemical analysis showed alveolar capillaries to be positive for CD31 in TG/+ mice at 6 weeks of age, while only relatively large vessels in the periphery of the lung were CD31-positive in wild type littermates (Figure 1 K,L). In the cardiovascular system, the heart and large vessels of 6-week-

old TG/+ mice was smaller (about 10% reduction in weigh, length, and diameter) than those of wild type litter mates (Figure 1M,N). Differences in vessel branching were observed in 6-week-old TG/+ mice at the surface of the brain, in which they generally had a wider branching angle than those of their wild type littermates (Figure 1O,P). All these anatomical findings suggest that the reduced formation of straight blood vessels, especially arteries, during tail development might be responsible for the short and deformed tail phenotype of the transgenic mice, which could be closely associated with the hyperbranching of the vascular system and resulting hypervascularity exhibited by the narrowed vessels in the peripheral part of all organs.

Capillaries sprouting from aortic walls of LYL1 transgenic mice exhibited an increased number of bifurcations

In order to observe vascular branching patterns of the LYL1 transgenic mice *in vitro*, aorta ring sections about 1 mm wide were freshly prepared from 6-week-old LYL1 transgenic mice and their wild type littermates and implanted into Matrigel or type 1 collagen gel and cultured until day 9 with endothelial cell growth medium 2 (EGM2) supplemented with several growth factors and 10% FBS in a CO_2 incubator. In this aorta ring assay, slightly delayed budding of capillaries from the aortic wall was seen in 6-week-old TG/+ mice. From the beginning of budding, formation of straight capillary tubes was greatly reduced in TG/+ mice. Capillaries from LYL1 transgenic mouse aortas showed an increased number of bifurcations that resulted in vessel hyperdensity near the aortic wall (Figure 2A,B). This hyperbranching phenotype of sprouting

vessels was also seen in day 9 culture when maturation and reorganization of sprouting capillaries had already begun (Figure 2C,D). The counting of unambiguous bifurcation points was done at day 7. In wild type mouse aorta ring cultures, the average number of branching points (bifurcations) per 10 arbitrary fields (1 field: 1.05 × 1.4 mm) was 48, while that of LYL1 transgenic mice was 90 (n = 3 each for wild type and TG/+) (Figure 2E,F and 2G,H). The branching angle was generally wider in TG/+ mice, consistent with the wider branching angle seen in vessels at the surface of the brain (Figure 2G,H). The branching pattern of blood vessels is largely determined by VEGFR-DLL4-Notch [38], and the oligomeric transcription factor consisting of TAL1, LMO2, LDB2, GATA2 was recently shown to be involved in regulation of VEGF-induced DLL4 upregulation in sprouting tip cells [30]. We speculated that forced overexpression of the LYL1 transcription factor (which also binds to LMO2) in endothelial cells affects DLL4 expression levels in endothelia by forming an aberrant transcription factor complex in the Dll4 promoter/enhancer. Firstly, overexpression of LYL1 in endothelia was confirmed in a qPCR assay using mRNA from aortic cultures at days 5 and 7. Overexpression of the LYL1 gene by about 100 times was observed in both samples. Since expression of DLL4 from the aortic wall prior to culture was very low, and that from aortic culture after day 5 was greatly influenced by the number of tip cells (DLL4 is the tip cell marker protein), we transfected the cultured HUVECs with the expression vector of LYL1 (EF-BOS-LYL1) singly or combined with LMO2 and evaluated the expression level of DLL4 at 48 hours after transfection by qPCR. A reduction of about 40% in DLL4 expression was observed for LMO2/LYL1

double transfection and 20% for LYL1 single transfection (Figure 2I,J). In mice of DLL4 haploinsufficiency, the hyperbranching phenotype was seen in hindbrain vessels and in a postnatal angiogenesis assay of P4-6 retina [39]. The effect of haploinsufficiency of DLL4 on blood vessel sprouting compared to control was assessed in an *in silico* model of capillary sprouting [40]. Relatively straight blood vessel sprouting was seen in control conditions, while a laterally spreading capillary network was seen in the DLL4+/− model. The round organs seen in TG/+ mice were consistent with this capillary sprouting pattern.

Downregulation of all members of the LMO2/TAL1 transcription factor complex except LYL1 strongly inhibits sprouting angiogenesis *in vitro*

In order to explore the role of the transcription factor consisting of LMO2 and TAL1 in vessel sprouting and subsequent tube formation *in vitro*, we studied the efficiency of capillary sprouting induced by VEGF-A in spheroids of cultured HUVECs in type 1 collagen gel culture. This 3D spheroid-based angiogenesis assay was used to assess the effect on tube formation of downregulation by siRNAs of LMO2 transcription factor complex members (LMO2, TAL1, LYL1, and GATA2). Prior to the assay, expression of each transcription factor was estimated by qPCR (Figure 3A: Relative expression level). About a 50% reduction in expression of the target transcription factor mRNA was achieved. Each spheroid of 500 HUVECs containing transfected siRNA was formed in methylcellulose and then transferred to a type 1 collagen gel to assess its capacity for vessel sprouting. After stimulation of

VEGF-A at a final concentration of 20 ng/ml in culture medium for 24 hours, the total length of capillary sprouts with lumens was counted under a microscope. Only sprouts that were continuous from the spheroid to the tip cells were counted and compared with those that were transfected with control siRNA (si-eGFP). As shown in Fig. 3A, the cumulative length of the vessel sprouts decreased to 14%, 25%, and 26% of control (eGFP) by transfection with LMO2, TAL1, and GATA2 siRNAs, respectively. In contrast, and most importantly, it was not influenced by transfection of LYL1 siRNA.

Combined downregulation of LMO2/TAL1 and LMO2/GATA2 further inhibits sprouting angiogenesis

The synergic effect of downregulation via knockdown (KD) of members of LMO2 and TAL1 transcription factor complex was assessed using the 3D angiogenesis assay described above. As shown in Fig. 3B, sprouting angiogenesis was almost completely abolished in both the LMO2/TAL1 and LMO2/GATA2 combined KDs. The latter result was in accordance with a previous report [28].

Angiopoietin 2 secretion levels correlate with vessel sprouting

Angiopoietin 2 has been identified as a downstream transcriptional target molecule of TAL1, LYL1, and LMO2 [29]. Angiopoietin 2 is expressed at high levels in tip cells and plays two important roles in sprouting angiogenesis. Firstly, it liberates endothelial cells by detachment of mural cells [38]. Secondly, it binds to integrins on endothelial cells such as tip cells that express low

levels of Tie2 and stimulates migration [34]. Migration of HUVECs into matrigel was measured after knockdown of LMO2, TAL1, LYL1, and GATA2. When LMO2 and TAL1 were downregulated in HUVECs, the number of cells that migrated into Matrigel significantly decreased (**$P < 0.01$) when compared with control si-eGFP-transfected cells (Fig. 4A). In parallel with the 3D angiogenesis assay, angiopoietin 2 secreted by HUVECs into the culture medium was measured by ELISA after 24 hours. Generally, angiopoietin 2 protein levels in the culture medium were reduced by downregulation of each member of the LMO2 transcription factor complex. As shown in Fig. 4B, the quantity of protein decreased to 52%, 47%, 65%, and 51% of that in the control (eGFP) by transfection of LMO2, TAL1, LYL1, and GATA2 siRNA, respectively. Interestingly, angiopoietin 2 levels in the supernatant showed a strong correlation with CSL in spheroids treated with each siRNA (correlation coefficient: r = 0.73). We concluded that the reduced sprout length and endothelial cell migration resulting from knockdown of LMO2 and TAL1 can at least partially be explained by lowered secretion of angiopoietin 2 from tip cells.

Downregulation of LMO2 or TAL1 suppresses expressions of the main VEGF receptors and co-receptors

In search of other downstream targets of the LMO2 transcription factor complex that are responsible for the inhibition of sprouting angiogenesis, we quantified the mRNA levels of the main VEGF receptors (VEGFR1, VEGFR2, and VEGFR3) and their co-receptors (neuropilin 1 and neuropilin 2) after

downregulation of LMO2 transcription factor complex members. The mRNA levels of VEGFR1, VEGFR2, and neuropilin 2 (NRP2) significantly decreased after LMO2 or TAL1 knockdown (Fig. 4C, D). In the case of GATA2 knockdown, only the VEGFR2 mRNA level decreased.

LYL1 overexpression inhibits endothelial cell migration and tube formation in sprouting angiogenesis

In order to estimate the effect of TAL1 and LYL1 overexpression (OE) on endothelial cell migration and tube formation in sprouting angiogenesis, we transfected the mammalian expression vectors, BOSTAL1 or BOSLYL1, into cultured HUVECs. After confirming overexpression of TAL1 or LYL1 in cultured HUVECs 3–5 times using real-time qPCR, transfected cells were used for 3D angiogenesis assays (Fig. 5A). The same RNAi assay as described above was used and sprouting from spheroids was stimulated by VEGF-A at a final concentration of 20 ng/ml. After incubation for 24 hours, CSL in transfected HUVECs was measured under a microscope and compared with those of control cells that had been transfected with EF-BOS empty vectors. As shown in Fig. 5A, CSL from the spheroids of the BOSLYL1-transfected HUVECs decreased to 50% of that of the control EF-BOS empty vector (BOSXba)-transfected HUVECs. Thus, increased expression of LYL1 in HUVECs inhibits sprouting angiogenesis. In contrast, increased expression of TAL1 in HUVECs did not affect vessel sprouting (Fig. 5A). Since vessel sprouting is affected by endothelial migration capacity, an endothelial invasion assay was performed. It was reported that TAL1 overexpression in

endothelium inhibited endothelial migration [7]. In our assay, the number of cells that migrated into Matrigel significantly decreased when TAL1 or LYL1 was overexpressed in HUVECs as compared with control (Fig. 5B). Therefore LYL1 overexpression resulted in a greater reduction in endothelial migration than did TAL1 overexpression.

TAL1-expressing cells preferentially occupy the tip cell position in vessel sprouts

In order to explore the effect of the components of LMO2 transcription factors in endothelial cells on the competition between tip and stalk cells in vessel sprouts, we performed 3D spheroid-based angiogenesis assays using chimeric spheroids of TAL1-expressing and LYL1-expressing HUVECs. To achieve higher labeling efficiency, we used a linear polyethyleneimine based jet PEI-HUVEC transfection method (Polyplus-transfection S.A., France) throughout this experiment. About 70% of HUVECs were positive in X-gal staining after the transfection of BOS β gal. Labeling of HUVECs was done by co-transfection of BOS β gal and the expression vector of each transcription factor. Two types of chimeric spheroid were prepared by 1:1 ratio mixing of cells after DNA transfection before spheroid formation in hanging drops. One type (pair1) consisted of non-labeled TAL1-expressing cells, obtained by single transfection of BOSTAL1, and β-galactosidase-labeled LYL1-expressing cells. The other type (pair2) consisted of β-galactosidase-labeled TAL1-expressing cells and non-labeled LYL1-expressing cells. Both pair1 and pair2 chimeric spheroids were stimulated by 20 ng/ml VEGF-A in type 1

collagen gel culture for 24 hours. The number and position of labeled endothelial cells in vessel sprouts was detected by whole mount X-gal staining. As shown in Figure 6 and Table 1, among a total of 77 pair1 chimeric spheroids, in only 1 case did LYL1-expressing cells occupy the tip position in sprouts growing from the spheroid. In contrast, the tip position was occupied by TAL1-expressing cells in 6 out of a total of 78 pair2 chimeric spheroids. The stalk position was occupied by LYL1-expressing cells in 10 out of 78 pair1 chimeric spheroids, while it was was occupied by TAL1-expressing cells in only 2 out of 77 pair2 chimeric spheroids (** $P = 0.0018$). This result shows that TAL1-expressing cells preferentially occupy the tip position in vessel sprouts while LYL1-expressing cells are preferentially located in the stalk position.

VEGFR1 expression is differentially regulated by TAL1 and LYL1

In order to explore the molecular mechanism specifying the location of TAL1- and LYL1-expressing cells in vessel sprouts, we quantified the mRNA levels of VEGFR1 and VEGFR2 by RT-PCR after transfection of the expression vectors BOSTAL1 and BOSLYL1 into HUVECs. These transfections were done in the same way as the 3D spheroid-based angiogenesis assay using chimeric spheroids and achieved more than 10 times upregulation of both transcription factors compared with non-transfected HUVECs. mRNA levels were measured and analyzed after 3 separate dual DNA transfection and PCR reactions ($n = 3 \times 2$). In BOSTAL1-transfected HUVECs, the relative expression level of VEGFR1 was

suppressed to 88% of that in non-transfected cells (**$P < 0.01$), while it increased to 116% in BOSLYL1-transfected HUVECs (**$P < 0.01$) (Fig. 7A). This result means that about a 1.3-times upregulation of VEGFR1 expression (**$P < 0.01$) was seen in LYL1-expressing cells compared with that in TAL1-expressing cells (Fig. 7B). No statistically significant upregulation or downregulation of VEGFR2 mRNA levels resulted from transfection of BOSTAL1 and BOSLYL1. According the results of Jakobsson et al., differential VEGFR levels affect tip cell selection and VEGFR1-downregulated cells (the TAL1-expressing cells in our experiment) dominated in the tip cell population in the assay using mosaic embryoid bodies [41]. We concluded that VEGFR1 downregulation by TAL1 OE and VEGFR1 upregulation by LYL1 OE in endothelial cells affects tip cell selection in vessel sprouts and results in TAL1-expressing cells preferentially locating to the tip cell position in the sprouts. Since VEGFR1 can function as a VEGF-A scavenging receptor [42,43], this result is likely to be associated with the inhibition of endothelial migration seen in Fig. 5.

Discussion

The basic helix-loop-helix proteins TAL1, LYL1 and the LIM-only protein LMO2 were identified from the breakpoints of chromosomal translocations associated with T cell acute leukemia. Among these three factors, both TAL1 and LMO2 are necessary for the generation of hematopoietic stem cells because the TAL1- and LMO2-knockout mouse phenotypes are bloodless. Formation of the canonical oligomeric transcription factor complex consisting of E2A-TAL1-LMO2-LDB1-LMO2-GATA2 in the nucleus of hemogenic endothelia is considered to be essential for the initiation of adult type hematopoiesis. Overexpression of both TAL1 and LMO2 in T cells results in leukemogenesis by formation of aberrant transcription factors such as E2A-TAL1-LMO2-LDB1-LMO2-TAL1-E2A that disrupt normal differentiation of T cells. Similarly, overexpression of LYL1 also produces leukemia/lymphoma in mice by trapping and inhibiting E2A proteins [36]. Aberrant expression of members of the LMO2 transcription factor complex results in defects in differentiation of blood cells that eventually lead to leukemogenesis [44].

With respect to cardiovascular development, LMO2, TAL1, and LYL1 are endothelium-specific transcription factors that have essential roles in embryonic angiogenesis. The forced expression of the LYL1 transcription factor in endothelia of 6-week-old LYL1 transgenic mice results in increased vessel branching in the aorta ring assay and hypervasuclarity in the peripheral regions of organs. Its short tail phenotype is associated with a relative paucity of straight arteries during embryonic development. We conclude that these findings are due to reduced expression of DLL4 in tip cells

as a result of overexpression of LYL1 disrupting the transcription factor complex consisting of LMO2 and TAL1 that acts on the DLL4 promoter. Qutub and Popel proposed a multiscale systems model that closely simulated the mechanism underlying sprouting angiogenesis and analyzed the effect of DLL4 haploinsufficiency on the pattern of vessel growth [40]. In accordance with our findings, vessel length is greater but more variable in $DLL4^{+/-}$-knockout mice compared with those in simulated controls, which is a result of increased numbers of sprout tips and more branching.

In *in vitro* spheroid-based angiogenesis assays combined with targeted gene knockdown using RNAi, siRNA that targeted LMO2, TAL1, and GATA2 inhibited sprouting angiogenesis whereas silencing LYL1 did not. The synergistic effect of LMO2/TAL1 and LMO2/GATA2 on vessel sprouting observed here, as well as in a previous study [28], strongly suggests that LMO2 acts as a member of a multimeric transcription factor complex. This positive role of the LMO2 transcription factor complex in vessel sprouting is partly explained by upregulation of a downstream direct target, angiopoietin 2, because its levels in supernatant from cultured endothelium showed a strong correlation with CSL. The presence of a sequence consisting of the E-box-11bp-GATA binding site in the angiopoietin 2 promoter proximal region strongly suggests that the LMO2 transcription factor complex consisting of TAL1-LMO2-GATA2 directly binds to this site and positively regulates angiopoietin 2 expression [29]. Among the main VEGF receptors, VEGFR2 (Flk-1) was first identified as a TAL1 and GATA2 downstream target and is considered the VEGF receptor that most influences sprouting angiogenesis.

Its enhancer has both the TAL1 and GATA binding sites [20]. Since our data showed that LMO2 also positively regulates its expression, the most likely conformation of the transcriptional regulators is TAL1-LMO2 or LMO2-GATA2, in which LMO2 can play a stabilizing role in the complex. Coma et al. showed that the VEGF co-receptor neuropilin 2 is a direct transcriptional target of the LMO2-GATA2 complex [28]. There is a GATA binding site in its promoter region that positively regulates its expression upon LMO2-GATA2 binding. Our data showed that a single LMO2 knockdown downregulated its mRNA level, suggesting that LMO2 acts to stabilize the protein complex. Thus, the LMO2 transcription factor complex (TAL1-LMO2, LMO2-GATA2, and TAL1-LMO2-GATA2) plays a positive role in sprouting angiogenesis by upregulating angiopoietin 2 and VEGFR2 with the involvement of neuropilin 2 in our VEGF-A (VEGF165)-induced sprouting angiogenesis assay. Our study shows that VEGFR1 and VEGFR2 are also regulated by TAL1, LYL1, and LMO2.

Expression of another direct transcriptional target, VE-cadherin, was also measured after RNAi knockdown. After LMO2 and TAL1 knockdown, VE-cadherin mRNA levels were reduced to 80% and 90%, respectively, of those of the transfection control, although they were not affected by transfection of siLYL1 or siGATA2. The reduction in VE-cadherin observed here was smaller than that in a previous study [11], possibly reflecting methodological differences in siRNA transfection and a reduction in mRNA levels. Another reason for this difference could be the relatively high expression levels of VE-cadherin in endothelial cells. Other transcriptional activators could also regulate its

expression. A recent study showed that there is a substantial reduction in the expression of 33 core angiogenesis genes, out of the 84 studied, after LMO2 knockdown [45]. Among which, sphingosine kinase 1 (SphK1) was identified as another downstream target of the transcription factor complex consisting of LMO2/TAL1. SphK1 is involved in generation of sphingosine 1 phosphate (S1P), an intracellular second messenger, which plays a key role in endothelial cell migration [46]. Further studies are needed to determine how expression levels of each downstream gene contribute to vessel sprouting.

Forced LYL1 expression in cultured endothelial cells also inhibited subsequent VEGF-A-induced sprouting angiogenesis in the 3D spheroid-based angiogenesis assay. This inhibition is likely to be related to the reduced endothelial migration of LYL1-expressing endothelial cells. VEGFR1, which has a higher affinity for VEGF-A but lower tyrosine kinase activity in its intracellular domain, functions as a scavenger receptor of VEGF-A and negatively regulates VEGF-A-induced endothelial migration in sprouting angiogenesis. In its promoter region, there is a CREB binding site and CREB binding to it positively regulates its expression [47]. LYL1, but not TAL1, can bind to CREB to form a heterodimer [48]. Indeed, LYL1 overexpression resulted in VEGFR1 upregulation while TAL1 overexpression downregulated VEGFR1 expression in our study. LYL1 overexpression and a corresponding VEGFR1 upregulation in endothelial cells could explain the decrease in endothelial migration and *in vitro* sprouting angiogenesis. According to the results of Jakobsson et al., tip cells are continuously competing for position during sprouting angiogenesis and differential VEGFR1 or VEGFR2 levels

affect tip cell selection [41]. VEGFR1 downregulation in TAL1-expressing cells and its upregulation in LYL1-expressing cells might give TAL1-expressing endothelial cells an advantage in this competitive positioning in our sprouting angiogenesis assay using 1:1 mixed endothelial cells. This sprouting inhibition by LYL1 OE is consistent with the vascular stabilizing effects of LYL1 described in a previous report [14].

In sprouting angiogenesis, vascular tree formation is a stepwise process of tip cell migration and vessel branching [49]. The former is chiefly regulated by the branching pattern generator consisting of VEGFR2-DLL4-Notch [38]. Endothelial cells, especially tip cells in the sprouts, also have an intrinsic transcriptional regulator that may regulate the transition from quiescent to angiogenic endothelium. In quiescent endothelium, which expresses both LMO2 and LYL1 but not TAL1, LMO2 is thought to primarily bind to LYL1 to form the LYL1-LMO2 complex. When LMO2 binds to LYL1, the downstream target angiopoietin 2 is only weakly activated in the quiescent endothelium. In the activated endothelium, which begins to express TAL1, the LMO2 binding partner shifts from LYL1 to TAL1 to form the classical TAL1-LMO2 or TAL1-LMO2-GATA2 transcription factor complexes that initiate vessel sprouting (Fig. 8). After TAL1 is activated in the angiogenic endothelium, the classical LMO2-TAL1 complex becomes a stronger activator of two tip cell proteins, angiopoietin 2 and DLL4, and at the same time suppresses expression of the VEGF-A scavenging receptor, VEGFR1. Moreover, the LMO2-TAL1 complex also upregulates VEGFR2 to augment endothelial cell migration induced by VEGF-A, which is necessary to form

relatively straight vessels. Overexpression of the LYL1 protein in tip cells leads to slowing of endothelial cell migration, firstly by upregulating VEGFR1, and secondly by downregulating DLL4 in tip cells, which results in increased branching at the peripheries of the vascular tree (Fig. 8).

As mentioned above, both TAL1-knockout and LMO2-knockout mice exhibited a bloodless phenotype because of the absence of hematopoietic stem cells (HSCs) that normally emerge from the hemogenic endothelium of the dorsal aorta [50]. TAL1 expression becomes dispensable once HSCs are generated [51]. We hypothesize that DLL4 expression on the surface of the hemogenic endothelium of the dorsal aorta stimulates Notch signaling in adjacent cells, future HSCs in this case, which activate the Notch downstream target molecule RUNX1 [52,53].

Figure legends

Fig. 1. LYL1 transgenic mice have a short tail and round organs.

Anatomical features of LYL1 transgenic mice were studied macroscopically and microscopically. A., B. Tail shortening was seen in LYL1 transgenic (TG/+) mice (B) compared with wild type littermates (A) at 6 weeks of age. C. The short tail phenotype was observed in the TG/+ P0 mice. D. LYL1 transgenic mice showed alopecia later in life, which was severe in homozygotes (TG/TG). E., F. The shape of organs in LYL1 transgenic mice was generally rounder and smaller than that of wild type mice. Spleens of 6-week-old TG/+ transgenic mice (F) were smaller (about 10% reduction in weight) and rounder than those of wild type littermates (E). G., H. The peripheral part of the TG/+ mouse spleen showed increased numbers of blood vessels (indicated by the arrow in H). They were usually smaller in diameter compared with those seen in wild type littermates (indicated by the arrow in G) in the section of the spleens stained with hematoxylin and eosin. I., J. The same histological analyses of the peripheral part of lung from a TG/+ mouse (J) and its littermate (I). Smaller alveolar spaces and vessels with smaller diameters were observed in TG/+ lungs (J). K., L. CD31 (PECAM) staining of a lung section from a TG/+ mouse (L) and its wild type littermate (K). In TG/+ lungs, smaller vessels and capillaries surrounding alveoli were positively stained (brown color) while only vessels of medium size were positive in wild type littermates. M., N. In the cardiovascular system, a smaller and rounder heart was seen in TG/+ mice (N: about 10% reduction of weight and axis length) compared with hearts in wild type littermates (M). O., P. TG/+ blood

vessels showed wider branching angles typical of those at the surface of the brain (P) compared with those from wild type littermates (O). Representative branching points are indicated by dots in the figure.

Fig. 2. LYL1 transgenic mouse aorta explants cultured in Matrigel showed hyperbranching of sprouting capillaries in the aorta ring assay.

Three paired aorta ring sections were dissected out at separate times from 6-week-old wild type littermates and LYL1 transgenic mice, embedded in Matrigel, and cultured until day 9. A., B. Representative low magnification images of aorta ring culture of wild type littermates (A) and LYL1 transgenic (TG/+) mice (B) at day 5. Capillary sprouting was slightly delayed in TG/+ aortas compared with wild type controls. Note the increased density of capillaries near the TG/+ aortic wall in B. C., D. Representative low magnification images of other paired cultures at day 9, by which remodeling of capillaries has begun. In TG/+ aorta culture (D), remodeled vessels showed higher frequencies of branching, which resulted in a relative decrease in the number of straight capillaries at the periphery compared with wild type aorta ring culture (C). E., F. Representative high magnification images of other paired (E: wild type, F: TG/+) aorta ring assays at day 7, in which capillaries from TG/+ aortas showed hyperbranching. G., H. Representative high magnification images of another paired (G: wild type, H: TG/+) aorta ring assay at day 7. Unambiguous bifurcations were indicated by white dots. A comparison of clear bifurcation points per 10 arbitrary fields showed that there were 48 in wild type and 90 in TG/+ aortas. The branching angle was

generally wider in TG/+ aorta explant culture. I. Relative expression level of DLL4 after double transfection of the expression vectors BOSLMO2/BOSTAL1 or BOSLMO2/BOSLYL1 into cultured HUVECs. Statistically significant (** $P < 0.01$) decreases in DLL4 expression compared with control HUVECs were seen after both combined transfections. J. Relative expression levels of DLL4 after a single transfection of the expression vector BOSTAL1 or BOSLYL1 into cultured HUVECs. Statistically significant (** $P < 0.01$) decreases of DLL4 compared with control HUVECs were seen after both single transfections.

Fig. 3. Knockdown of LMO2, TAL1, and GATA2 leads to inhibition of endothelial sprouting and tube formation *in vitro,* but LYL1 knockdown does not.

Knockdown of components of the LMO2 complex was achieved using siRNA with HUVEC spheroids. A. Representative images from 3D spheroid-based *in vitro* angiogenesis assays and a statistical summary of CSL measurements from spheroids. Cultured HUVECs were transfected with siRNAs of eGFP, LMO2, TAL1, LYL1, and GATA2 as indicated in the figure. siRNA of eGFP was used as a transfection control. Relative mRNA levels (average of 2 separate transfections) of HUVECs transfected with each siRNA compared with that of si-eGFP-transfected cells are indicated below the names of the RNAi target genes below the statistical summary of CSL measurements. Note that sprouts with continuous tubes from the spheroids are counted in this analysis. Statistically significant (** $P < 0.01$) decreases in CSL were observed

in spheroids transfected with siRNAs for LMO2, TAL1, and GATA2. B. Cultured HUVECs were transfected with siRNAs of eGFP, LMO2 combined with TAL1, and LMO2 combined with GATA2 as indicated in the figure. siRNA of eGFP was used as a transfection control. Statistically significant (** $P < 0.01$) decreases in CSL were observed in spheroids transfected with LMO2/TAL1 and LMO2/GATA2 siRNAs (combined transfection). Combined knockdowns of LMO2/TAL1 and LMO2/GATA2 almost abolished sprouting from spheroids.

Fig. 4. Effects of LMO2, TAL1 silencing on endothelial cell migration and their downstream targets' expression.

Knockdown of components of the LMO2 complex was achieved using siRNA with cultured HUVEC. A. Number of invading HUVECs transfected with siRNA per field is indicated in the figure. Cell counting was done in four randomly selected fields after 2 separate DNA transfections. Invasion of LMO2, TAL1 knockdown HUVECs into a Matrigel extracellular matrix was significantly (** $P < 0.01$) reduced compared with the transfection control (si-eGFP). B. Cultured HUVECs were transfected with the indicated siRNA. Angiopoietin 2 (ANG-2) secretion into the culture medium after 24 hour incubation was measured by ELISA. Statistically significant (* $P < 0.05$) decreases in ANG-2 secretion were observed in culture media obtained from si-LMO2-, si-TAL1-, and si-GATA2-transfected HUVECs, but not in that from si-LYL1-transfected HUVECs. C., D. Cultured HUVECs were transfected with siRNA of LMO2, TAL1, LYL1, GATA2, and the transfection control si-

eGFP. Relative mRNA expression levels of VEGFR1 (C), VEGFR2 (C), and NRP2 (D) compared with those of si-eGFP transfection controls are shown. They were measured by real-time qPCR after normalization using GAPDH as an internal reference. Statistically significant (*$P < 0.05$) decreases are indicated.

Fig. 5. Overexpression of LYL1 inhibits endothelial cell migration and sprouting angiogenesis in a 3D spheroid-based assay.

Overexpression of TAL1 or LYL1 was achieved using DNA transfection with HUVEC spheroids. A. Cultured HUVECs were transfected with mammalian expression vectors of TAL1 and LYL1. The empty mammalian expression vector BOSXba was used as a transfection control. Statistically significant (**$P < 0.01$) decreases in CSL were observed in spheroids transfected with BOSLYL1. Representative images of the analyses are also shown. B. Number of invading HUVECs (per field) transfected with expression vectors are indicated in the figure. Cell counting was done in four randomly selected fields after 2 separate DNA transfections. Invasion of TAL1, LYL1 OE HUVECs into Matrigel extracellular matrix was significantly (**$P < 0.01$) reduced compared with the transfection control (BOSXba).

Fig. 6. TAL1-expressing cells preferentially occupy the tip position over LYL1-expressing cells in vessel sprouts *in vitro*.

Cultured HUVECs were transfected with a mammalian expression vector encoding LYL1, TAL1, and β-galactosidase. In pair1, non-labeled TAL1-

expressing cells were mixed at 1:1 ratio with β-galactosidase-labeled LYL1-expressing cells. In pair2, β-galactosidase-labeled TAL1-expressing cells were mixed at 1:1 ratio with non-labeled LYL1-expressing cells. Both chimeric spheroids were cultured in type 1 collagen gel for 24 hours with 20 ng/ml VEGF-A stimulation. Labeled cells were detected by whole mount X-gal staining immediately after culture. Cells at the tip position in the sprouts are indicated by black arrowheads and those at the stalk position by white arrowheads. A., C., E., G., I. Representative images of X-gal-stained pair1 spheroids. The labeled cells (LYL1-expressing cells in this pair) were preferentially located in the stalk position. B., D., F., H., J. Representative images of X-gal-stained pair2 spheroids. The labeled cells (TAL1-expressing cells) tend to occupy the tip position. The distributions of labeled cells in all chimeric spheroids are shown in Table 1.

Fig. 7. Differential VEGFR1 expression with TAL1 or LYL1 overexpression.

Cultured HUVECs were transfected singly with the mammalian expression vectors encoding TAL1 (BOSTAL1) or LYL1 (BOSLYL1). After 48 hours of DNA transfection, mRNA was prepared for RT-PCR based quantification of mRNA levels of VEGFR1 and VEGFR2 using HPRT1 as an internal reference. Statistical analysis of the relative expression levels of both mRNAs was carried out after 3 separate transfections and dual real-time PCR reactions ($n = 3 \times 2$). A. A statistically significant ($^{**}P < 0.01$) decrease in VEGFR1 expression level was observed after BOSTAL1 transfection while an increase was seen after BOSLYL1 transfection. B. An approximately x1.3 upregulation

of VEGFR1 expression levels was observed in response to LYL1 overexpression compared with TAL1 overexpression.

Fig. 8. Hypothetical mechanism of transcriptional regulation of hierarchical vascular tree formation.

In sprouting angiogenesis, activated endothelial cells upregulate TAL1 to form the transcription factor complex consisting of LMO2 and TAL1 in the nucleus, which upregulates angiopoietin 2, VEGFR2, and DLL4. After detachment of mural cells in response to angiopoietin 2, these cells then preferentially occupy the tip position in vessel sprouts (indicated by red cells). DLL4 on tip cells binds to Notch on adjacent cells to downregulate VEGFR2 (indicated by the dashed line). In contrast, endothelial cells in which LMO2 chiefly binds to LYL1 in the nucleus to transcriptionally upregulate VEGFR1 tend to occupy the stalk position (indicated by blue cells). LYL1 overexpression in tip cells downregulates DLL4 expression and upregulates VEGFR1, which results in hyperbranching of capillaries and slowing of tip cell migration at the periphery of the vascular tree.

Table 1. Number of spheroids in which the labeled cells occupied the indicated positions after sprouting in collagen gel

	Pair1 spheroids	Pair2 spheroids
Tip position	1	6
Stalk position	10	2
Internal position	66	70
Total	77	78

$^{**}P = 0.0018$

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Author's contribution

Originator of Project: YY, THR

Participated in research design: YY, THR

Conducted experiments: YY, YZ, SM, AT

Performed data analysis: YY, SM, AT, THR

Wrote or contributed to the writing of the manuscript: YY, THR

Conflict of interest statement

The authors have no conflict of interest to declare.

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Figures

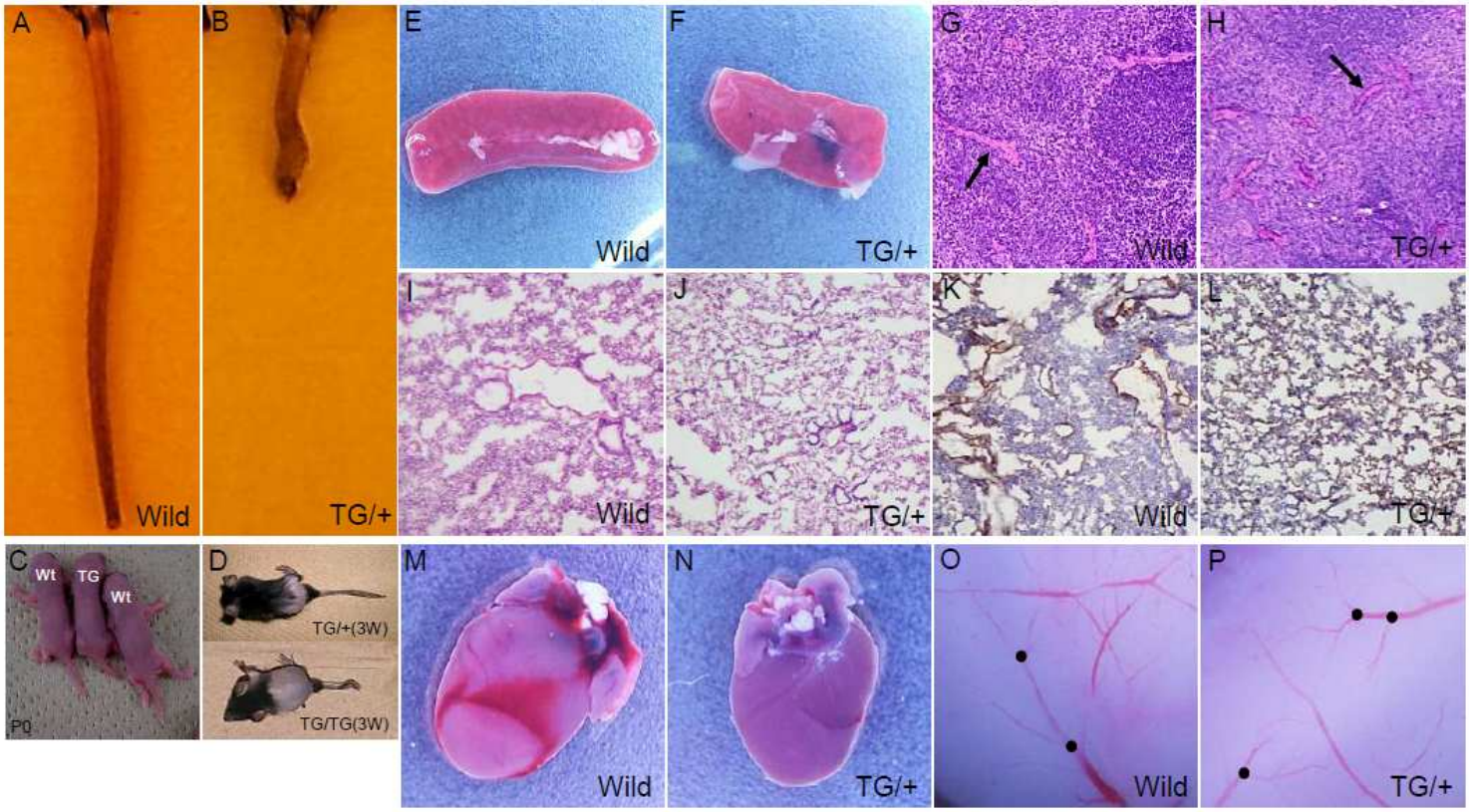

Figure 1

LYL1 transgenic mice have a short tail and round organs.

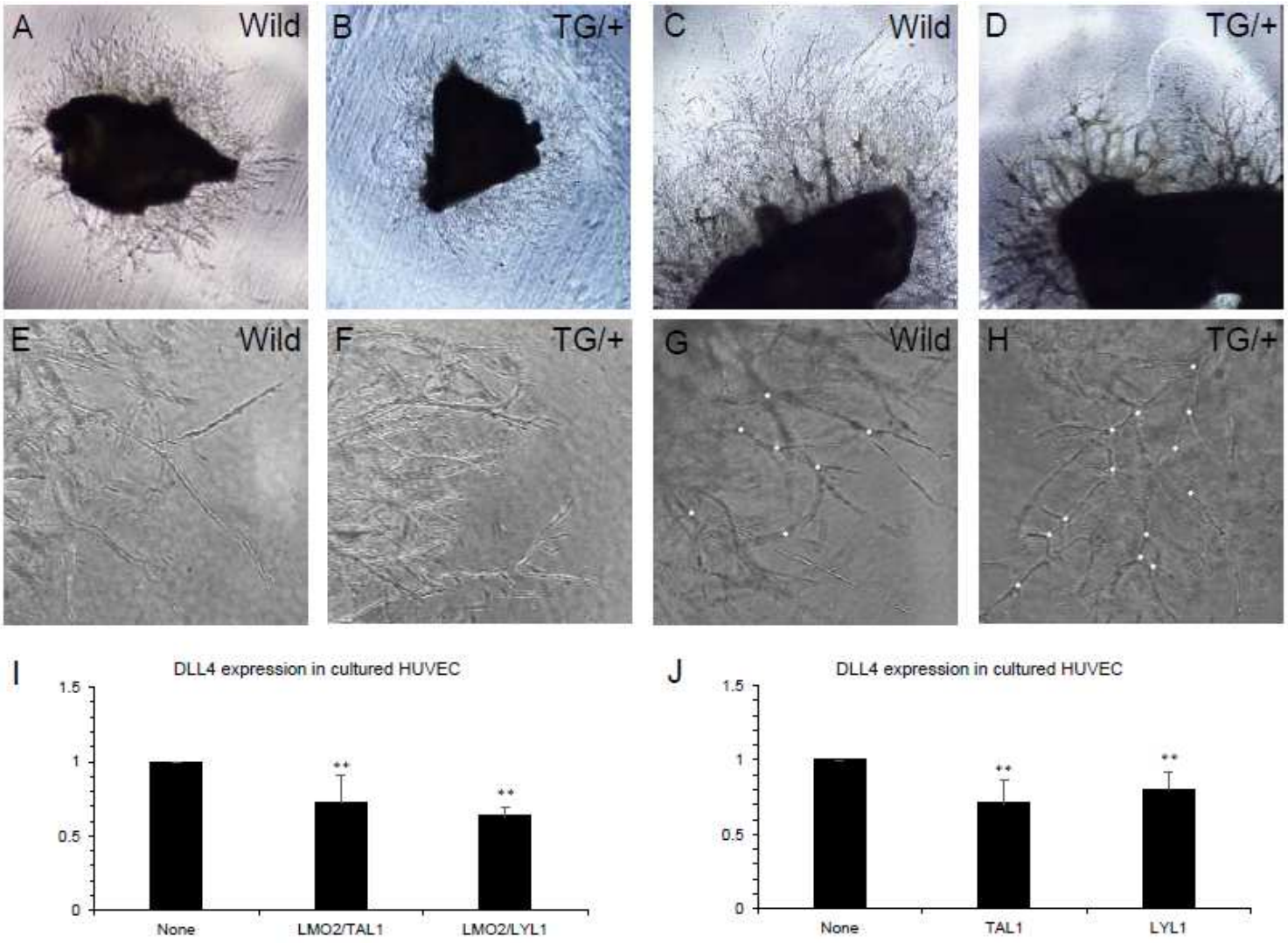

Figure 2

LYL1 transgenic mouse aorta explants cultured in Matrigel showed hyperbranching of sprouting capillaries in the aorta ring assay.

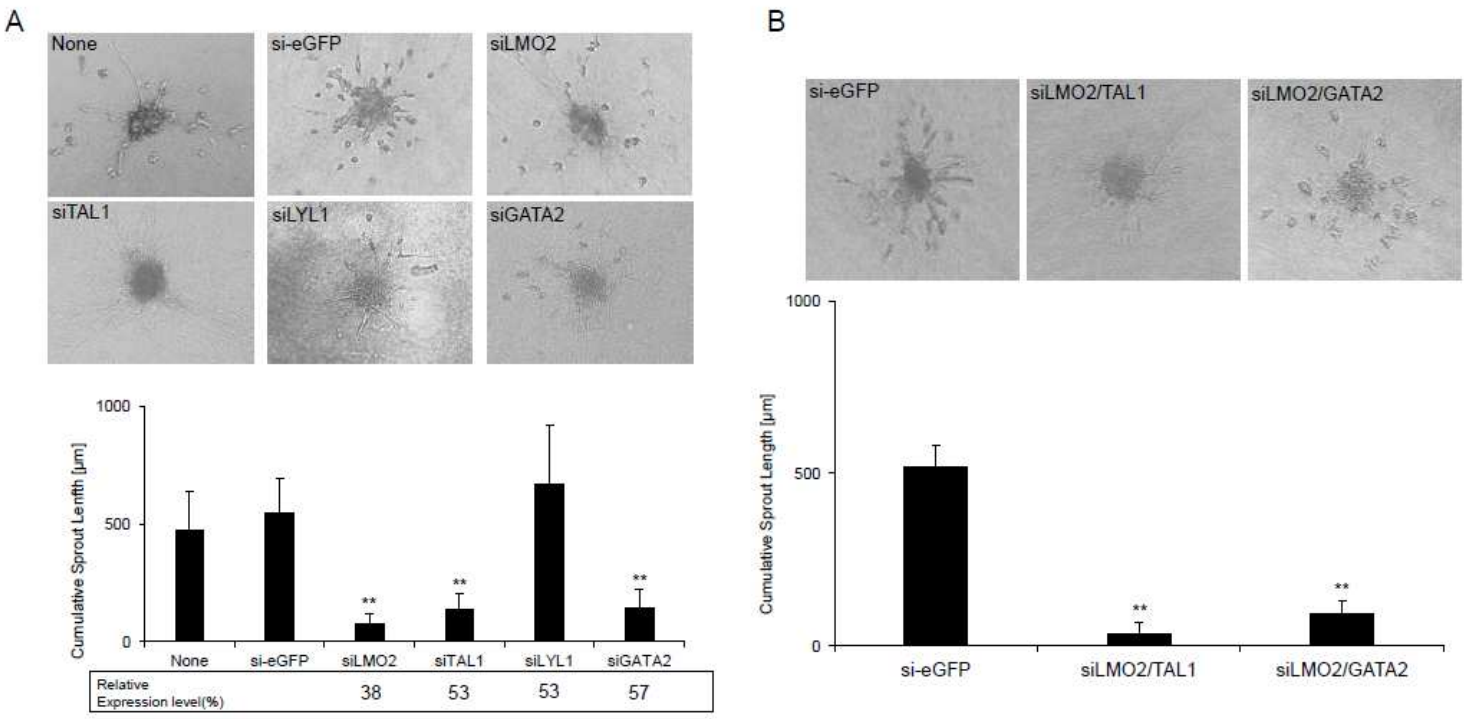

Figure 3

Knockdown of LMO2, TAL1, and GATA2 leads to inhibition of endothelial sprouting and tube formation in vitro, but LYL1 knockdown does not.

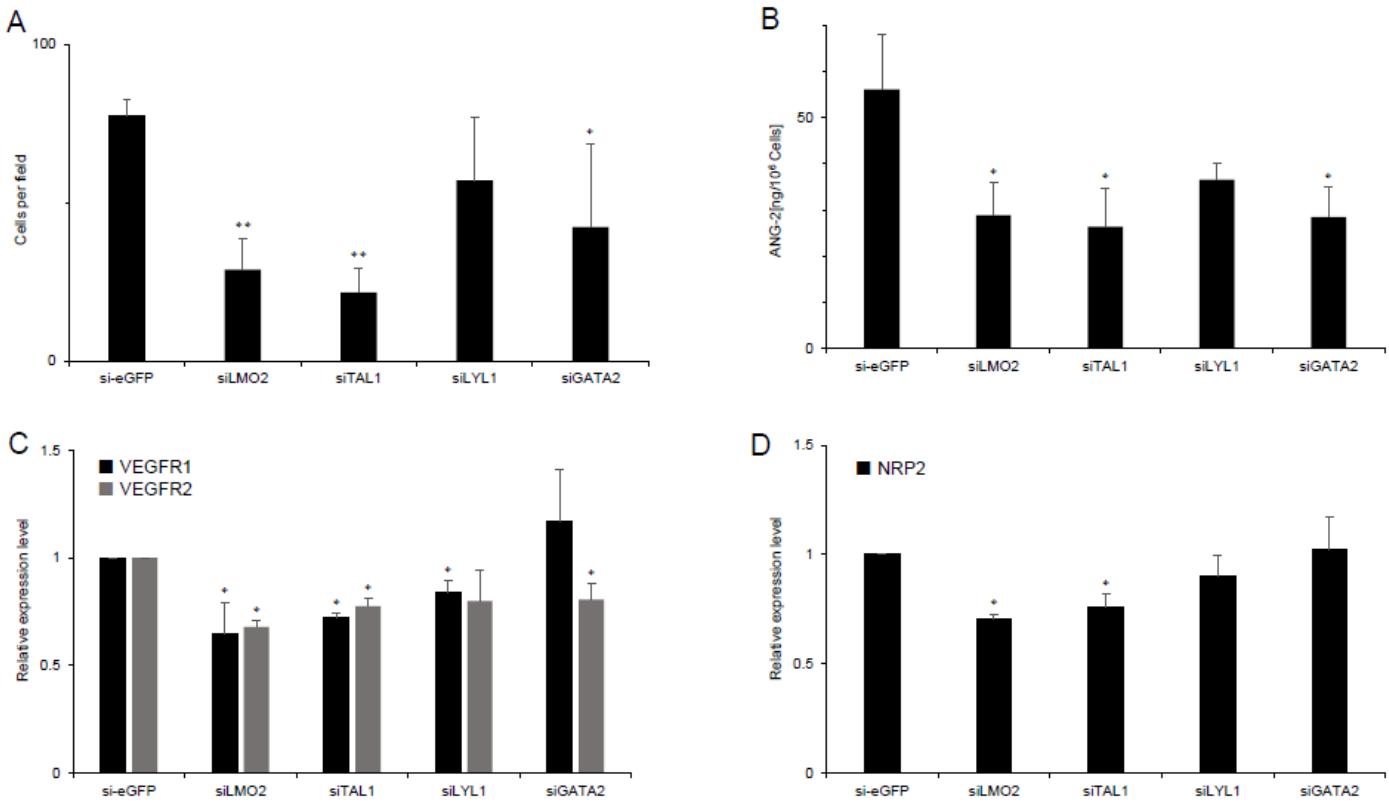

Figure 4

Effects of LMO2, TAL1 silencing on endothelial cell migration and their downstream targets' expression.

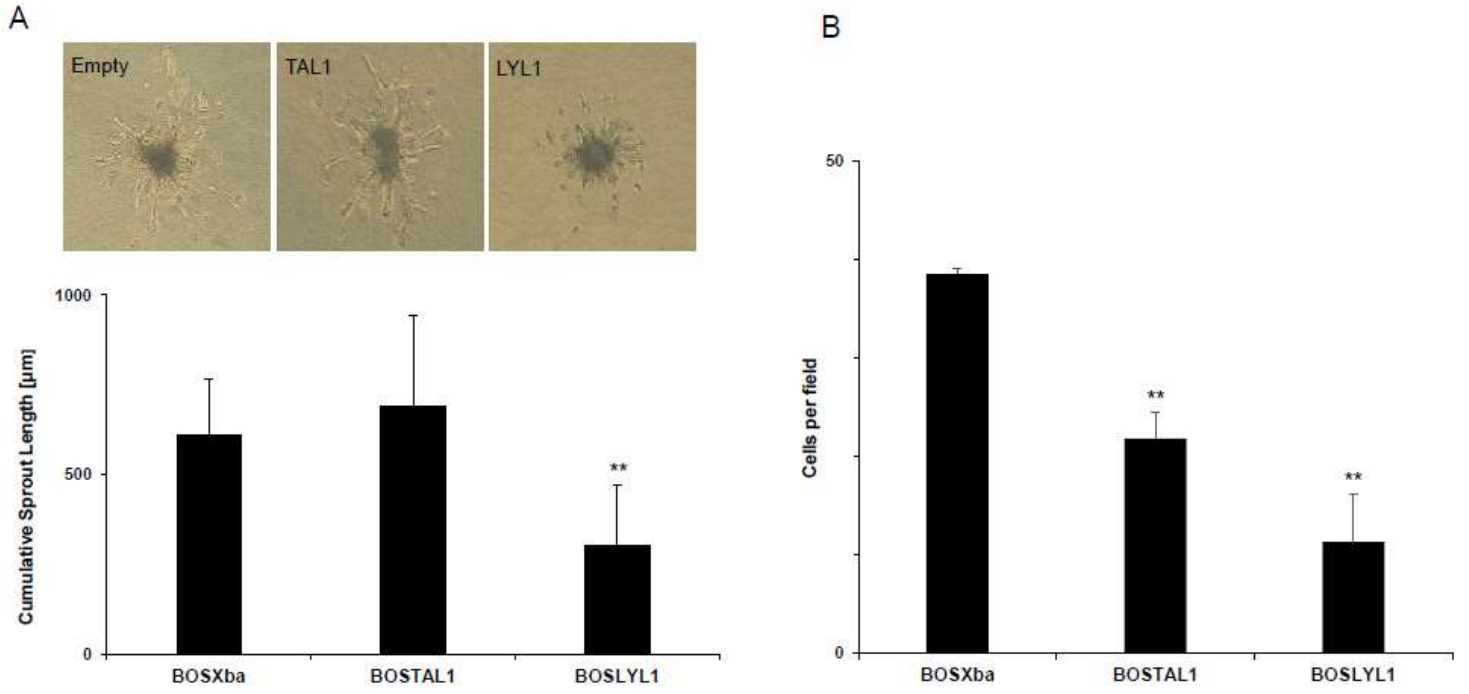

Figure 5

Overexpression of LYL1 inhibits endothelial cell migration and sprouting angiogenesis in a 3D spheroid-based assay.

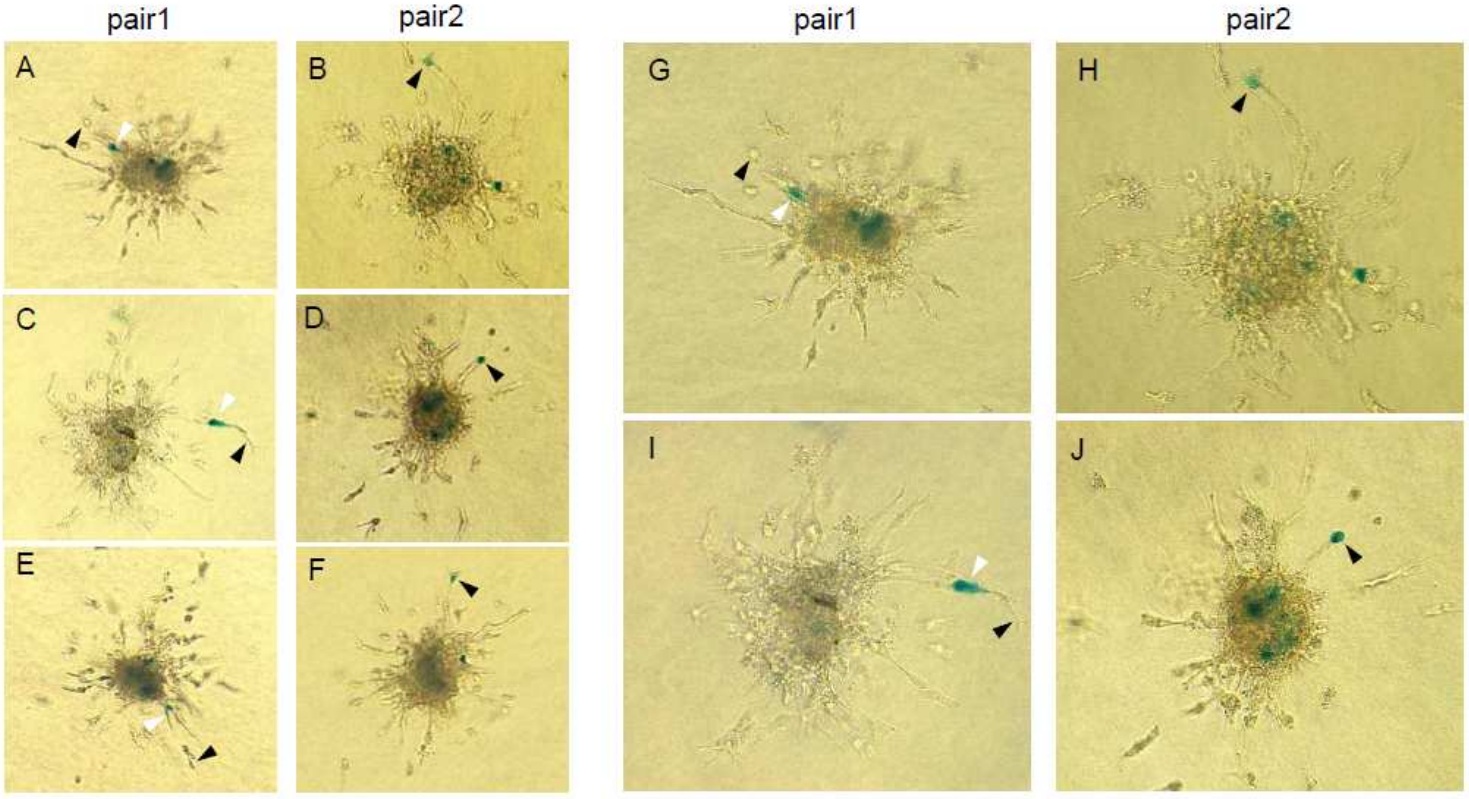

Figure 6

TAL1-expressing cells preferentially occupy the tip position over LYL1- expressing cells in vessel sprouts in vitro.

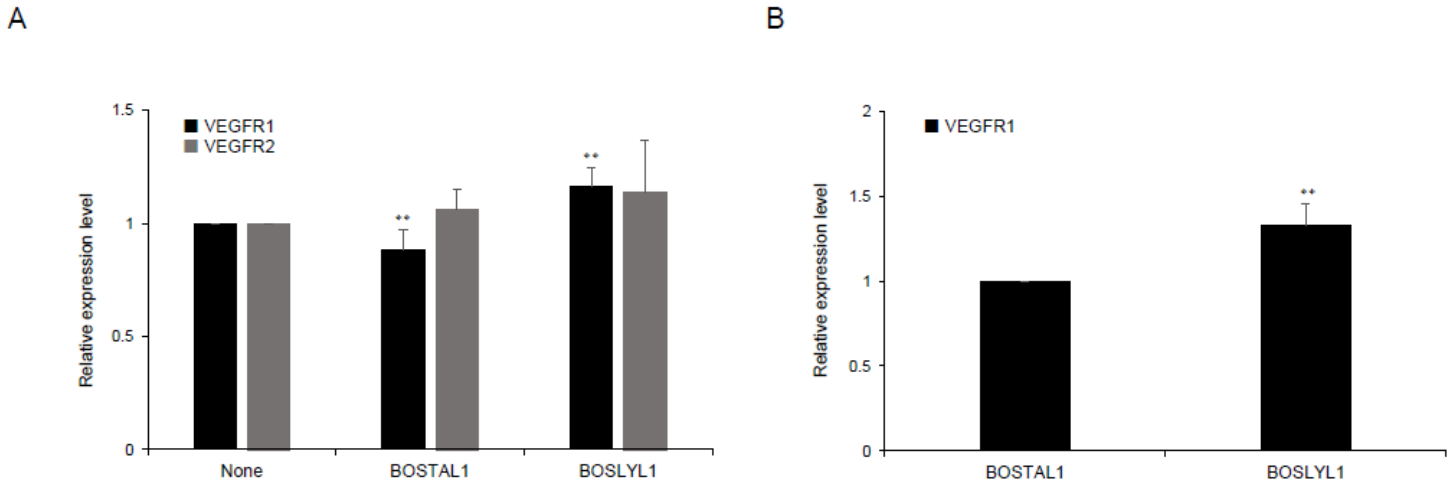

Figure 7

Differential VEGFR1 expression with TAL1 or LYL1 overexpression.

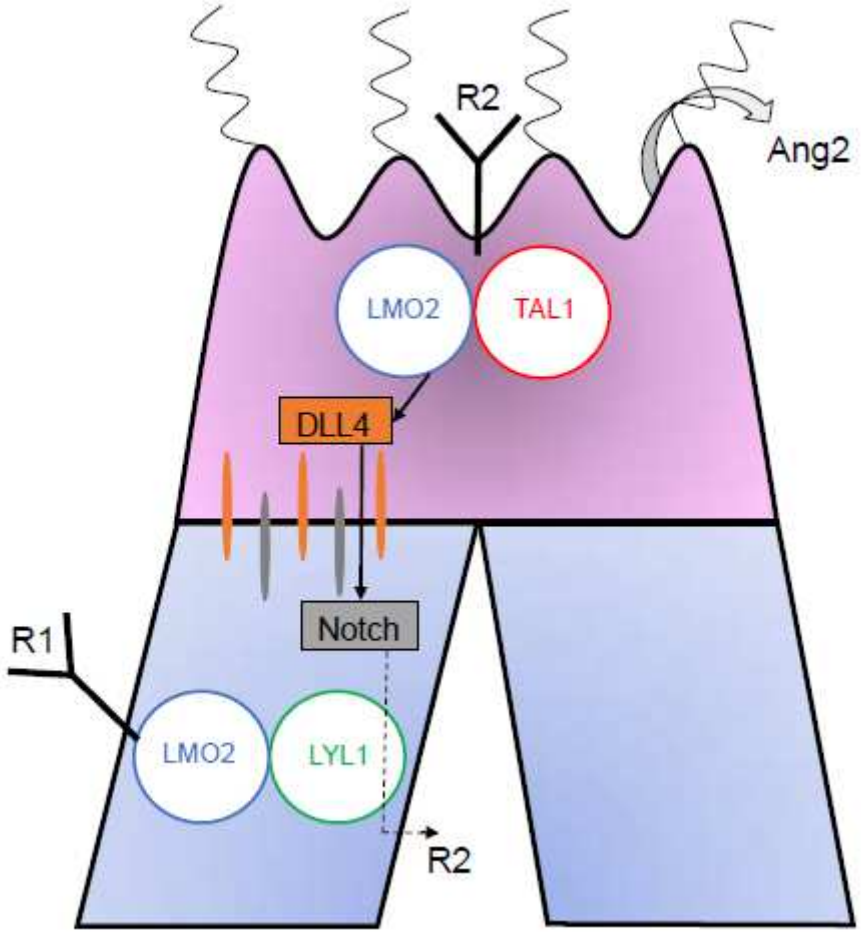

Figure 8

Hypothetical mechanism of transcriptional regulation of hierarchical vascular tree formation.