ORAI1 Activates Proliferation of Lymphatic Endothelial Cells in Response to Laminar Flow Through Krüppel-Like Factors 2 and 4

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**Rationale:** Lymphatic vessels function to drain interstitial fluid from a variety of tissues. Although shear stress generated by fluid flow is known to trigger lymphatic expansion and remodeling, the molecular basis underlying flow-induced lymphatic growth is unknown.

**Objective:** We aimed to gain a better understanding of the mechanism by which laminar shear stress activates lymphatic proliferation.

**Methods and Results:** Primary endothelial cells from dermal blood and lymphatic vessels (blood vascular endothelial cells and lymphatic endothelial cells [LECs]) were exposed to low-rate steady laminar flow. Shear stress–induced molecular and cellular responses were defined and verified using various mutant mouse models. Steady laminar flow induced the classic shear stress responses commonly in blood vascular endothelial cells and LECs. Surprisingly, however, only LECs showed enhanced cell proliferation by regulating the vascular endothelial growth factor (VEGF)-A, VEGF-C, FGFR3, and p57/CDKN1C genes. As an early signal mediator, ORAI1, a pore subunit of the calcium release–activated calcium channel, was identified to induce the shear stress phenotypes and cell proliferation in LECs responding to the fluid flow. Mechanistically, ORAI1 induced upregulation of Krüppel-like factor (KLF)-2 and KLF4 in the flow-activated LECs, and the 2 KLF proteins cooperate to regulate VEGF-A, VEGF-C, FGFR3, and p57 by binding to the regulatory regions of the genes. Consistently, freshly isolated LECs from Orail knockout embryos displayed reduced expression of KLF2, KLF4, VEGF-A, VEGF-C, and FGFR3 and elevated expression of p57. Accordingly, mouse embryos deficient in Orail, Klf2, or Klf4 showed a significantly reduced lymphatic density and impaired lymphatic development.

**Conclusions:** Our study identified a molecular mechanism for laminar flow–activated LEC proliferation.

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**Key Words:** calcium channel ■ capillary ■ cell proliferation ■ lymphatic vessels ■ vascular endothelial growth factor A

The lymphatic system is composed of lymphatic vascular networks and lymphoid tissues and is essential for tissue fluid homeostasis, immune cell trafficking, and lipid absorption and transport. Lymphatic vessels may be largely classified into 2 distinct compartments: capillary versus collecting vessels. Lymphatic capillaries function to uptake tissue fluids, lipids, large molecules, and cells, collectively called lymph fluid, from the interstitial space. During this lymph fluid uptake, lymphatic endothelial cells (LECs) lining the capillaries may experience interstitial fluid flow with a basal-to-apical direction at their intercellular junctions. Drained lymph fluid then flows toward downstream-collecting lymphatics, imposing laminar shear stress on the luminal surface of capillary LECs. It has been recently reported that, as the embryos develop, accumulating interstitial fluid gradually increases fluid pressure and creates fluid flow that imposes shear stress on the luminal LECs in developing...
Nonstandard Abbreviations and Acronyms

<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>BECs</td>
<td>blood vascular endothelial cells</td>
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<td>HUVECs</td>
<td>human umbilical venous endothelial cells</td>
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<td>KLF</td>
<td>Krüppel-like factors</td>
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<td>LECs</td>
<td>lymphatic endothelial cells</td>
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What Is Known?
- Fluid flow profoundly influences lymphatic development and remodeling.
- Interstitial pressure-driven lymphatic drainage may activate lymphatic expansion.

What New Information Does This Article Contribute?
- Our study revealed that steady laminar flow selectively enhances endothelial cells from lymphatic vessels but not from blood vessels.
- We defined a molecular mechanism by which steady laminar flow activates lymphatic endothelial cell proliferation.
- A major function of lymphatic vessel is to drain tissue fluid. Although lymphatic vessels are essential for fluid flow, fluid flow also controls lymphatic vessel development. This reciprocal feedback control mechanism seems to be critical for tissue fluid balance and distribution in our body. Here, we investigated this reciprocal feedback control, specifically by deciphering a molecular mechanism explaining how physical force generated by fluid flow can be translated to biological phenomenon, namely, cell proliferation. Our study uncovered that fluid flow enhances the intracellular calcium level and thus activates key transcriptional regulators. These regulators induce the expression of genes involved in endothelial growth. Our study provides a mechanistic understanding on flow-induced lymphatic growth.

Lymphatic capillaries, on the contrary, the collecting lymphatic vessels function to transport the lymph to the lymph nodes and back to the circulation. LECs lining the collecting vessels may more frequently experience oscillatory flows. Studies showed that this type of fluid flow serves as a key signal for development of the luminal valves, a hallmark of collecting lymphatic vessels.

It has been known that hemodynamics deliver a profound influence on vascular morphogenesis throughout development. Because interstitial fluid drainage is a primary function of lymphatic vessels, fluid flow force and pattern have been hypothesized to play key roles in lymphatic development as important nonbiological stimuli. Indeed, previous studies reported that interstitial flow associated with functional drainage acts as a critical lymphangiogenic mediator by controlling LEC migration, vascular endothelial growth factor (VEGF)-C expression, and lymphatic capillary network formation. Increased embryonic fluid drainage was demonstrated to coincide with and promote initial lymphatic development, possibly serving as an embryonic signal for lymphatic expansion. Here, we investigated a mechanism by which steady laminar flow can trigger lymphatic expansion. Our study revealed that low-rate steady laminar flow activates a highly selective calcium channel ORAI1 to upregulate Krüppel-like factors (KLF) 2 and 4, which directly regulate lymphatic expansion but also provides important insights into the mechanisms whereby endothelial cells incorporate hemodynamic signals into their biological responses.

Methods

Cell Culture, Related Reagents, and Flow Application
Human primary dermal blood vascular endothelial cells (BECs) and LECs were isolated from human foreskins with approval by the Institutional Review Board, University of Southern California (PI: Y.-K.H.) and cultured in Endothelial Basal Media (Lonza)-based media as previously described. Primary human umbilical venous endothelial cells (HUVECs) were purchased and cultured in Endothelial Basal Media-based media (EGM Bullet Kit; Lonza). Steady laminar flow was applied using culturing media as previously reported on monolayer cells for indicated times at 2 dynes/cm² for all experiments in this study. Sources of antibodies are listed in Method section in the Online Data Supplement.

Animal-Related Work
Animal-related works were approved by the University of Southern California Institutional Animal Care and Use Committee (PI: Y.-K.H.), Prox1-CreER T2 previously described. Prox1-CreER T2 mouse was a gift from Dr Taija Mäkinen (Uppsala University, Sweden). Cdh5(PAC)-CreER T2 mouse was generated and provided by Dr Ralf Adams (University of Münster, Germany). Flexed Klf2 mouse was kindly provided by Dr Kristin Hogquist (University of Minnesota), and floxed Klf4 mouse (B6.129S6-Klf4tm1Klk/Kmmh Klf4) was obtained from the Mutant Mouse Regional Resource Centers. All mutant mice were maintained outbred for the experiments.

Isolation of Mouse LECs
Mouse embryonic dermal and postnatal lymph node LECs were isolated from embryos harvested at embryonic day (E) 16.5 as described in Method section in the Online Data Supplement.

Statistical Analyses
Error bars in all graphs represent the mean ± SD, unless otherwise stated. Normally distributed continuous variables between the experimental and control groups were compared by 2-tailed t test. Statistical significance between the 2 groups was calculated as $P$ value using Microsoft Excel (Microsoft Office) and GraphPad PRISM6 (GraphPad Software, Inc). A $P$ value <0.05 is considered to be statistically significant.

Results

Steady Laminar Flow Selectively Activates Proliferation of Lymphatic Endothelial Cells
Studies have shown that steady laminar flow imposes an antiproliferative effect to blood vessel–derived endothelial cells through mechanisms involving p21Waf1 and p53. In comparison, the impact of steady laminar flow on developing lymphatic vessels has not been fully understood. We, therefore, investigated the effect of steady laminar flow on proliferation of LECs and its underlying molecular basis. As the precise flow shear force levels in developing lymphatic vessels in vivo are unknown, we first evaluated the effect of different doses...
of shear force (0.25, 0.5, 1, 2, and 5 dyne/cm²) on cultured human dermal LECs and then searched for the effective or preferable force level, also known as the set point, that triggers the classic in vitro endothelial shear stress responses. Specifically, we focused on the cellular (cell elongation and alignment along the flow direction), molecular (upregulation of the key shear response regulators, KLF2 and KLF4), and biochemical (activation of intracellular calcium influx) responses. These pilot studies revealed that elongation and alignment of LECs could be clearly triggered by steady laminar flow at ≥2 dyne/cm² (Online Figure IA). In comparison, upregulation of KLF2 and KLF4 was detectable from the lowest shear force examined (0.25 dyne/cm²) and progressively increased as the force level increased (Online Figure IB and IC). Moreover, activation of calcium uptake by the laminar flow was clearly detectable at the force levels of 2 and 5 dyne/cm² (Online Figure ID). On the basis of these studies, laminar flow force at 2 dyne/cm² was chosen for our experiments in this study. Under this shear force condition, laminar flow triggered human dermal LECs and BECs to become elongated and aligned to the direction of flow (Figure 1A). HUVECs did not show as much clear changes in their cell morphology even after 48 hours probably because the applied shear force (2 dyne/cm²) was much lower than the reported

Figure 1. Low-rate steady laminar flow selectively activates proliferation of lymphatic endothelial cells (LECs). A, Steady laminar flow (LF, 2 dyne/cm²) induced elongated cell morphology and alignment in LECs and blood vascular endothelial cells (BECs), with marginal changes in human umbilical venous endothelial cells (HUVECs). Bar=50 µm. B and C, Western blot assays showing upregulation of KLF2 (B) and KLF4 (C) in LECs, BECs, and HUVECs in response to steady laminar flow (2 dyne/cm²) for indicated time. D, Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) showing upregulation of eNOS in LECs, BECs, and HUVECs by steady laminar flow (2 dyne/cm²) for 8 and 16 h. E, Total cell number increase after static culturing or laminar flow exposure. Equal number of LECs, BECs, and HUVECs were plated and subjected or not to laminar flow (2 dyne/cm²). After 24 h, total cell number was counted, and the percent increase from the initial cell numbers was graphed. F, LECs, BECs, and HUVECs were cultured under the static or flow condition (2 dyne/cm²), and the relative percentage of cells in the S-phase was determined using flow cytometry. G, BrdU (5-bromo-2´-deoxyuridine)-incorporation assays showing the percent of BrdU-positive cells under the static or flow condition for 48 h. H, Western blot assays showing LEC-specific downregulation of p57 by laminar flow (2 dyne/cm²). I, ELISA-based cell death assays showing that laminar flow (2 dyne/cm²) commonly reduced cell death in all cell types. Error bars in the graphs represent the SD of the mean. Laminar flow was steadily applied at 2 dyne/cm² as previously described. Using 2-tailed t test, statistical significance was calculated between the static vs laminar flow conditions, and the significance level was expressed as follows: *P<0.05; **P<0.01; ***P<0.001.
set point for HUVECs. Nonetheless, all endothelial cells upregulated the established shear stress genes, KLF2, KLF4, and eNOS, in response to this low level of shear force (Figure 1B through 1D). PROX1 expression in LECs was not altered by the current flow condition (Online Figure II), suggesting that the LEC identity was not compromised by this shear force condition.\(^6\) Importantly, steady laminar flow significantly stimulated LEC proliferation, while expectantly suppressing the growth of BECs and HUVECs, as determined by 3 independent assays measuring total cell numbers, the relative number of cells in the S phase, and the relative percentage of BrdU (5-bromo-2’-deoxyuridine)-incorporated cells (Figure 1E through 1G). When different doses of shear force were applied from 0 to 5 dyne/cm\(^2\), a force level stronger than 1 dyne/cm\(^2\) was required to activate proliferation of cultured LECs, whereas 2 dyne/cm\(^2\) yielded the highest activation of LEC proliferation (Online Figure IIIA). Consistent with these data, steady laminar flow at 2 dyne/cm\(^2\) suppressed the expression of cyclin-dependent kinase inhibitor 1C (CDKN1C/p57) and, notably, this flow-induced p57 downregulation was only detectable in LECs but not in BECs and HUVECs (Figure 1H). We also verified these results using freshly isolated mouse lymph node LECs as another source of LECs: Consistent with human LECs, mouse lymph node LECs displayed enhanced cell proliferation, upregulation of KLF2 and KLF4, and down-regulation of p57 in response to steady laminar flow (Online Figures IIIIB and IVA, IV, and IVF). Notably, the laminar flow suppressed cell death of LECs, as well as of BECs and HUVECs, as previously reported\(^14,32\) (Figure 1H). Together, these results demonstrate that, although LECs, BECs, and HUVECs largely display comparable classic shear stress responses in response to steady laminar flow, only LECs exhibit the unique progrowth phenotypes.

**Molecular Players in the Laminar Flow–Induced Lymphatic Cell Proliferation**

We next set out to identify the molecular players in laminar flow–activated LEC proliferation and found that laminar flow at 2 dyne/cm\(^2\) commonly upregulated VEGF-A in LECs, BECs, and, as reported,\(^14,33\) in HUVECs\(^14,33\) (Figure 2A). Interestingly, however, the flow force induced the expression of VEGF-C and FGFR3 selectively in LECs, not in BECs and HUVECs (Figure 2B and 2C). A progressive upregulation of VEGF-A, VEGF-C, and FGFR3, as well as a gradual downregulation of p57, were detectable in LECs by the increasing laminar flow forces (Online Figure IE through IVH). This flow-induced regulation of VEGF-A, VEGF-C, and FGFR3 was also confirmed in freshly isolated mouse LECs (Online Figure IVB through IVD). Moreover, steady laminar flow increased phosphorylation of VEGF2R and FGFR3 in LECs without changing the total protein levels (Figure 2D and 2E). Notably, previous studies reported ligand-independent activation of VEGF2R\(^24\) and FGFR3\(^31\) by fluid shear stress to induce eNOS activation and arterial remodeling, respectively. We, therefore, investigated whether the laminar flow–induced phosphorylation of these VEGFRs was dependent on the presence of their ligands. Pretreatment of LECs with anti–VEGF-A antibody and soluble VEGF3 protein significantly reduced the flow-induced phosphorylation of both VEGF2R and FGFR3 (Figure 2F).

These data suggest that VEGF-A and VEGF-C, which are upregulated by laminar flow (Figure 2A and 2B), are necessary for the flow-induced phosphorylation of VEGF2R and FGFR3 in LECs. Moreover, small chemical-based inhibition of VEGF2R, VEGFR3, and FGFR3, but not of CXCR2 (chosen as a negative control), reduced the flow-activated LEC proliferation (Figure 2G). To exclude potential off-target effects of these chemical inhibitors, nonchemical blocking reagents were also used: An anti–VEGF-A neutralizing antibody and a soluble VEGFR3 protein, individually or together, profoundly inhibited the laminar flow–induced LEC proliferation (Figure 2H). Similarly, siRNA (small interfering RNA)-mediated knockdown of FGFR3 reduced the flow-activated LEC growth (Figure 2I), confirming a significant contribution of FGFR3 to the flow-activated LEC proliferation. Together, our studies demonstrate that low-rate steady laminar flow upregulates VEGF-A, VEGF-C, and FGFR3 in LECs, which together play important roles in the flow-activated proliferation of LECs.

**ORA11 Mediates the Laminar Flow–Induced Calcium Influx and KLF2/4 Regulation in LECs**

We next investigated a mechanism that may contribute to LEC-specific activation of cell proliferation by laminar flow. Because intracellular calcium increase is known to be an immediate response of endothelial cells on the onset of laminar flow,\(^7\) through which shear stress upregulates KLF2,\(^35\) we investigated whether the calcium signaling may play a role in orchestrating the laminar flow–induced LEC phenotypes. Time-lapse calcium imaging using a protein calcium reporter GCaMP3\(^36\) showed that low-rate steady laminar flow activated the calcium influx in LECs within the first minute of the flow onset (Online Figure VA and VB),\(^37\) consistent with previous studies.\(^37,38\) Moreover, this calcium influx was efficiently blocked by a low concentration of SKF-96365,\(^39\) a chemical inhibitor of the store-operated Ca\(^{2+}\) entry.\(^40\) In addition, SKF-96365 prevented the laminar flow–induced cell elongation in both LECs and BECs (Figure 3A). To be more specific, we next knocked down the expression of ORA11, a pore subunit of the highly selective calcium release–activated calcium channel on the plasma membrane.\(^16,19\) Importantly, siRNA-mediated ORA11 inhibition significantly blocked or delayed the laminar flow–induced cellular elongation of both cell types (Figure 3A). Moreover, ORA11 knockdown significantly reversed the flow-induced upregulation of KLF2 and KLF4 in LECs with much less impact to BECs (Figure 3B and 3C). Using freshly isolated mouse embryonic LECs, we also confirmed that SKF-96365 treatment reversed the flow-induced regulation of KLF2 and KLF4 (Online Figure IV through IVD). Together, our studies identified ORA11 as an essential calcium channel for the laminar flow–induced calcium uptake, cell morphology change, and regulation of KLF2 and KLF4 in LECs.

**ORA11 Plays a Key Role in the Laminar Flow–Induced LEC Proliferation**

In addition, ORA11 knockdown in LECs strongly reduced the flow-induced expression of VEGF-C and FGFR3, with a marginal suppression of VEGF-A only at 12 hours. (Figure 4A; Online Figure IV through IVL). In comparison, ORA11 knockdown in
Figure 2. Molecular players in laminar flow-induced lymphatic endothelial cell (LEC) proliferation. A–C, Steady laminar flow (LF) upregulated vascular endothelial growth factor (VEGF)-A (A) in LECs, blood vascular endothelial cells (BECs), and human umbilical venous endothelial cells (HUVECs), while activating the expression of VEGF-C (B) and FGFR3 (C) specifically in LECs, determined by ELISA (A and B) and Western blot assays (C). D and E, Laminar flow increased phosphorylation of VEGFR2 (D) and VEGFR3 (E) in LECs. LECs were exposed to laminar flow and then subjected to immunoprecipitation (IP) for VEGFR2 or VEGFR3, followed by immunoblotting (IB) for phosphorylated tyrosine (pY). As controls, LECs under the static condition were treated with VEGF-A (20 ng/mL) or VEGF-C (20 ng/mL) for 15 min before cell harvest. F, Western blot assays showing the ligand dependency of the flow-induced phosphorylation of VEGFR2 and VEGFR3 in LECs. LECs were preincubated for 10 min with a VEGF-A–neutralizing antibody (α-VEGF-A, 20 ng/mL) and soluble VEGFR3 receptor (sVEGFR3, 20 ng/mL), followed by static culturing or laminar flow for 24 h. Western blots were performed using antibodies against phospho-VEGFR2, whole VEGFR2, phospho-VEGFR3, whole VEGFR3, and β-actin. As controls, LECs under the static condition were treated with VEGF-A (20 ng/mL) or VEGF-C (20 ng/mL) for 15 min before cell harvest. G–I, BrdU (5-bromo-2´-deoxyuridine)-incorporation assays were performed to estimate the roles of VEGFRs and FGFR3 in the laminar flow–induced LEC proliferation. LECs were pretreated for 10 min with chemical inhibitors of FGFR3 (FGFRi, 50 µmol/L of PD 166866), VEGFR2 (Ki, 50 µmol/L of Ki8751), VEGFR3 (MAZ, 50 µmol/L of MAZ51), or CXCR2 (SB, 50 µmol/L of SB225002), followed by static culturing or laminar flow exposure for 24 h before BrdU assays. H, LECs were preincubated for 10 min with a VEGF-A–neutralizing antibody (α-VEGF-A, 20 ng/mL) and soluble VEGFR3 receptor (sVEGFR3, 20 ng/mL), followed by 24-h exposure to static culturing or laminar flow and then subjected to the BrdU assays. I, LECs were transfected with 2 different siRNAs (small interfering RNAs) for FGFR3 (siFGFR3-1, siFGFR3-2), or control siRNA (siCTR), overnight before static culturing or laminar flow for 24 h and then subjected to BrdU assay. Laminar flow was applied at 2 dyne/cm² as previously described. 14 Error bars indicate the SD of the mean. Using 2-tailed t test, statistical significance was calculated between the static vs laminar flow conditions (A and B) or between the control vs treated groups (G–I). Statistical values: **P<0.01; ***P<0.001.
BECs mainly reversed the VEGF-A upregulation without much impact on the expression of VEGF-C and FGFR3 (Figure 4B). When calcium influx in LECs was chemically blocked by Bapta-AM or SKF-96365, or genetically inhibited by ORAI1 knockdown, the laminar flow–induced p57 downregulation was significantly abolished (Figure 4C and 4D; Online Figure IVM). Similarly, inhibition of calcium influx in freshly isolated mouse LECs with SKF-96365 reversed the laminar flow–induced regulation of VEGF-A, VEGF-C, FGFR3, and p57 (Online Figure IVA through IVD). Consistent with these molecular phenotypes,
ORAI1 knockdown also prevented the laminar flow–activated LEC proliferation (Figure 4E). In comparison, however, ORAI1 knockdown did not reverse the flow-mediated suppression of BEC growth. Moreover, freshly isolated mouse embryonic LECs from wild-type versus Orai1 KO embryos displayed differential responses to laminar flow: genetic deletion of Orai1 largely abrogated the above-observed flow-induced regulation of KLF2, KLF4, VEGF-A, VEGF-C, FGFR3, and p57 (Online Figure VI), further verifying an essential role of ORAI1 in the flow-induced regulation of these genes. Therefore, our studies
show that ORAI1 is responsible for the shear stress–induced intracellular calcium influx in LECs and plays an essential role in the laminar flow–activated LEC proliferation.

**ORAI1 Deletion Reduces Lymphatic Vessel Density During Development**

We next investigated lymphatic phenotypes in Orai1 KO mice to validate our in vitro findings. Heterozygote Orai1 KO (Orai1+/−) mice16,17 were intercrossed with the Prox1-tdTomato lymphatic reporter mice,15 which allows a convenient visualization of lymphatic vessels because of the expression of the tdTomato reporter under the direction of the Prox1 promoter. From this genetic cross, we obtained Prox1-tdTomato/Orai1 KO embryos (Prox1-tdTomato; Orai1−/−) along with their control heterozygote embryos (Prox1-tdTomato; Orai1+/−) at E14.5. Indeed, Orai1 KO significantly inhibited embryonic lymphatic development with a notable reduction in lymphatic vessel area (Figure 5A through 5D; Online Figure VIIA). Moreover, Orai1 deletion also profoundly reduced lymphatic sprouting, which was documented in detail in a separate study.37 The reduced number of LECs was also detectable in the trachea of rarely surviving postnatal Orai1 KO mice, where the tracheal lymphatics were significantly smaller in diameter, compared with those in the wild-type littermates (Figure 5E through 5H; Online Figure VIIIB). To confirm the ORAI1–regulated gene expression phenotypes, we determined the expression levels of the flow-regulated molecular players in LECs and BECs freshly isolated from the back skins of control and Orai1 KO embryos. Indeed, genetic deletion of Orai1 caused a reduced expression of VEGF-A, VEGF-C, KLF2, KLF4, and FGFR3, as well as upregulation of p57, in Orai1 KO LECs (Online Figure VIII). These in vivo

![Figure 5. ORAI1 is required for embryonic lymphatic development. A–D. Developing dermal lymphatic vessels were visualized in Orai1 heterozygote (+/-) and homozygote (-/-) KO embryos (E14.5) using the Prox1-tdTomato lymphatic reporter:15 Compared to lymphatic vessels of Orai1 heterozygote embryos (A, C, n=5), those of homozygote KO embryos (B, D, n=4) displayed a significantly reduced number of LECs and impaired lymphatic vessel formation in the dorsolateral (A, B) and dorsal midline (C, D) areas. E–H. Lymphatic vessels in the trachea of rarely surviving 3-week-old Orai1 KO mouse (F, H, n=3) were abnormally thinner, compared to those of heterozygote littermates (E, G, n=6). Boxed areas in panels E and F are enlarged in panels G and H, respectively. Scale bars; 100 µm (A-D, G, H), 500 µm (E, F). Relative lymphatic vascular areas were shown in Online Figure VII A, B.](http://circres.ahajournals.org/content/143/1433)
expression signatures are consistent with the in vitro gene expression profiles seen in the ORAI1-depleted cultured LECs (Figures 3 and 4). In BECs, by comparison, Orai1 KO altered expression of VEGF-A and KLF4 only. Together, our studies suggest that Orai1 deletion prevents the flow-enhanced LEC proliferation and thus impairs lymphatic development because of dysregulation of the molecular players involved in the flow-induced LEC proliferation.

**KLF2 and KLF4 Directly Regulate the Molecular Players in Flow-Induced LEC Proliferation**

Previous studies have genetically placed KLF proteins upstream of the VEGF signaling in the shear-exposed vascular endothelial cells. We, therefore, aimed to establish the genetic relationships among these molecular players. Individual knockdown of KLF2 or KLF4 using 2 different siRNA complexes markedly altered the flow-induced regulation of VEGF-A, VEGF-C, FGFR3, and p57 (Figure 6A; Online Figure IXA through IXD). Consistent with this regulation, when mouse LECs freshly isolated from Klf2 KO embryos were subjected to the laminar flow, they displayed defective regulation of these genes (Online Figure IXE). Combined knockdown of KLF2 and KLF4 caused largely additive effects on the flow-induced regulation of the genes (Figure 6A; Online Figure IXF).

Conversely, adenoviral overexpression of KLF2 or KLF4 in LECs significantly upregulated VEGF-A, VEGF-C, and FGFR3, while downregulating p57 (Figure 6B and 6C). Chromatin immunoprecipitation assays were performed to investigate whether KLF2 and KLF4 proteins directly bind to the regulatory sequences of these genes. Many KLF proteins have been reported to bind to similar DNA sequences, known as the KLF consensus binding motif, presumably because of a high homology in their zinc finger domains. Because KLF2 and KLF4 proteins were previously reported to bind to the VEGF-A gene and regulate its expression, we focused our study on the other 3 genes, VEGF-C, FGFR3, and p57. To find the functional enhancer regions of the VEGF-C and FGFR3 genes, we took advantage of the epigenetic signatures reported by the Encyclopedia of DNA Elements (ENCODE) Consortium and identified several regions with the enhancer histone marks (high H3K4Me1 and H3K27Ac and low H3K4Me3) upstream of the VEGF-C and FGFR3 genes (Online Figure X). We then investigated whether KLF2 and KLF4 proteins are physically associated with these putative enhancer regions by chromatin immunoprecipitation assays. Indeed, both KLF2 and KLF4 were found to bind to the 210-kb upstream area of the VEGF-C gene, and these bindings were profoundly increased by laminar flow (Figure 6D; Online Figure X). Notably, neither protein bound to the 130-kb and 50-kb upstream regions. Similarly, when LECs were exposed to steady laminar flow, KLF2 and KLF4 were recruited to a putative enhancer region present 34-kb upstream of the FGFR3 coding sequence (Figure 6D; Online Figure X). In comparison, both KLF proteins occupy the proximal promoter of p57 under the static condition, and the laminar flow only slightly increased their binding to the region (Figure 6D).

We next investigated the cellular phenotypes of LECs after the individual or combined knockdown of KLF2 and KLF4. Single knockdown of each gene did not clearly alter the flow-induced activation of cell cycle progression of LECs or BECs (Figure 6E). However, simultaneous knockdown of both KLF proteins decreased the S-phase population in LECs but not in BECs. On the contrary, the flow-enhanced cell survival was reversed in both cell types by combined inhibition of KLF2 and KLF4 (Figure 6F). We next studied whether overexpression of KLF2 and KLF4 could reverse the dysregulation of VEGF-A, VEGF-C, FGFR3, and p57 in ORAI1-depleted LECs. To address this, ORAI1 was knocked down first and then KLF2 and KLF4 were adenovirally expressed in LECs. These LECs were then subjected to laminar flow or cultured under the static condition. As expected, laminar flow upregulated VEGF-A, VEGF-C, and FGFR3 and suppressed p57 expression (Online Figure XIA through XID), and this flow-induced gene regulation was abrogated by ORAI1 knockdown as seen above (Figures 3 and 4). Importantly, when KLF2 and KLF4 were ectopically expressed in the ORAI1-depleted, flow-exposed LECs, the flow-mediated regulations of VEGF-A, VEGF-C, FGFR3, and p57 were significantly restored. The expected expression levels of ORAI1, KLF2, and KLF4 were also verified (Online Figure XIE through XIG). Together, these data demonstrate that KLF2 and KLF4, which are downstream targets of ORAI1, directly regulate VEGF-A, VEGF-C, FGFR3, and p57 and play key roles in the laminar flow–induced activation of LEC proliferation.

**Abnormal Lymphatic Development by Tissue-Specific Deletion of Klf2 or Klf4**

We next studied the in vivo roles of KLF2 and KLF4 in developing lymphatic vessels through targeted deletion. Mice harboring the floxed Klf2 alleles (Klf2/208) were crossed with the Cdh5(PAC)-CreER72 mice (also known as VE-CadCreER72) expressing the tamoxifen-responsive Cre in endothelial cells. Resulting pregnant females were IP injected with tamoxifen at E11.5 and E13.5 to induce endothelium-specific deletion of Klf2 (Klf2 ECKO), and the embryos were harvested at E15.5 for vascular analyses. Indeed, the Klf2 ECKO embryos revealed defective lymphatic network formation, characterized with reduced lymphatic branching, irregular vessel thickness, and round-end sprouts, without similar defects in blood vessel development (Figure 7A through 7D; Online Figure XIIA). Image analyses revealed that the relative lymphatic area is significantly reduced in Klf2 ECKO embryos, compared with their control litter embryos (Online Figure VIIC).

Similarly, the Klf4 gene was deleted selectively in developing lymphatic vessels in embryos using the Proxl-CreERT2 driver line. Compared with those in the control litter embryos, the back skins of Klf4 ECKO embryos displayed profound defects in lymphatic branching morphogenesis and hierarchical network formation with a decreased lymphatic density (Figure 7E through 7G; Online Figure VIID). Cd31-positive blood vessels were unaffected as expected because of the Proxl-CreERT2–driven lymphatic deletion (Online Figure XIIB). Finally, we asked whether overexpression of KLF4 could increase lymphatic density using endothelium-specific Klf4 transgenic mouse (Klf4 ECI-Tg), where KLF4
is ectopically expressed under the direction of the Cdh5/VE-Cad promoter. Indeed, lymphatic vessels were significantly enlarged in the ear skins of the Cdh5-Klf4 mouse compared with those in their control litter mates (Figure 7K and 7L), indicating that ectopic KLF4 expression may increase lymphatic density presumably by activating LEC proliferation. Together, the outcome of these animal-based studies was consistent with our in vitro studies as described above and further demonstrated the important roles of KLF2 and KLF4 in lymphatic development.

Figure 6. KLF2 and KLF4 regulate molecular players in flow-induced LEC proliferation. A. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) data showing the effects of KLF2, KLF4 or combined knockdown on the expression of VEGF-A, VEGF-C, FGFR3 and p57 in LECs exposed to laminar flow. Knockdown was performed for 24 hr. prior to the onset of laminar flow (2 dyne/cm²) for 12 or 24 hr. Data were obtained by using 2 independent siRNA for KLF2 (siKLF2-1, siKLF2-2) and KLF4 (siKLF4-1, siKLF4-2), individually and together, and displayed here and Online Figure IX A-D. Expression levels of KLF2 and KLF4 after individual or combined knockdowns are shown in Online Figure IX F. B and C, qRT-PCR assays showing the expression of these genes in LECs that were infected for 48 hr. with adenovirus expressing KLF2 (Ade-KLF2; B), or KLF4 (Ade-KLF4; C). Ade-CTR, control adenovirus. D, ChiP assays showing association of KLF2 and KLF4 proteins to the regulatory regions of the VEGF-C, FGFR3, and p57 genes. LECs were exposed to laminar flow (2 dyne/cm²) for 6 hr. and ChiP assays were performed using normal IgG, anti-KLF2 or anti-KLF4 antibody and the primers against the indicated upstream sequences (UPS) regions. Detailed locations of these regions can be found in Online Figure X. E and F, Effects of the individual or combined knockdown of KLF2 and KLF4 on the percent of cells in the S phase (E) and on the cell death (F) of LECs. LECs were transfected with control siRNA (siCTR), KLF2 siRNA (siKLF2), and KLF4 siRNA (siKLF4) for 24 hr under the static condition, followed by flow cytometry-based measurement of the S-phase cell population (E) or by ELISA-based measurement of cell death (F). Statistical significance was calculated using 2-tailed t test between the siCTR vs siKLF2/siKLF4 groups (A, E, and F) or between the Ade-CTR vs Ade-KLF2/KLF4 groups (B and C). Statistical values: *P<0.05; **P<0.01; ***P<0.001.
Figure 7. Defective lymphatic development by targeted deletion of Klf2 or Klf4. A–D, Developing dermal lymphatic and blood vessels were stained using anti-Lyve1 (A and C) and anti-Cd31 (B and D) antibodies, respectively, in the control embryos (Klf2+/+; Cdh5(PAC)-CreER<sup>2</sup>) or endothelium-specific inducible Klf2 KO embryos (Klf2<sup>fl/fl</sup>; Cdh5(PAC)-CreER<sup>2</sup>). Tamoxifen-responsive Cre was activated by IP injection of tamoxifen (1.5 mg) into pregnant females at E11.5 and E13.5, and their embryos were harvested at E15.5 for vascular analyses. Relative vascular areas (%) are shown in Online Figure VII C. More than 6 embryos were analyzed per genotype. 

E–J, Dermal lymphatic and blood vessels were visualized in the control embryos (Klf4+/+; Prox1-CreER<sup>2</sup>; Prox1-tdTomato) or lymphatic-specific Klf4 knockout (KO) embryos (Klf4<sup>fl/fl</sup>; Prox1-CreER<sup>2</sup>; Prox1-tdTomato) at E15.5. Tamoxifen-responsive Cre was activated in the same way as for the Klf2 deletion described above. Lymphatic vessels were visualized using the tdTomato reporter. Enlarged images of the boxed regions are shown in the specified part figures. Relative vascular areas (%) are shown in Online Figure VII D. More than 6 embryos were analyzed per genotype. K and L, The ear lymphatics of wild-type (WT) or Cdh5-KLF4 transgenic adult mice<sup>46</sup> were stained with anti-Lyve1 antibody (K). Relative lymphatic vascular area (%) in wild-type and Cdh5-KLF4 transgenic mice (n>3) were quantitated (L). Error bars display the SD of the mean. Statistical values: *P<0.05. Bars=500 µm (A–N and S) and 100 µm (O–R).
Discussion

Because oxygen delivery is a major function of blood vessels, oxygen deficiency serves as a strong nonbiological stimulus for blood vessel growth. Similarly, lymphatic vessels function to drain tissue fluid, fluid flow generated by interstitial fluid drainage triggers lymphatic vessel expansion and remodeling.1 Because of their distinct physiological roles, blood and lymphatic vessels are expected to differentially respond to various patterns and forces of fluid flow. Studies have shown that laminar flow suppresses proliferation of blood vessel–derived cells through mechanisms involving p21Cip1 and p53.24–29 However, the effects of laminar flow on lymphatic development and function need to be better understood. In this study, we investigated whether and how low-rate steady laminar flow triggers lymphatic expansion and remodeling, particularly focusing on proliferation and survival of LECs. On the basis of the outcome of our study, we built a working model for a molecular mechanism underlying the laminar flow–induced LEC proliferation and survival (Online Figure XIII). In this model, the calcium release–activated calcium channel ORAI1 is an early and essential mediator of the laminar flow–induced LEC proliferation. In response to laminar flow, ORAI1 activates intracellular calcium influx and upregulates KLF2 and KLF4. These 2 KLF proteins together promote the cell cycle progression of LECs through upregulation of VEGF-A, VEGF-C, and FGFR3 and concurrent downregulation of the cell cycle inhibitor, p57. Secreted VEGF-A and VEGF-C may deliver their activities through autocrine and paracrine manners, especially onto those present immediately downstream. In comparison, upregulated FGFR3 may make the cells more sensitive to its limited ligands, such as FGF2. We, therefore, conclude that the interplay of ORAI1 and KLF2/4 proteins may direct the laminar flow–induced lymphatic expansion and remodeling by activating the proliferation and survival of LECs.

The precise shear force level in developing lymphatic networks is not known, and it will be technically challenging to determine the force level. Previously, several reports estimated shear levels in certain postnatal lymphatics. The shear force level in a collecting lymphatic vessel was found to be ≈0.64 dyne/cm² under the normal physiological condition.47 Shear levels for mouse tail capillaries48 and human skin capillaries 49 were ≈0.001 dyne/cm² and ≈0.003 dyne/cm², respectively. Compared with these quiescent mature postnatal lymphatics, developing lymphatics in the rapidly expanding embryos are likely to experience significantly elevated levels of shear force to deal with the overwhelming amount of embryonic tissue fluid. In fact, an elegant study by Planas-Paz et al2 demonstrated the presence of functional fluid drainage and flow as early as E11.5 by showing the inter-relationship among embryonic fluid accumulation, fluid pressure increase, stretching of LECs, VEGFR3 phosphorylation, proliferation of LECs, and functional fluid drainage. In comparison, an antiproliferative effect on BECs and HUVECs, which was previously seen by the higher, physiologically more relevant shear levels (10–30 dyne/cm²),24–30 was also detected by our low-rate shear force (2 dyne/cm²).

ORAI1 is a pore component of a calcium-selective ion channel on the plasma membrane and activates the store-operated Ca²⁺ entry process.40 Although it was initially discovered from studying defective Ca²⁺ entry in T cells that is associated with severe combined immune deficiency, ORAI1 has been found to be expressed in several different cell types, including arterial, venous, and capillary endothelial cells, and to play essential roles in various molecular and cellular responses toward physiological stimuli and pathologic insults.30,31 A recent study convincingly demonstrated that the intracellular calcium dynamics in cultured LECs depends on the magnitude of the shear stress and also that blockage of calcium release–activated calcium channels significantly reduced the calcium mobilization.38 Consistent with this study, we identified ORAI1 as an important calcium release–activated calcium channel responsible for the store-operated Ca²⁺ entry process activated by laminar flow in LECs. When ORAI1 was chemically or genetically inhibited in cultured LECs, laminar flow could no longer activate the classic shear stress responses. Moreover, the ORAI1 inhibition in LECs abolished the flow-induced regulation of VEGF-A, VEGF-C, FGFR3, and p57 and efficiently reversed the cell proliferation activated by laminar flow. Consistent with these cellular phenotypes, Orai1 KO embryos displayed significant defects in lymphatic development with reduced numbers of LECs. Our data suggest that KLF2 and KLF4 are downstream effectors of ORAI1 for the laminar flow–induced lymphatic phenotypes.

KLF2 and KLF4 transcription factors have been shown to act as critical regulators of endothelial homeostasis. Because of their closely related structures, functions, and expressions,53–55 they are believed to play shared and overlapping roles in vascular development and maintenance. Notably, numerous previous studies show that KLF2 and KLF4 negatively regulate vasculogenesis and angiogenesis.51 KLF2 inhibits VEGF-mediated angiogenesis,56 and laminar shear stress inhibits endothelial cell metabolism via KLF2-mediated repression of PFKFB3.57 KLF2 induces a gene expression pattern that can be seen in functionally quiescent endothelial cells58 and suppresses angiogenesis of liver endothelial cells through ERK1/2.59 KLF2 decreased the hypoxia-induced VEGF protein level in HUVECs.40 Loss of epigenetic KLF4-mediated transcriptional suppression was found to be crucial for upregulation of VEGF-A in breast cancer cells.41 However, their positive roles in vascular development have also been documented. KLF2 was shown to activate VEGF/VEGFR2 signaling and survival of HUVECs in response to laminar flow.14 This study, however, did not report any mitogenic activity of the flow-activated VEGF/VEGFR2 signaling in HUVECs.14 KLF2 cooperates with a ETS family protein ERG to activate Flk1/VEGFR2 expression during vascular development.60 KLF2 and KLF4 genetically interact to maintain endothelial integrity in mouse embryonic vasculogenesis.53 Considering these debated roles of KLF2 and KLF4 in vascular development, it is unexpected and unique to find that KLF2 and KLF4 concerted activation can activate the cell cycle progression of LECs as regulated by the expression of VEGF-C, FGFR3, and p57 in response to laminar flow. Especially, the 2 KLF proteins bind to the enhancer areas present as far as ≈210 kb and ≈34 kb from the coding sequences of VEGF-C and FGFR3, respectively. In comparison, the 2 KLF proteins bind to the proximal promoter of p57 to suppress its expression, suggesting that differential transcriptional programs regulate VEGF-C/FGFR3 versus p57 in response to the laminar flow. Together, KLF2 and KLF4 proteins may,
individually and concertedy, regulate vascular development and maintenance in different manners depending on the physiological and pathological settings of the cells.

One important question in our study was why the low-rate steady laminar flow delivers distinct cell proliferative effects to LECs and BECs. Although different types of endothelial cells may prefer different levels of flow rates, or set points, to initiate their remodeling program, our data showed that LECs, BECs, and HUVECs commonly displayed the molecular signatures of shear stress responses, most clearly the upregulation of KLF2, KLF4, and ENOS, in response to the low-rate laminar flow condition. In addition, higher rate laminar flows, which are comparable to blood flow, instead suppress proliferation of vascular endothelial cells. Therefore, we speculate that the flow-induced cell proliferation program is unique to LECs, but absent in BECs, and that distinct pathways may operate to trigger the seemingly opposing proliferative responses between LECs and BECs. It seems that the flow-responsive cell proliferation program in LECs uses the ORAI1-KLF pathway to regulate VEGF-A, VEGF-C, FGFR3, and p57 and to activate the cell cycle progression of LECs. Accordingly, ORAI1 inhibition abrogated the flow-induced cell cycle progression of LECs, while not affecting the flow-induced growth suppression of BECs.

In summary, we demonstrated that low-rate laminar flow activates proliferation of LECs. We also identified the important molecular mediators and players involved in the laminar flow–induced LEC proliferation. We propose that this phenotype and the underlying mechanism are unique to LECs, as proliferation of blood vessel–derived endothelial cells was not stimulated by the same condition, despite the comparable upregulation of KLF2 and KLF4. These findings are consistent with the function and physiology of lymphatic vessels, as LECs would experience an extensive shear stress during functional interstitial fluid drainage. Recent studies showed that the pore-forming subunit of a mechanosensitive ion channel is required for vascular development and plays a key role in integrating vascular architecture with physiological force. It will be interesting to define the flow-sensing mechanism in LECs and to study how the flow sensors activate ORAI1 and downstream genes.

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Disclosures
None.

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ORAI1 Activates Proliferation of Lymphatic Endothelial Cells in Response to Laminar Flow Through Krüppel-Like Factors 2 and 4
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SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHOD

Reagents

Sources of antibodies are as follows: anti-KLF2 (Santa Cruz Biotechnology, SC-18690), anti-KLF4 (R&D Systems, AF3640), anti-p57 (Santa Cruz Biotechnology, SC-56341), anti-FGFR3 (Santa Cruz Biotechnology, SC-123), anti-Prox1 (rabbit polyclonal antibody generated by the authors), anti-β-actin (Sigma-Aldrich, AC-15), anti-VEGF-A (R&D Systems, MAB293-SP), anti-VEGFR2 (R&D Systems, AF357), anti-pVEGFR2 (Cell Signaling, #2478, anti-phospho Tyr1175), anti-VEGFR3 (Santa Cruz Biotechnology, SC-321), anti-pVEGFR3 (Cell Applications, CY1115, anti-phospho-Tyr1230/1231), soluble VEGFR-3 (ReliaTech, RLT-S01-018-C050), anti-Cd31 (BD Bioscience, MEC13.3), anti-BrdU (Santa Cruz Biotechnology, SC-56258), anti-LYVE1 (AngioBio, 11-034), anti-podoplanin (Iowa Hybridoma Bank, 8.1.1) and anti-Tyr (Sigma-Aldrich, p5872). Sources of other reagents are as follows: Tamoxifen Free Base (MP Biomedicals), inhibitors for FGFR3 (PD 166866), VEGFR2 (Ki8751), VEGFR3 (MAZ51) and CXCR2 (SB225002) from Calbiochem. Tamoxifen (MP Biomedicals) was dissolved in Dimethyl sulfoxide (DMSO), mixed with sunflower seed oil, and intraperitoneally injected (final 1.5 mg) into pregnant females at E11.5 and 13.5. Adenoviruses expressing mouse Klf2 and human KLF4 were kindly provided Drs. Guillermo Garcia-Cardena (Harvard Medical School) and Chunming Liu (University of Kentucky College of Medicine), respectively.

Isolation of Mouse Embryonic Dermal LECs and Adult Lymph Node LECs

For isolation of embryonic dermal LECs, embryos were harvested at E16.5 and genotyped. Their back skins were then collected, chopped into pieces, and incubated with dispase and collagenase (1mg/ml, Hoffmann-La Roche, Ltd), collagenase II (50 U/mL, Worthington Biochemical, Lakewood, NJ) and DNase I (1,000 U/mL, New England Biolabs, Ipswich, MA) in phosphate buffered saline (PBS) at 37 °C for 1 hr. The enzymatically treated back skins were triturated through a needle (18.5G) to harvest dermal cell mixtures, which was filtered through a cell strainer, centrifuged, resuspended in EBM-based
culturing media, seeded on a culture dish, and incubated 37 °C. After 4 hr., the cells were washed twice with PBS, trypsinized, and incubated with an anti-LYVE1 rabbit antibody (Angiobio, 11-034) and an anti-Cd34 rat antibody (BD Pharmingen, 550537) at 4 °C for 1 hr. Mouse BECs were first collected using Dynabeads Sheep anti-Rat IgG and directly subjected to RNA isolation using Trizol reagents (Ambion). Next, mouse LECs were isolated from the remaining cell suspension using Dynabeads sheep anti-rabbit IgG and plated on a collagen pre-coated 6-well plate. For qRT-PCR, the cells were immediately subjected to RNA isolation without plating.

For isolation of adult lymph node LECs (LN-LECs), brachial, superficial cervical and axillary lymph nodes were harvested from Prox1-EGFP or Prox1-tdTomato mice and then incubated in DMEM with Penicillin/Streptomycin (2,000 U/mL) at 4 °C overnight. They were then incubated in a digestive enzyme solution (1 ml) in one well of 24-well plate, cut into small pieces with surgical scissors and incubated at 37 °C for 1 hour. The digestive enzyme solution is a mixture of dispase and collagenase (1 mg/ml, Hoffmann-La Roche, Ltd), collagenase II (50 U/mL, Worthington Biochemical, Lakewood, NJ) and DNase I (1,000 U/mL, New England Biolabs, Ipswich, MA) in PBS. The enzymatically treated lymph nodes were then triturated through an 18.5-gauge needle and the dissociated cells were filtered through a 40 μm-cell strainer. Subsequently, the cells were centrifuged and resuspended in media (EGM™ BulletKit™ with 20% FBS).

**Gene and Protein Expression**

Standard protocols were employed for quantitative real-time RT-PCR (qRT-PCR) and western blot assays. Nucleic acid sequences of primers, probes and siRNA duplexes will be available upon request. Plasmids and siRNA were transfected into primary endothelial cells using HMEC-L Nucleofector Kit (Lonza, VPB1003) and PBS 1, respectively. Sequences of siRNA are KLF2 (#1, CCAAGAGUUCGCAUCUGAATT; #2, AGACCUACACCAAGAGUUCUU), KLF4 (#1, GGACUUUAUUCUCUCAAUdTdT; #2, CCUUACACAUGAAGAGGCAdTdT), FGFR3 (#1, GGACUUUAUUCUCUCAAUdTdT; #2, CCUUACACAUGAAGAGGCAdTdT),
CACGACCUGUACUGUACUAdTdT; #2, UGCACAACCUCGUACUAdTdT), ORAI1 (#1, UCACUGGUUAGCCAUAAGA; #2, GCUCACUGGUUAGCCAUAAdTdT). Protein concentration of VEGF-A and VEGF-C was determined using Human VEGF Standard ELISA Development Kit (Peprotech) and Human VEGF-C ELISA Kit (AbCam). Whole-mount and tissue section immunofluorescent staining of mouse tissues were performed as previously described 2.

Cell Proliferation and Death Assays

5-Bromo-2′-deoxyuridine (BrdU, Sigma-Aldrich Co.)-based cell proliferation assay was performed as previously described 3. Briefly, BrdU (final 100 μM) was added to the culture media 2 hr. prior to harvest. Cells were detached using Trypsin-EDTA, fixed in ethanol (70%) for 4 hr. at - 20°C and subjected to the standard BrdU assay. Cell death assay was performed using Cell Death Detection ELISA (Roche). Source of chemical inhibitors for VEGFRs, FGFRs and CXCR2 were previously described 4. SKF 96365 hydrochloride and Bapta-AM were purchased from Tocris Bioscience and Sigma-Aldrich, respectively.

Chromatin Immunoprecipitation (ChIP) assays

ChIP assay was performed as previously described 4. Sequences of the primers used for the following ChIP assays are as follows: VEGF-C 210-kb UPS (CCCTCTCCAACCTGGATTCA/ATCGGACATTTTGCAAGACC), VEGF-C 130-kb UPS (GACCTGAAAGGACCTGTGGC/TGGCTAACAGGAAACCCTCC), VEGF-C 50-kb UPS (ATTGCACAAGGCCAAAAATC/GCCTACTGTGCTTGCATTGA), FGFR3 34-kb UPS (GGGACTTCCCACACTCGTAA/GCCTCAGTGTGCTTGCATTGA), p57 (CAGGCTCACCTGAGATAGGG/CAGGCCAGACCAAAAGAGAC).

Confocal laser-scanning fluorescence microscopy for calcium imaging
Human primary LECs were transiently transfected on μ-Slides (Ibidi GmbH, Germany) with a GCamP3-expressing vector for 24 hr. and then exposed to steady laminar flow (2 dyne/cm²) generated with a syringe pump using either culturing media with or without SKF-96365 (10 μM), or PBS lacking Ca²⁺ and Mg²⁺. Calcium signals were captured using a Leica TCS SP5 AOTF MP confocal microscope system (Leica Microsystems, Germany). Florescent images were collected in time series (xyt, 1 s per frame) with the Leica LAS AF imaging software and fluorescence intensity was determined by the Leica LAS lite.

SUPPLEMENTAL INFORMATION REFERENCES


ONLINE FIGURE LEGENDS

Online Figure I. Cellular, molecular and biochemical effects of laminar flow at various shear force levels on cultured LECs. Primary human LECs were subjected to steady laminar flow at 0, 0.25, 0.5, 1, 2, or 5 dyne/cm². (A) Cellular morphology change was imaged at 0 (static), 6, 12 and 24 hr. under the flow. Scale bars: 50 μm. (B-C) qRT-PCR assays showing the expression levels of KLF2 (B) and KLF4 (C) in LECs that were subjected to laminar flow at various forces for 24 hr. (D) Intracellular calcium influx was measured in LECs that were plated on μ-Slides (Ibidi, GmbH), loaded with Fluo-4 and subjected to laminar flow at the indicated force levels. (E-H) qRT-PCR assays showing the expression levels of VEGF-A (E), VEGF-C (F), FGFR3 (G), and p57 (H) in LECs after exposure to the indicated levels of laminar flow for 24 hr. All qRT-PCR expression levels were normalized against the level of β-actin. Error bars: the standard deviations (SD) of the mean. Statistical values: *, p < 0.05; **, p < 0.01; ***, p < 0.001.

Online Figure II. Prox1 expression was not altered in LECs by steady laminar flow at 2 dyne/cm². Human primary LECs were subjected to steady laminar flow at 2 dyne/cm² for the indicated time. PROX1 mRNA levels were quantified using qRT-PCR and normalized against the level of β-actin. Error bars: the standard deviations (SD) of the mean.

Online Figure III. Activation of proliferation of human and mouse LECs by laminar flow. (A) Human primary LECs were exposed to steady laminar flow at 0, 0.25, 0.5, 1, 2, or 5 dyne/cm² for 24 hr. and cell proliferation was measured by BrdU-incorporation assay. Percent BrdU-positive cells are shown against the static (0 dyne/cm²) culture. (B) LECs freshly isolated from lymph nodes of adult mice were subjected or not to steady laminar flow (LF, 5 dyne/cm²) for 24 hr. and the relative amount of cells in the S-phase was determined by flow cytometry. Error bars: the standard deviations (SD) of the mean. Statistical values: *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Online Figure IV. ORAI1 is essential for the laminar flow-induced gene regulation in LECs. (A-F)
Mouse dermal LECs were freshly isolated from the back skin of wild type embryos (E16.5) and cultured. After 2 days, culture media were changed with fresh media containing PBS (CTR) or SKF-96365 (SKF, 10 μM) before laminar flow (LF, 2 dyne/cm²) was applied or not for 24 hr. Subsequently, qRT-PCR was performed to determine the expression levels of p57 (A), VEGF-A (B), VEGF-C (C), FGFR3 (D), KLF2 (E), AND KLF4 (F). (G-M) Human primary dermal LECs were transfected overnight with control siRNA (siCTR) or a second set of ORAI1 siRNA (siORAI1-2), which is different from the first set (siORAI1-1) used for Figs.3 & 4. Cells were then subjected to static culturing or laminar flow (LF, 2 dyne/cm²) for 24 hr. before qRT-PCR analyses. Relative expression levels of ORAI1 (G), KLF2 (H), KLF4 (I) VEGF-A (J), VEGF-C (K), FGFR3 (L), and p57 (M) were normalized again β-actin and expressed in the graphs. Error bars: the standard deviations (SD) of the mean. Statistical values: **, p < 0.01; ***, p < 0.001.

Online Figure V. Laminar flow-activated calcium influx in LECs is inhibited by SKF-96365. Time-lapse images (A) and relative signal intensity graph (B) showing the intracellular calcium mobilization in LECs upon the onset of steady laminar flow (LF) at 2 dyne/cm². Calcium influx was detected by the calcium reporter protein, GCaMP3. LECs were transfected with a GCamP3-expressing vector for 24 hr. and then exposed to laminar flow using culture media (CTR), culture media containing SKF-96365 (SKF, 10 μM), or PBS without (w/o) Ca²⁺ and Mg²⁺. Error bars: the standard deviations (SD) of the mean. Statistical values: *, p < 0.05; **, p < 0.01; ***, p < 0.001.

Online Figure VI. Orai1 is required for the regulation of the laminar flow-responsive genes in mouse LECs. Mouse dermal LECs were freshly isolated from wild type (WT) or Orai1 KO mutant embryos and subjected or not to laminar flow (LF, 2 dyne/cm²) for 24 hr. qRT-PCR assays were performed to determine the expression levels of Klf2, Klf4, Vegf-A, Vegf-C, Fgr3, and p57. Error bars: the standard deviations (SD) of the mean. Statistical values: *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Online Figure VII. Quantitation of the lymphatic density in the control and mutant embryos.
Relative lymphatic area was determined in the wild type and various mutant embryos. (A,B) Relative lymphatic vessel area in the embryonic back skins (A) and young adult trachea (B) of Orai1 heterozygote (Prox1-tdTomato; Orai1 Het) and KO (Prox1-tdTomato; Orai1 KO) animals shown in Fig.5. (C,D) Relative lymphatic vessel area in the embryonic back skins of wild type and KO embryos lacking Klf2 (C) or Klf4 (D), as shown in Fig.7 (A,C) and (E,H), respectively.

Online Figure VIII. Expression of laminar flow-responsive genes is dysregulated in the isolated mouse LECs from Orai1 KO embryo. qRT-PCR analyses showing the mRNA level of Vegf-A (A), Vegf-C (B), Klf2 (C), Klf4 (C), Fgfr3 (E) and p57 (F) in primary embryonic LECs (mLECs) and BECs (mBECs) that were freshly isolated using anti-Lyve1 and anti-Cd34 antibodies, respectively, from Orai1 wild type embryos (WT, n=4) or littermate KO embryos (KO, n=4) (E16.5). Expression of each gene was measured and normalized against that of β-actin. Each data point was derived from one embryo. Results were expressed as mean and the standard error of the mean (SEM). Statistical values: *, p < 0.05; **, p < 0.01; ***, p < 0.001; n.s., not significant.

Online Figure IX. KLF2 and KLF4 is necessary for the regulation of the laminar flow-responsive genes in LECs. (A-D) qRT-PCR data showing the effects of knockdown of KLF2 (A,B) or KLF4 (C,D) on the expression of KLF2, KLF4, VEGF-A, VEGF-C, FGFR3 and p57 in LECs exposed to laminar flow. Knockdown was performed with two different siRNAs for KLF2 (siKLF2-1 (A) and siKLF2-2 (B)), or two different siRNAs for KLF4 (siKLF4-1 (C) or siKLF4-2 (D)), along with control siRNA (siCTR) for 24hr. prior to the onset of laminar flow (2 dyne/cm²) for 24 hr. (E) Mouse dermal LECs were freshly isolated from wild type (WT) and Klf2 KO mutant embryos and then subjected or not to laminar flow (LF, 2 dyne/cm²) for 24 hr. qRT-PCR assays were performed to determine the expression levels of VEGF-A, VEGA-C, FGFR3 and p57. (F) qRT-PCR assays showing the knockdown efficiency of KLF2 and KLF4.
in the experiments of Fig.6A. Error bars: the standard deviations (SD) of the mean. Statistical values: *, p < 0.05; **, p < 0.01; ***, p < 0.001.

Online Figure X. Relative locations of the putative enhancers of VEGF-C and FGFR3. Red boxes mark the putative upstream sequences (UPS) that may function as enhancers in VEGF-C and FGFR3. Binding of KLF2 and KLF4 to these regions were determined by ChIP assays shown in Fig.6D. The transcriptional start site, the direction of the coding sequences (CDS) as well as the ENCODE histone tracks (H3K4Me1, H3K27Ac and H3K4Me3) are also shown.

Online Figure XI. Overexpression KLF2 and KLF4 rescues the compromised flow-induced gene expression pattern caused by Orai1 knockdown. LECs were transfected with control siRNA (siCTR) or ORAI1 siRNA (siORAI1-1) overnight, infected simultaneously with control adenovirus (C) or with Ade-KLF2/ Ade-KLF4 (2/4), and followed by static culturing or laminar flow (2 dyne/cm²) for 24 hr. before qRT-PCR analyses. Relative expression levels of VEGF-A (A), VEGF-C (B), FGFR3 (C), p57 (D) ORAI1 (E), KLF2 (F), and KLF4 (G) were normalized against β-actin and expressed in the graphs. Error bars: the standard deviations (SD) of the mean. Statistical values: **, p < 0.01; ***, p < 0.001, n.s., not significant.

Online Figure XII. Low-power images of the back skin of wild type, Klf2ECKO, and Klf4ECKO embryos. (A) The images of developing dermal lymphatic and blood vessels in the control embryos (Klf2+/+; Cdh5(PAC)-CreER²) or endothelial-specific inducible Klf2 KO embryos (Klf2flo/flo; Cdh5(PAC)-CreER²) at E15.5. Lymphatic and blood vessels were stained with anti-Lyve1 and anti-Cd31 antibodies, respectively. (B) Dermal lymphatic and blood vessels were visualized in the control embryos (Klf4+/+; Prox1-CreER²; Prox1-tdTomato) or lymphatic-specific Klf4 KO embryos (Klf4flo/flo; Prox1-CreER²; Prox1-tdTomato) at E15.5. Lymphatic vessels were visualized using the tdTomato reporter and blood vessels were stained with anti-Cd31 antibody. Scale bars: 100 μm.
Online Figure XIII. Working model for the laminar flow-induced LEC proliferation. Low-rate steady laminar flow activates ORAI1, which increases the intracellular calcium influx and upregulates KLF2 and KLF4 in LECs. Increased KLF2 and KLF4 proteins individually and/or concertedly stimulate the gene expression of VEGF-A, VEGF-C and FGFR3, and suppress the p57 expression, promoting LEC proliferation and survival.
A

<table>
<thead>
<tr>
<th>Force of laminar flow (dyne/cm²)</th>
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<tr>
<td>0.25</td>
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<tr>
<td>Static</td>
</tr>
<tr>
<td>LF 6hr</td>
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<tr>
<td>LF 12hr</td>
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<td>LF 24hr</td>
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</tbody>
</table>

B

**KLF2**

![Graph showing relative mRNA level of KLF2](image)

C

**KLF4**

![Graph showing relative mRNA level of KLF4](image)

**Online Figure I (Part 1)**
Online Figure I (Part2)
Online Figure II
Online Figure III
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Online Figure IX (Part 1)
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Online Figure IX (Part 3)
Online Figure IX (Part 5)
Online Figure IX (Part6)
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Online Figure XI (Part 2)
Online Figure XII
Lymphatic Endothelial Cells

Online Figure XIII