# SUPPLEMENTAL MATERIAL

## Supplemental Methods

### Cell Culture and Reagents

Human primary lymphatic endothelial cells (LECs) were isolated from neonatal foreskins with an approval (PI: Hong) by the Institutional Review Board, University of Southern California, and cultured in a complete media as previously described [1, 2]. LECs less than 8-population doubling (passage) were used for all experiments in this study. 9-cis retinoic acid (RA), all-trans RA, TTNPB and AM580 were purchased from Sigma-Aldrich (St. Louis, MO) and soluble FGFR3 was purchased from R & D Systems (Minneapolis, MN). Control and Prox1 adenovirus were previously described[1]. Sources of antibodies for western blot analyses were anti-p27 (Santa Cruz Biotechnology, sc-528), anti-p57 (Santa Cruz Biotechnology, KP39), anti-Prox1 (rabbit polyclonal antibody generated by the authors), anti-β-actin (Sigma-Aldrich, AC-15), anti-aurora kinase A (Cell Signaling, 1G4), anti-aurora kinase B (Abcam, Cambridge, MA; ab2254), anti-phospho-AKT (Cell Signaling, 9271), anti-phospho- $p27^{Kip1}$ (Serine 10)(Invitrogen) and anti-AKT (Cell Signaling, 9272). Sources of antibodies used for immunohistochemistry were anti-CD31 (BD Bioscience, , San Jose, CA; MEC13.3), anti-LYVE-1 (Abcam, ab14917) and anti-podoplanin (Hybridoma bank, 8.1.1).

### WST-1-Based Proliferation Assay

Endothelial cell proliferation assay was performed using a Premixed WST-1 Cell Proliferation Assay kit (TaKaRa, Japan), following the manufacturer's instruction. In brief, human primary LECs were seeded at a concentration of $2 \times 10^4$ cells per well in 24-well plates and cultured overnight. The media were then replaced with a low serum (1% FBS) media containing vehicle (ethanol, 0.1%) alone, or vehicle with 1 μM RA derivatives. In order to inhibit the PI3K/Akt signaling, LY294002 (Sigma-Aldrich) was pretreated for 30 minutes and then 9-cisRA (1 μM)

was added to the media. Cells were allowed to grow for 48 hours and then the cell proliferation reagent WST-1 was added to each well and incubated for additional 4 hours. Optical absorbance was subsequently measured at 450 nm using a microplate reader (Hidex Chameleon V, Finland) and the proliferated cell number was estimated based on the standard curve that was simultaneously prepared. The receptor inhibition studies were performed by incubating LECs with vehicle or 9-cisRA in the presence of DMSO or inhibitors for FGF receptor (PD173074)[3], VEGFR-2 (Ki8751)[4] and VEGFR-3 (MAZ51)[5], which were purchased from CalBiochem (Gibbstown, NJ).

**Migration Assay**

Cell migration assay was performed using HTS Fluoroblok™ Multiwell Insert System (BD Falcon, Franklin Lakes, NJ). Bottom side of the Fluoroblok inserts was coated with collagen (50 µg/mL) solution containing 0.01% delipidized BSA (Sigma-Aldrich) for 30 minutes. 200 µL basal media containing 0.2% delipidized BSA and 5 x $10^4$ cells of human primary LECs were added into the upper chamber. 500 µL of basal media containing 0.2% delipidized BSA was added in the lower chamber along with either vehicle (ethanol), 9-cisRA (1 µM), FGFR inhibitor (PD173074, 50 nM) or 9-cisRA plus FGFR inhibitor, PI3K inhibitor (LY294002 ,100nM) or 9-cisRA plus LY294002. After 3-hour incubation at 37°C, the migrated cells to the bottom side of the insert were stained with 2 µM Calcein AM (Invitrogen) at 37°C for 10 minutes. The Fluoroblok™ inserts were then washed in PBS and fixed with 4% paraformaldehyde (PFA). Fluorescence intensity was measured using a fluorometer (Hidex Chameleon V) and images were captured using a Zeiss fluorescent microscopy.

**Endothelial Cord Formation and Scratch Assays**

Human dermal LECs were pre-treated with vehicle (ethanol) or 9-cisRA (1 µM) in a low serum media (1% FBS) for 24 hours. Separately, 200 µL of growth factor-depleted Matrigel (BD

Bioscience) were placed into a pre-chilled 12-well plate and solidified for 30 minutes at 37°C. Pre-treated LECs (5 x $10^4$) cells were overlaid on the matrigel in a low serum (1% FBS) media containing vehicle or 9-cisRA (1 μM). Cord formation was observed from 6 hours and the representative images were randomly taken at 24 hours, and total length of formed cord therein was measured using the NIH Image J program. For scratch assay, primary human dermal LECs (4 x $10^5$) were seeded in 60-mm dishes and pre-treated with vehicle or 9-cis-RA (1 μM) in a low (1%) serum media for 24 hours. Confluent monolayer was scratched using a 1000-μL pipette tip and incubated for additional 24 hours. The migrated cell images were captured and analyzed to determine the average remaining wounded area using the NIH Image J program.

**Immunofluorescence Analyses**

Immunofluorescence analyses were performed as previously described [1, 2]. Cells attached to collagen-coated cover slips were washed in PBS and fixed with 4% PFA for 20 minutes. The cells were incubated in a blocking buffer (0.5% bovine serum albumin in PBS) for 1 hour at room temperature, followed by overnight incubation with anti-Prox1 (ReliaTech, Germany), anti-p27 or anti-p57 (Santa Cruz Biotechnology) antibodies. Cells were then rinsed with PBS, incubated with fluorescence-conjugated secondary antibodies (Invitrogen), rinsed three times with PBS and mounted on glass slides using Vectashield medium (Vector Laboratories, Burlingame, CA) containing DAPI (4,6-diamidino-2-phenylindole). Images were obtained from a fluorescence microscope (Zeiss, Germany).

**Chromatin Immunoprecipitation (ChIP), Gene Expression, Luciferase Assays**

ChIP assays were performed following a published protocol [6]. Briefly, genomic DNA of LECs were fragmented by sonication using a Misonix model XL2000 sonicator and the pre-cleared extracts were immunoprecipitated with a rabbit anti-Prox1 (generated by the authors) or a normal rabbit IgG (Sigma Aldrich) antibody at 4ºC overnight. DNA was isolated from precipitated

complexes, de-crosslinked and analyzed using PCR primers for human $p57^{Kip2}$ promoter (primer set #1: ATCATGGCTTTTGGTTCCAC/ CAGGGAAGCGAGATCTGAAG; primer set #2: CAGGCTCACCTGAGATAGGG/ CAGGCCAGACCAAAAGAGAC), human aurora kinase A (CTCGCCAGGTAAACAGAAGC/ CCTTAGTTCGCCTCTGCATC), human aurora kinase B (CCTGAGGACAGGAGTTCGAG/ AATGGTGCTATCTGGGCTTG), human survivin (CCCAGCTCCAGAAGTGACTC/ GGGCCACTACCGTGATAAGA), human Cyclin B1 (GGCTTGAATGCAAGAAGAGG/ TCAAAACACACCAGCAGCTC) and human Cyclin B2 (ACCCCAACACACCAGAAGAG/ TCTCATTGGCCAACACAAGA). Quantitative real-time RT-PCR (qRT-PCR) was performed by using TaqMan EZ RT-PCR Core Reagent (Applied Biosystems). Each reaction was multiplexed for both target gene and the internal control β-actin for normalization. Conventional RT-PCR was performed using Superscript II (Invitrogen) and Taq polymerase (Finnzymes, Finland). Primer sequences will be provided upon request. All luciferase assays were carried out in HEK293T in triplicates at 48-hours after transfection and the luciferase activity was measured using the Bright-Glo reagent (Promega) and normalized by total protein amount. Luciferase reporter constructs harboring the promoters of $p57^{Kip2}$ were generous gifts from Dr. Sam Okret [7] (Huddinge University Hospital, Sweden). Microarray assays were performed by Los Angeles Childrens Hospital Genome Core on Human Genome U133 Plus 2.0 Array from Affymetrix using total RNAs isolated from human primary LECs transfected with siRNA for PROX1 (GCAAAGAUGUUGAUCCUUCdTdT) and/or RXRα (GACCCUGUCACCAACAUUUGC) for 48 hours.

**Corneal Micropocket Assay**

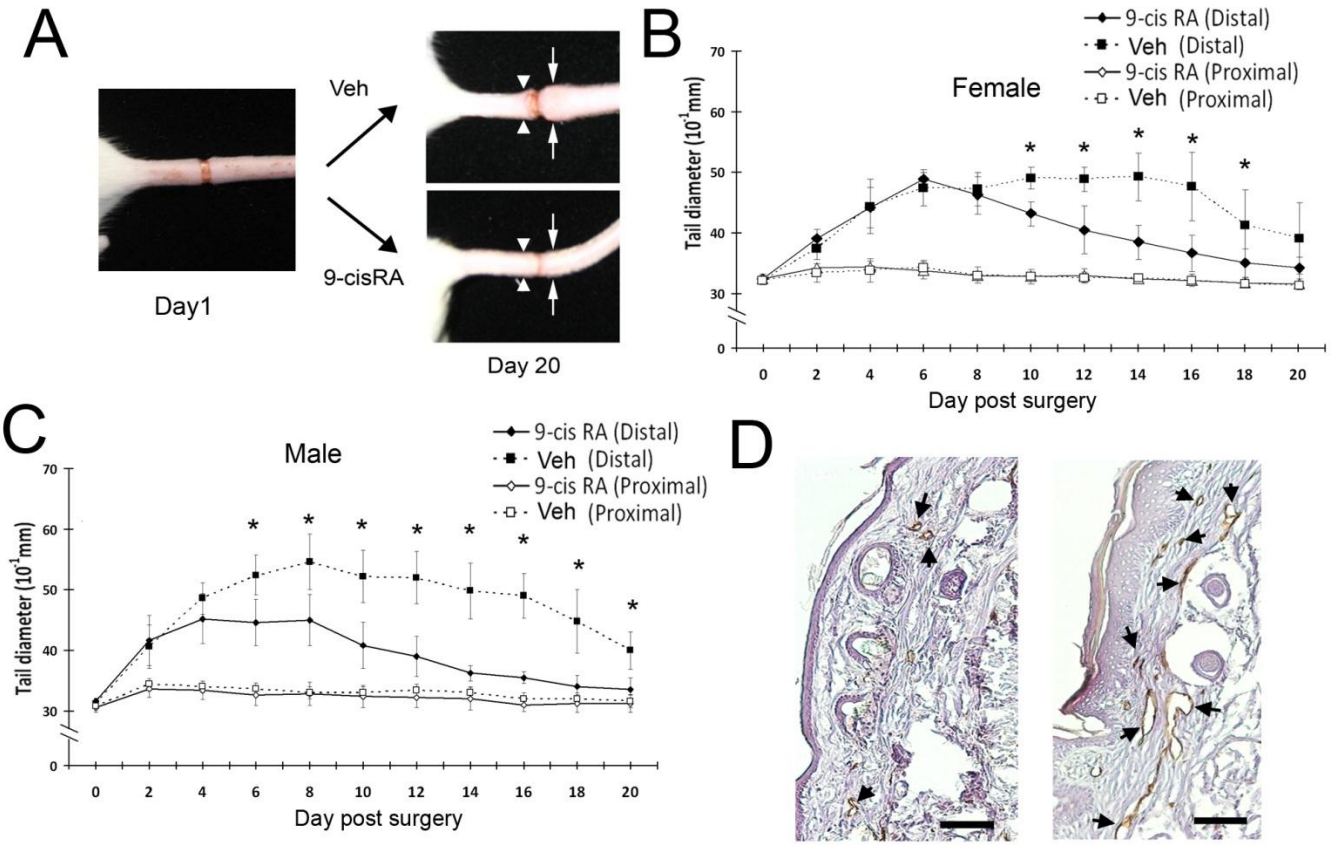
Six to 8-week-old male BALB/c mice (Jackson Laboratory, Bar Harbor, ME) were used for the experiments. All mice were treated according to ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and all protocols were approved by the Animal Care and Use Committee, University of California, Berkeley. Mice were anesthetized using a mixture of

ketamine, xylazine, and acepromazine (50 mg, 10 mg, and 1 mg/kg body weight, respectively) for each surgical procedure. The mouse corneal micropocket assay was performed as described previously [8]. Briefly, corneal micropockets were created 1.0 mm apart from the limbal vascular arcade using a modified von Graefe Knife. A slow-release pellet of uniformed size (0.3 mm) was implanted into each pocket. The pellet was made of sucralfate (Sigma-Aldrich) and hydron polymer (Sigma-Aldrich) containing either vehicle (ethanol) alone (10 mice) or vehicle with 9-cisRA or all-trans RA (0.3 mg each, 13 animals per group). Antibiotic ointment (tetracycline) was applied to the eye after pellet implantation and the pellet was left in place for 14 days. Freshly excised corneas were fixed in acetone for immunofluorescent staining. Nonspecific staining was blocked with anti-Fc CD16/CD32 antibody (BD Biosciences). The samples were stained overnight with purified rabbit anti-mouse LYVE-1 antibody (Abcam), which was visualized by a rhodamine-conjugated donkey anti-rabbit secondary antibody. Samples were covered with Vector Shield mounting medium (Vector Laboratories) and examined by an epifluorescence deconvolution microscope. Vascular structures stained LYVE-1+ were defined as lymphatic vessels. Lymphatic vessels were graded and analyzed using the NIH Image J software as described previously [9 10]. Basically, the lymphatic area was normalized to the total corneal area to obtain a percentage coverage score for each sample. The lymphatic area refers to the fraction of the corneal area in which lymphatic vessels are present and the total corneal area was measured by outlining the innermost lymphatic vessels of the limbal arcade. The statistical significance was evaluated using Mann-Whitney test with GraphPad Prism® software (GraphPad Software, Inc., La Jolla, CA).

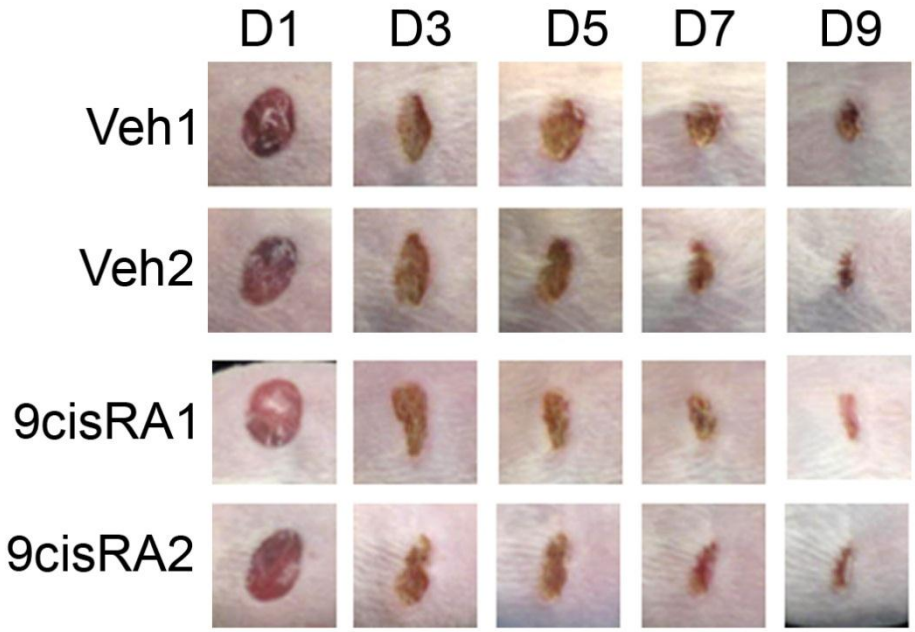
**Mouse Trachea and Matrigel Plug Assays**

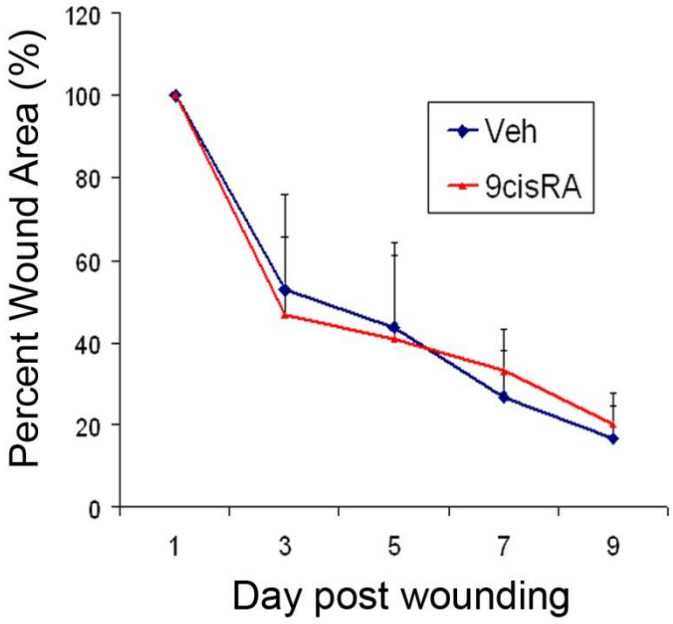
For mouse trachea assay, vehicle (ethanol) (15 µL) alone or vehicle containing 9-cisRA (1 mM) was injected daily into each nostril of normal BALB/c mice (five mice per group) under anesthesia and, after 5 days, the trachea was harvested and stained for CD31 and LYVE-1. For

matrigel plug assay, 250 µL of growth factor-depleted matrigel (BD Bioscience) premixed with vehicle alone or vehicle containing 9-cisRA (10 µM) was subcutaneously injected into normal BALB/c (five mice per group) and, after a week, harvested for immunohistochemical analyses against podoplanin. In a separate experiment, 250 µL of growth factor-depleted matrigel was subcutaneously injected into the lymphatic-specific fluorescent transgenic mice [11] and, after two weeks, the implants were recovered to directly visualize the newly formed lymphatic networks.

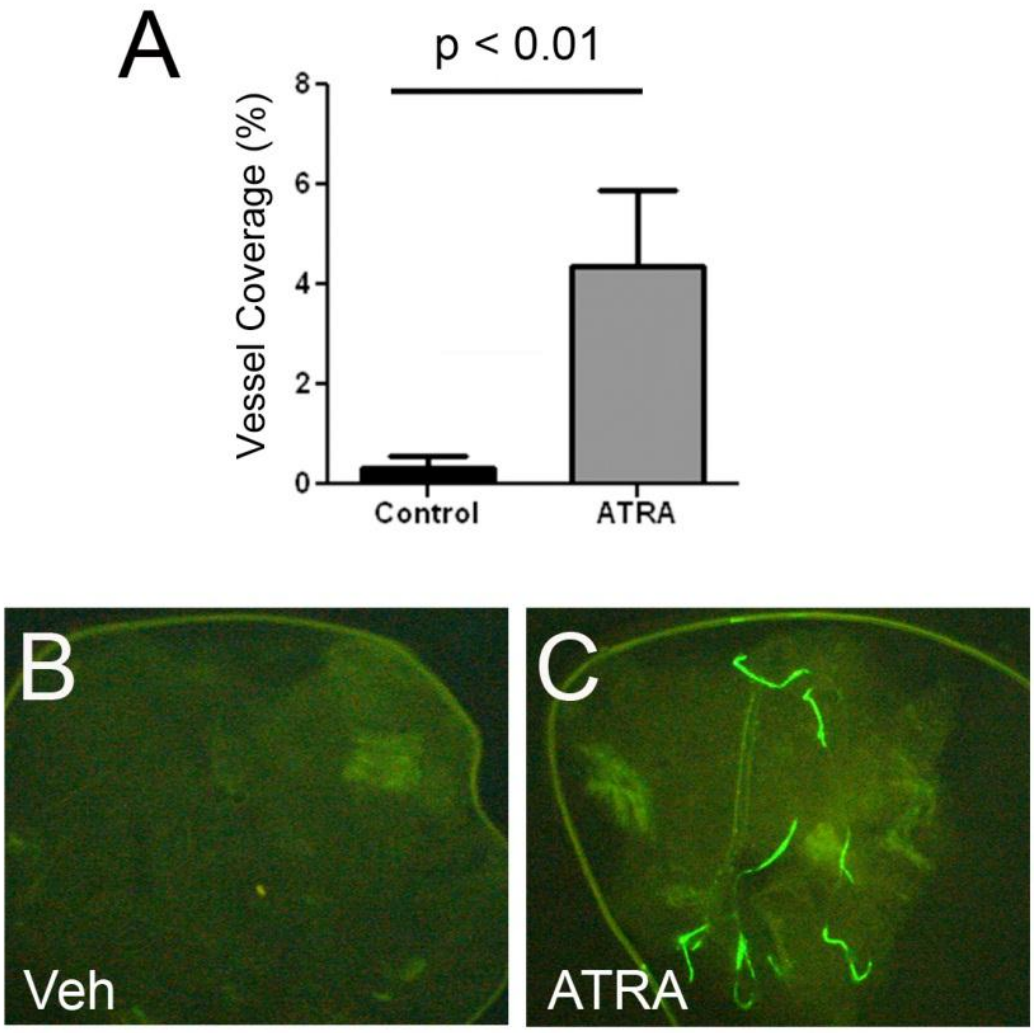



Supplemental Figure 1

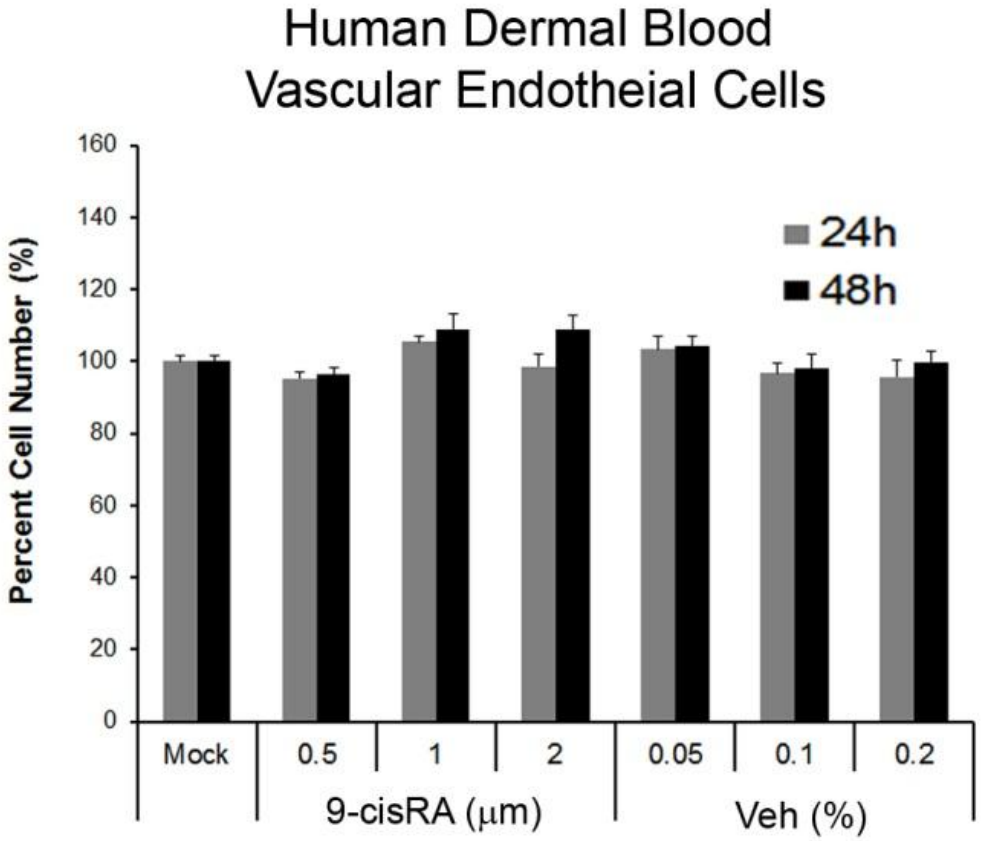


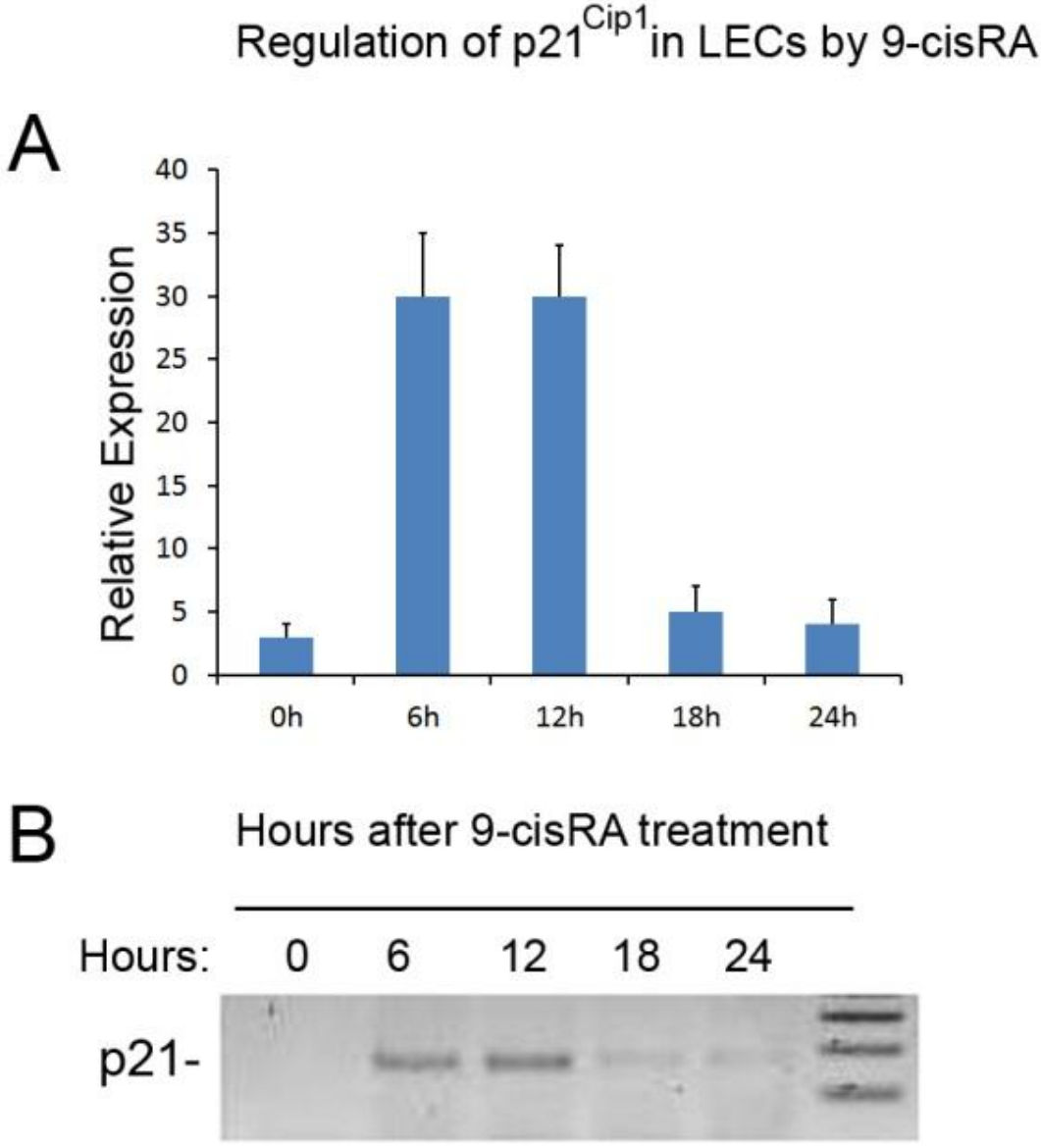

Supplemental Figure 2



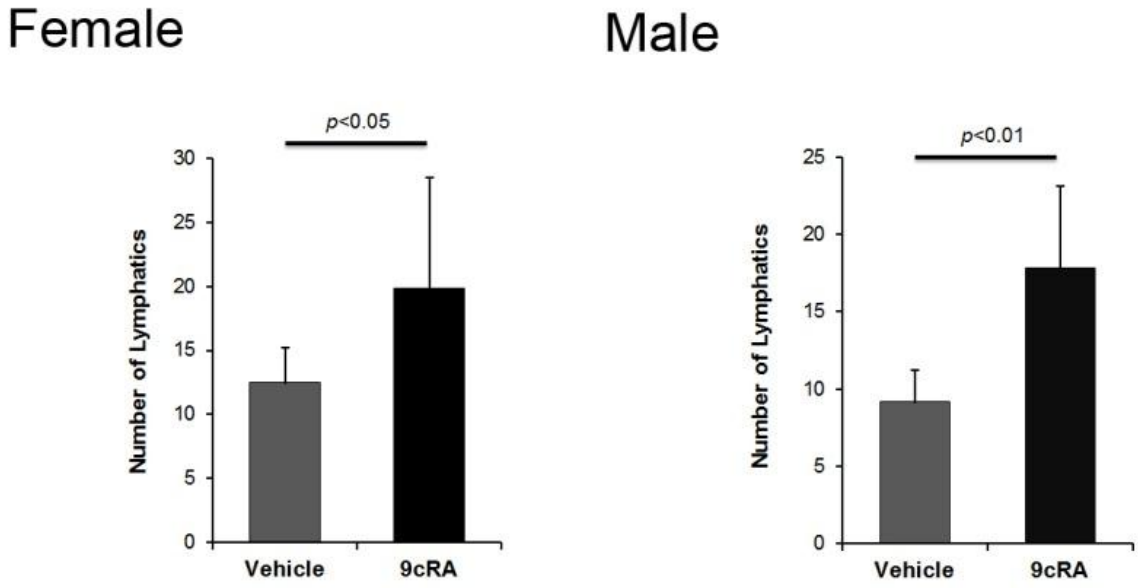

Supplemental Figure 3




Supplemental Figure 4




Supplemental Figure 5




Supplemental Figure 6

**Legends of Supplemental Figures**

**Supplemental Figure 1. Therapeutic effect of 9-cisRA on secondary lymphedema and lymphatic vessel regeneration of BALB/c mice** Experimental lymphedema was surgically induced in the tail of BALB/c mice and, from day 2, vehicle (ethanol/seed oil) or vehicle with 9-cisRA was i.p. injected daily. (A) Images of secondary lymphedema before and after treated with vehicle or 9-cisRA for 20 days. Diameter of tail at the distal (white arrows) and proximal (white arrowheads) sides of the wound was measured every other day for female (B) and male (C) mice. The numbers of mice used are as follows: vehicle-treated female (7 mice), 9-cisRA-treated female (8 mice), vehicle-treated male (6 mice) and 9-cisRA-treated male (7 mice). Asterisk, $p < 0.01$. (D) Regeneration of lymphatic vessels in the wounded area after 20 days of treatment with vehicle alone (left) and vehicle containing 9-cisRA (right). Black arrows point newly generated lymphatic vessels.

**Supplemental Figure 2. Insignificant effect of 9-cisRA on wound healing in mouse.** Two incisional wounds were made on the back skin of each mouse (BALB/c) by using a 6-mm biopsy punch and treated either vehicle (ethanol) alone or vehicle containing 9-cisRA (0.8 mg/kg) daily for 9 days. Images of the wounded area were captured every other day and the percent wound area was determined by measuring the wound sizes by using the Image J software. Three mice were used for each group and representative wound images of vehicle and 9-cisRA-treated group are shown on the top. Data in the bottom graph are shown by mean ± standard deviation.

**Supplemental Figure 3. Pro-lymphangiogenic activity of all-trans RA (ATRA).** (A) Mouse cornea pocket assay: Activation of lymphatic vessel growth into the mouse cornea by all-trans RA (0.3 mg) after 14 days. The degree of newly formed lymphatics was expressed by percent lymphatic vessel coverage. (B,C) Matrigel plug assay: Matrigel premixed with vehicle (Veh) (B)

or vehicle containing all-trans RA (ATRA,10 μM) (C) was injected into the skin of the lymphatic-specific GFP mice [11] (two matrigel plugs per mouse, 5 mice each group) and isolated after two weeks. Lymphatic vessels within the matrigels were directly visualized under a fluorescent stereoscope.

**Supplemental Figure 4. Insignificant effect of 9-cisRA on proliferation of human dermal blood vascular endothelial cells (BECs).** Primary human BECs were cultured in a low serum media (1% FBS) in the presence of vehicle (ethanol) or 9-cisRA and the percent cell number was determined after 24 or 48 hours.

**Supplemental Figure 5. Regulation of CDKN1A/p21$^{Cip1}$ by 9-cisRA in LECs.** Primary human LECs were treated by 9-cisRA for various time points and the expression of CDKN1A/p21$^{Cip1}$ was determined by quantitative real-time RT-PCR (A) or by conventional RT-PCR (B).

**Supplemental Figure 6. Increased number of lymphatic vessels in the lymphedema mice treated with 9-cisRA (9cRA).** From the immunohistochemistry analyses shown in Figure 6C and F, the number of LYVE-1-positive lymphatic vessels per image (100X) was counted and charted to compare the degree of lymphangiogenesis between the vehicle- versus 9-cisRA-treated groups.

## Supplemental References


1. Shin JW, Min M, Larrieu-Lahargue F, Canron X, Kunstfeld R, Nguyen L, Henderson JE, Bikfalvi A, Detmar M, Hong YK. Prox1 promotes lineage-specific expression of fibroblast growth factor (fgf) receptor-3 in lymphatic endothelium: A role for fgf signaling in lymphangiogenesis. *Mol Biol Cell*. 2006;17:576-584
2. Lee S, Kang J, Yoo J, Ganesan SK, Cook SC, Aguilar B, Ramu S, Lee J, Hong YK. Prox1 physically and functionally interacts with coup-tfii to specify lymphatic endothelial cell fate. *Blood*. 2009;113:1856-1859
3. Panek RL, Lu GH, Dahring TK, Batley BL, Connolly C, Hamby JM, Brown KJ. In vitro biological characterization and antiangiogenic effects of pd 166866, a selective inhibitor of the fgf-1 receptor tyrosine kinase. *Journal of Pharmacology and Experimental Therapeutics*. 1998;286:569-577
4. Kubo K, Shimizu T, Ohyama S, Murooka H, Iwai A, Nakamura K, Hasegawa K, Kobayashi Y, Takahashi N, Takahashi K, Kato S, Izawa T, Isoe T. Novel potent orally active selective vegfr-2 tyrosine kinase inhibitors: Synthesis, structure-activity relationships, and antitumor activities of n-phenyl-n'-{4-(4-quinolyloxy)phenyl}ureas. *J Med Chem*. 2005;48:1359-1366
5. Kirkin V, Thiele W, Baumann P, Mazitschek R, Rohde K, Fellbrich G, Weich H, Waltenberger J, Giannis A, Sleeman JP. Maz51, an indolinone that inhibits endothelial cell and tumor cell growth in vitro, suppresses tumor growth in vivo. *Int J Cancer*. 2004;112:986-993
6. Nelson JD, Denisenko O, Bomsztyk K. Protocol for the fast chromatin immunoprecipitation (chip) method. *Nat Protoc*. 2006;1:179-185

7. Alheim K, Corness J, Samuelsson MK, Bladh LG, Murata T, Nilsson T, Okret S. Identification of a functional glucocorticoid response element in the promoter of the cyclin-dependent kinase inhibitor p57kip2. *J Mol Endocrinol*. 2003;30:359-368

8. Rogers MS, Birsner AE, D'Amato RJ. The mouse cornea micropocket angiogenesis assay. *Nat Protoc*. 2007;2:2545-2550

9. Ecoiffier T, Yuen D, Chen L. Differential distribution of blood and lymphatic vessels in the murine cornea. *Invest Ophthalmol Vis Sci*. 2010;51:2436-2440

10. Zhang H, Tse J, Hu X, Witte M, Bernas M, Kang J, Tilahun F, Hong YK, Qiu M, Chen L. Novel discovery of lyve-1 expression in the hyaloid vascular system. *Invest Ophthalmol Vis Sci*. 2010;51:6157-6161

11. Choi I, Chung HK, Ramu S, Lee HN, Kim KE, Lee S, Yoo J, Choi D, Lee YS, Aguilar B, Hong YK. Visualization of lymphatic vessels by prox1-promoter directed gfp reporter in a bacterial artificial chromosome-based transgenic mouse. *Blood*. 2011;117:362-365