Spreds Are Essential for Embryonic Lymphangiogenesis by Regulating Vascular Endothelial Growth Factor Receptor 3 Signaling[▽]

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Spred/Sprouty family proteins negatively regulate growth factor-induced ERK activation. Although the individual physiological roles of Spred-1 and Spred-2 have been investigated using gene-disrupted mice, the overlapping functions of Spred-1 and Spred-2 have not been clarified. Here, we demonstrate that the deletion of both *Spred-1* and *Spred-2* resulted in embryonic lethality at embryonic days 12.5 to 15.5 with marked subcutaneous hemorrhage, edema, and dilated lymphatic vessels filled with erythrocytes. This phenotype resembled that of *Syk*^{-/-} and *SLP-76*^{-/-} mice with defects in the separation of lymphatic vessels from blood vessels. The number of LYVE-1-positive lymphatic vessels and lymphatic endothelial cells increased markedly in *Spred-1/2*-deficient embryos compared with WT embryos, while the number of blood vessels was not different. Ex vivo colony assay revealed that *Spred-1/2* suppressed lymphatic endothelial cell proliferation and/or differentiation. In cultured cells, the overexpression of Spred-1 or Spred-2 strongly suppressed vascular endothelial growth factor-C (VEGF-C)/VEGF receptor (VEGFR)-3-mediated ERK activation, while *Spred-1/2*-deficient cells were extremely sensitive to VEGFR-3 signaling. These data suggest that Spreds play an important role in lymphatic vessel development by negatively regulating VEGF-C/VEGFR-3 signaling.

Vessels of the lymphatic system are highly permeable and specialized for the uptake of fluid and macromolecules from the interstitium and their return to venous circulation (2, 21). Embryonic development of the lymphatic vessels starts when a subset of endothelial cells in the cardinal vein commits to the lymphatic lineage and sprouts to form the primary lymph sacs (2, 21, 22). Recent studies have identified specific transcription factors and growth factors required to regulate the development of lymphatic vessels. In mice, the lymphatic vasculature starts to develop at embryonic day 10.5 (E10.5), when the cardiovascular system is already functioning. After the formation of the initial lymph sacs, the peripheral lymphatics are generated by centrifugal sprouting. Several genes have been shown to play essential roles in embryonic lymphatic vessel development. The Prox1 homeobox transcription factor, vascular endothelial growth factor C (VEGF-C), and its receptor, vascular endothelial growth factor receptor 3 (VEGFR-3), are essential for the generation of lymphatics (2, 21). Hematopoietic intracellular signaling proteins, Syk, SLP-76, and PLCγ2, are important for the separation of lymphatic vessels from blood vessels (1, 30). Furthermore, the final patterning and maturation of lymphatic vasculature require angiopoietin 2, neuropilin 2, and podoplanin/T1 (2, 21). However, regulatory

genes for lymphatic vessel development have not been identified yet.

Recently, Sprouty/Spred family proteins were identified as negative regulators for growth factor- and cytokine-induced ERK activation (5, 9, 14, 29). In mammals, four Sprouty homologues and three Spred (for Sprouty-related *Ena/VASP* homology 1 domain-containing proteins) homologues have been identified (6, 14, 29). Spred-1 has been implicated in hematopoiesis, since bone marrow-derived mast cells and eosinophils from *Spred-1*^{-/-} mice were more sensitive to interleukin 3 and interleukin 5, respectively, than those from wild-type (WT) mice (11, 20). In *Spred-2*^{-/-} mice, embryonic hematopoiesis was enhanced in the aorta-gonad-mesonephros region compared with WT mice (19). However, except for a shortened face and slight growth retardation, neither *Spred-1*^{-/-} nor *Spred-2*^{-/-} mice showed strong developmental abnormalities in most organs (11, 19).

To define overlapping functions of Spred-1 and Spred-2, we generated *Spred-1/Spred-2* double-knockout (DKO) mice. We found that Spreds are key regulators of embryonic lymphangiogenesis and that they can specifically regulate VEGF-C signaling by suppressing VEGFR-3-mediated ERK and Akt activation.

MATERIALS AND METHODS

Animals. *Spred-1*^{-/-} and *Spred-2*^{-/-} knockout mice backcrossed into the C57BL/6J background have been previously reported (11, 19). *Spred-1/2* DKO embryos (*Spred-1*^{-/-}/*Spred-2*^{-/-}) and corresponding WT control embryos with the same genetic background (C57BL/6J) as the knockout embryos were used. Timed mating was accomplished in the late afternoon, followed by a plug check

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the next morning. The point of a detected plug was counted as E0.5. The mice were housed in an environment with a 12-h light/dark cycle and a controlled temperature. Animal care and all experiments were conducted in accordance with the institutional guidelines of Kyushu University, Fukuoka, Japan.

Plasmids and viruses. Human *VEGFR-3* short-form cDNA was cloned from human embryonic kidney (HEK) 293T cells by PCR (25) and cloned into the pMX retroviral vector (kindly provided by Toshio Kitamura, University of Tokyo, Tokyo, Japan). Flag-*Spred-1*, Flag-*Spred-2*, Flag-*Sprouty4*, and *VEGFR-2* plasmids, as well as Sendai virus vector carrying *Spred-1* or *GFP*, have been described previously (16, 23).

Cell culture, transfection, and infection. HEK 293T cells and mouse embryonic fibroblasts (MEFs) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin. OP9 stromal cells were cultured in α -modified Eagle's medium supplemented with 20% FBS, penicillin, and streptomycin. To generate 293T cells expressing *VEGFR-3*, 293T cells were transfected with pMX-*VEGFR-3* using FuGENE 6 (Roche Molecular Biochemicals), and cells were selected with 1 μ g/ml puromycin (Sigma). To generate MEFs expressing *VEGFR-3*, MEFs were infected with the retroviruses produced by PLAT-E transfected with pMX-*VEGFR-3*. Infected cells were selected with 1 μ g/ml puromycin. CD45⁺ CD31⁺ endothelial cells sorted by the magnetic activated cell-sorting (MACS) system (Miltenyi Biotec) were infected with the Sendai virus at a multiplicity of infection of 2 and cultured for 3 to 14 days until they were used for experiments. Green fluorescent protein (GFP) indicated that more than 95% of the cells were infected. *VEGFR-3* proteins in 293T cells and MEFs were detected by Western blotting analysis with an anti-*VEGFR-3* antibody (Ab).

Antibodies and reagents. The anti-mouse lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) monoclonal Ab (MAb) (ALY2) and rabbit polyclonal Ab anti-LYVE-1 have been described previously (17, 18). Other Abs used in this experiment were as follows: anti-PECAM-1/CD31 (clone 390) and anti-*VEGFR-3* (AFL4) (eBioscience); anti-CD34 (RAM34), anti-PECAM-1/CD31 (MEC13.3), and anti-CD45 (clone 104) (PharMingen); anti-Prox1 and anti-podoplanin (Angio Bio); anti-vWF (DAKO); anti- α -smooth muscle actin (SMA) (Sigma); anti-phospho-ERK1/2 (P-ERK1/2) (no. 9106 and 9101), anti-FLAG (M2), anti-phospho-Akt (no. 4058), and anti-Akt (no. 9272) (Cell Signaling Technology); and anti-ERK2 (C-14), anti-*VEGFR-2* (A-3), and anti-Myc (9E10) (Santa Cruz Biotechnology). Human VEGF-C C156S, which acts on *VEGFR-3* but not on *VEGFR-2*, was purchased from R&D Systems. VEGF-C C156S was used at a concentration of 400 ng/ml, according to the manufacturer's recommendation. VEGF-A and PDGF-BB were purchased from Peprotech.

Immunohistochemistry. E14.5 mouse embryos were stained by whole-mount immunohistochemistry with 1:200-diluted anti-mouse LYVE-1 Ab (ALY2) or anti-PECAM-1/CD31 (MEC13.3) as previously described (17). The skin tissues from mouse embryos were fixed with 4% paraformaldehyde to make paraffin tissue specimens or were embedded in an OCT compound (SAKURA Finetechnical Co., Ltd.) to make frozen tissue specimens, and then they were sectioned at 10 μ m. Samples were stained with anti-LYVE-1-biotin, antipodoplanin, anti-vWF, anti- α -SMA, and anti-PECAM-1/CD31-PE (eBioscience) at 4°C overnight. Rabbit immunoglobulin G (heavy plus light chains)-biotin and streptavidin-fluorescein isothiocyanate were used to detect anti-LYVE-1 Ab. Immunofluorescence images were acquired using an Olympus BX51 microscope with a fluorescent attachment (Olympus, Tokyo, Japan). When horseradish peroxidase-conjugated immunoglobulin was used as the secondary Ab, samples were incubated in diaminobenzidine at room temperature to visualize reactive samples.

FACS analysis. To identify the characterization of lymphatic endothelial cells (LECs), we performed fluorescence-activated cell sorter (FACS) analyses of mouse embryo cells with FACSaria (Becton Dickinson), as previously described (17). After removing the embryonic liver and spleen microscopically, E14.5 mouse embryos were dissected and treated with 2.4 U/ml Dispase (Gibco) and 0.05% collagenase S-1 (Nitta Gelatin Co. Ltd.) at 37°C for 30 min to produce a single-cell suspension. To exclude erythrocytes from this preparation prior to analysis with FACSaria, lymphoprep (Axis-Shield PoCAS) treatment was performed as previously described (17). Before the cells were stained, Fc receptors were blocked with an anti-mouse CD16/CD32 Fc receptor (PharMingen). First, all cells were stained with anti-mouse CD45-PerCP Cy5.5, CD31-fluorescein isothiocyanate, CD34-phycoerythrin, and biotinylated ALY2 at 4°C for 30 min and washed three times. Subsequently, the cells were reacted with allophycocyanin-conjugated streptavidin (PharMingen) to visualize LYVE-1-positive cells at 4°C for 30 min and then were washed three times. The cells were sorted and analyzed by FACSaria.

RT-PCR analysis. Total RNA was extracted from sorted LECs, blood endothelial cells (BECs), and LYVE-1 and CD34 double-positive cells (DPs) using TRIzol (Invitrogen) as previously reported (17). Total RNA was reverse tran-

scribed using the Reverse Transcription Kit (Roche), and the product was used for further analysis. The sequences of primers for reverse transcription (RT)-PCR were previously described (11, 17, 19). PCR products were separated on a 2.0% agarose gel and stained with ethidium bromide. Quantitative real-time PCR was performed using SYBR Green Real-time PCR Master Mix (Toyobo) and the ABI 7000 sequence detector system (Applied Biosystems). The comparative threshold cycle method and an internal control (glyceraldehyde-3-phosphate dehydrogenase) were used to normalize the expression of the target genes.

Primary LEC coculture on OP9 feeder layers. To examine the effect of *Spred* on LEC growth, MACS sorted CD45⁺ CD31⁺ endothelial cells from E14.5 (5.0×10^4 /well in six-well plates) (see Fig. 4B). Unsorted cells from E12.5 (1.0×10^6 /well in six-well plates) (see Fig. 4C and D) embryos were plated on OP9 stromal cells in RPMI1640 (Gibco) with 10% FBS and 10^{-5} mol/liter 2-mercaptoethanol (Gibco) as previously reported (17). Seven or 14 days later, the dishes were immunostained with anti-LYVE-1 MAb (ALY2). To quantify the effect of *Spred* on the colony formation of LECs, we counted the LYVE-1⁺ endothelial cell colonies, which formed the compact type.

Immunohistochemical detection of P-ERK. Immunohistochemical detection of P-ERK was performed according to the procedure described previously (26). For section preparation, E14.5 embryos were incubated with or without 400 ng/ml human VEGF-C C156S in 2 ml serum-free Dulbecco's modified Eagle's medium for 30 min at 37°C. Then, samples were washed with phosphate-buffered saline and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer at 4°C overnight. The samples were dehydrated, embedded in paraffin wax, sectioned into specimens 7 μ m thick, and mounted onto 3-aminopropyltriethoxysilane-coated slides. The sections were deparaffinized, rinsed in Tris-buffered saline, microwaved for 5 min, and then treated with 3% H₂O₂ for 10 min. The sections were then incubated with rabbit polyclonal anti-P-ERK1/2 (no. 9101; 1:200 dilution; Cell Signaling Technology) in Tris-buffered saline containing 1% bovine serum albumin at 4°C overnight. Detection was done with the ENVISION+ System-HRP (DAKO) according to the manufacturer's instructions. All sections were counterstained with hematoxylin.

Luciferase assay. Elk-1 activation was measured as described previously (14). HEK 293T cells (2.0×10^5 /well) were seeded into six-well plates. The cells were transfected by the calcium phosphate precipitate method 12 h after being seeded with each expression vector (0.01 to 0.1 μ g), as indicated. The total DNA concentration was kept constant by supplementation with empty-vector DNAs. Luciferase activity was determined using the Promega luciferase assay system. The β -galactosidase vector (0.05 μ g) was used for normalizing transfection efficiencies. The values shown are the averages of one representative experiment in which each transfection was performed in triplicate.

Capillary formation assay. Human lymphatic endothelial cells (HULECs), which were isolated from human neonatal dermis, were purchased from Angio-Bio Co. and cultured in EGM2-MV (from Cambrex [formerly Clonetics]) supplemented with 20% off-clot human serum instead of FBS. The HULECs were infected with the Sendai virus carrying *GFP* or *Spred-1* at a multiplicity of infection of 2 and then were cultured for 3 days in EGM2-MV medium. ERK and Akt activation was measured by Western blotting after stimulation with or without 400 ng/ml VEGF-C C156S for 5 and 15 min. Infected HULECs were seeded on precoated 24-well plates with collagen/laminin (Matrigel; BD Biosciences) and incubated at 37°C for 24 h.

MTT assay and wound-healing assay. Microtiter tetrazolium (MTT) assays and wound-healing assays in the presence of 400 ng/ml VEGF-C C156S were performed as previously described (16).

Statistical analysis. Data are expressed as the mean $\pm$ standard deviation or the mean $\pm$ SEM. Statistical analysis was conducted using Student's *t* test. Statistical significance was defined as a *P* value of <0.05.

RESULTS

***Spred-1/Spred-2* DKO embryos showed severe subcutaneous hemorrhage and edema.** *Spred-1* and *Spred-2* have many structural similarities, and we have not found any functional differences between the two proteins in vitro (29). Furthermore, using in situ hybridization and RT-PCR analysis, we and others demonstrated that *Spred-1* and *Spred-2* were expressed in largely overlapping tissues during embryonic development (8; data not shown). Thus, we suspected a physiological and functional redundancy between them. To define overlapping functions of *Spred-1* and *Spred-2*, we generated *Spred-1/*

FIG. 1. Lethal phenotypes of *Spred-1/Spred-2* DKO embryos. (A to C) Gross appearance of *Spred-1/2* DKO (A), *Spred-1^{-/-} Spred-2^{-/-}* (B), and control (C) embryos at E13.5 to E14.5. (D and E) Higher-magnification views of dilated vessels in the cervical (D) and the abdominal (E) regions of a *Spred-1/2* DKO embryo. (F to H) LECs in *Spred-1/2* DKO (F and G) and control (H) embryos at E14.5 were analyzed by whole-mount immunohistochemical staining with anti-LYVE-1 MAb. Higher-magnification views of vessels in the cervical (F and H) and abdominal (G) regions. BECs in *Spred-1/2* DKO (I) and control (J) embryos at E14.5 were analyzed by whole-mount immunohistochemical staining with anti-CD31 MAb. Higher-magnification views of vessels in the cervical regions are shown.

Spred-2 DKO mice. We crossed *Spred1^{-/+} Spred2^{-/+}* male and female mice. The *Spred-1^{-/+} Spred-2^{-/-}* mice were smaller than the *Spred1^{-/+} Spred2^{-/+}* mice but were healthy and fertile. *Spred-1^{-/-} Spred-2^{-/+}* mice were rarely born, but several natal mice appeared sick; most died within a few months for unknown reasons. We could not obtain any *Spred-1^{-/-} Spred-2^{-/-}* pups among >200 pups, indicating that the deletion of both *Spred-1* and *Spred-2* genes resulted in embryonic lethality.

We found that most of the *Spred-1/2* DKO embryos died of severe subcutaneous hemorrhage and edema between E12.5 and E15.5 (Fig. 1A to C). *Spred-1^{-/-} Spred-2^{-/+}* embryos often showed bleeding similar to that of *Spred-1/2* DKO embryos (Fig. 1B). A subcutaneous hemorrhagic appearance in *Spred-1/2* DKO embryos was the most striking phenotype; it was also observed in mouse embryos lacking *Syk*, *SLP-76*, or *PLCγ2* (1, 30). Whole-mount immunohistochemical staining with the anti-LYVE-1 MAb confirmed that dilated and irreg-

ular vessels in *Spred-1/2* DKO embryos were LYVE-1-positive lymphatic vessels (Fig. 1D to H). On the other hand, whole-mount immunohistochemical staining with anti-PECAM-1/CD31 MAb confirmed that blood vessels were almost normal in *Spred-1/2* DKO embryos compared with control embryos (Fig. 1I and J). Other organs, such as the liver, heart, and placenta, showed no apparent abnormalities (data not shown). These data strongly suggest that lymphatic vessel development is abnormal in *Spred-1/2* DKO embryos.

Abnormal lymphatic vessel development in *Spred-1/2* DKO embryos. Histologically, in *Spred-1/2* DKO embryos, a number of nucleated red blood cells were observed in subcutaneous hyperplastic dilated vessels, which were LYVE-1 and podoplanin (lymphatic endothelial markers) positive but were von Willebrand factor (vWF) (a blood endothelial marker) and α-SMA (a pericyte and smooth muscle cell marker) negative, confirming that these dilated vessels were lymphatic vessels (Fig. 2A to F). This hyperplastic lymphatic vessel pattern closely resembled that of *VEGF-C* transgenic mice (28). In control embryos, red blood cells were observed only in blood vessels, but not in lymphatic vessels (Fig. 2G). Consistent with the whole-mount staining with LYVE-1 and CD31 Abs shown in Fig. 1, although the numbers of vWF-positive blood vessels of control and *Spred-1/2* DKO embryos were the same, LYVE-1-positive lymphatic vessels in the skin of *Spred-1/2* DKO embryos increased compared with the control (Fig. 2H).

To further characterize LYVE-1-positive cells, we performed double immunofluorescence staining of LECs and BECs in the skin of E12.5 embryos with anti-LYVE-1 Ab and anti-CD31 Ab, respectively. CD31 is a panendothelial marker, but it has been reported that blood vessels were stained strongly with the anti-CD31 Ab while newly formed LYVE-1-positive lymphatic vessels were only weakly stained (3). As shown in Fig. 2I and J, compared with WT embryos, the number of CD31-positive BECs in the skin of *Spred-1/2* DKO embryos was not significantly different, but there were more LYVE-1-positive LECs. Surprisingly, LYVE-1 and CD31 double-positive dilated vessels, which were never seen in WT embryos, were occasionally observed in the neck region of E12.5 *Spred-1/2* DKO embryos (Fig. 2K and L). The number of LYVE-1 and CD31 double-positive vessels in *Spred-1/2* DKO embryos was much higher than that in WT embryos (Fig. 2M). These data suggest that the developing lymphatic circulation in embryos lacking both *Spred-1* and *Spred-2* might communicate with blood circulation. Thus, LYVE-1 and CD31 double-positive dilated vessels could represent an incomplete separation of lymphatic vessels from blood vessels.

Expression of *Spred-1* and *Spred-2* in LECs and BECs. To determine the expression of *Spred-1/2* in developing lymphatics, we isolated LECs and BECs by using a cell sorter with anti-LYVE-1 and anti-CD34 Abs from E14.5 WT mouse embryos, as previously described (17) (Fig. 3A), and quantitative real-time PCR analysis was performed. As shown in Fig. 3B, LYVE-1⁺ CD34^{low/-} cells expressed the LEC markers *Prox1* and *podoplanin*, but not the BEC markers *neuropilin 1* (*Nrp-1*), *CD44*, and *VEGFR-1*, while LYVE-1⁻ CD34⁺ cells expressed BEC markers but not LEC markers. Thus, LECs and BECs were well separated by this procedure. Both *Spred-1* and *Spred-2* were expressed in LECs but not expressed in BECs (Fig. 3B). A small number of LYVE-1⁺ CD34⁺ DPs were

FIG. 2. Lymphatic vessel development in *Spred-1/2* DKO embryos. (A to G) Sections of skin tissues of a *Spred-1/2* DKO embryo (A to F) and a control embryo (G) at E14.5. Shown are hematoxylin and eosin staining (A, B, and G) and immunostaining for anti-LYVE-1 (C), anti-podoplanin (D), anti-vWF (E), and anti- α -SMA (F) Abs. In panels A and B, arrows and arrowheads indicate red blood cells inside and outside, respectively, of the subcutaneous dilated vessels. In panel G, the arrow and arrowheads indicate lymphatic and blood vessels, respectively. Scale bars, 200 μ m (B to G) and 1 mm (A). (H) vWF-positive vessels and LYVE-1-positive vessels in the skin (0.4 by 0.4 μ m) of control and *Spred-1/2* DKO embryos were counted. The graphs represent the mean $\pm$ SEM ($n = 3$). *, $P < 0.05$. (I to L) Immunostaining with DAPI (blue), CD31 (red), and LYVE-1 (green) in the skin (I and J) or neck (K and L) of control and *Spred-1/2* DKO embryos at E12.5. (L) LYVE-1 and CD31 double-positive vessels in the neck (white square) of *Spred-1/2* DKO embryos. Scale bars, 100 μ m. (M) The LYVE-1 and CD31 double-positive vessels in the necks (0.3 by 0.2 μ m) of control and *Spred-1/2* DKO embryos were counted. The graph represents the mean $\pm$ SEM ($n = 5$). *, $P < 0.05$.

observed in E14.5 embryos (Fig. 3B). DPs expressed both LEC and BEC markers, suggesting that this DP fraction could represent a transition state of LECs from BECs. *Spred-1* and *Spred-2* expression was high in this DP fraction (Fig. 3B). In contrast, *Sprouty2* (*Spry2*) and *Sprouty4* (*Spry4*), which have been implicated in angiogenesis (10, 15), were expressed in both LECs and BECs, but their expression was higher in BECs than in LECs (Fig. 3B).

Inhibition of in vitro LEC colony and capillary formation by *Spreds*. Next, we examined the effect of *Spred* overexpression

and loss of expression on the proliferation and differentiation of LECs. MACS-sorted CD45⁻ CD31⁺ endothelial cells from E14.5 embryos were cocultured on OP9 stromal cells in which VEGF-C/D was shown to be expressed (17). First, we confirmed that the efficacy of the infection, which was estimated by GFP, was almost 100%. Sendai virus-mediated *Spred-1* gene transfer resulted in several-times-higher *Spred-1* expression in endothelial cells than in control GFP-infected cells (Fig. 4A). Overexpression of *Spred-1*, but not control GFP, by the Sendai virus (16) resulted in a strong reduction of LYVE-1-positive

FIG. 3. Expression of *Spred-1* and *Spred-2* in LECs and BECs. (A) FACS profile of CD45⁻ CD31⁺ cells from E14.5 embryos. (B) The relative mRNA levels of the indicated genes determined by quantitative real-time PCR. Total RNA (1 μ g) isolated from approximately 5.0×10^4 FACS-sorted LECs, BECs, and DPs was analyzed ($n = 3$). The error bars indicate SEM.

LEC colonies (Fig. 4B). Conversely, LYVE-1-positive colony formation from E12.5 *Spred1/2* DKO embryos was two to three times more than that from WT embryos (Fig. 4C). To confirm that the increase in LYVE-1-positive colonies in *Spred-1/2* DKO embryos was specifically due to the lack of Spreds, we reduced LYVE-1-positive colonies in *Spred*-deficient cells by transducing exogenous *Spred-1* into *Spred*-deficient embryonic cells (Fig. 4D). These data suggest that *Spred-1* and *Spred-2* negatively regulate the proliferation and/or differentiation of LECs.

We also examined the effect of *Spred* overexpression on VEGF-C signaling and lymphatic capillary formation in HULECs. As shown in Fig. 5, *Spred-1* overexpression resulted in the suppression of ERK with or without VEGF-C and Akt phosphorylation with VEGF-C (Fig. 5A), as well as capillary

formation of HULECs on Matrigel (Fig. 5B). These data suggest that *Spred* negatively regulates VEGF-C signaling and lymphatic vessel formation.

The VEGF-C/VEGFR-3 pathway is negatively regulated by Spreds. It has been shown that the VEGF-C/VEGFR-3 pathway is essential for embryonic lymphatic vessel development (13). To determine whether *Spred-1* and *Spred-2* affect VEGF-C/VEGFR-3 signaling in vivo, we examined ERK activation by VEGF-C in situ using immunohistochemistry with Abs against P-ERK. Although we could not detect ERK phosphorylation in either E14.5 WT or *Spred-1/2* DKO embryos at basal levels, stronger ERK activation by VEGF-C was observed in the LECs of the skin of *Spred-1/2* DKO embryos than in those of WT embryos (Fig. 6A). The expression of VEGFR-3 and that of *Prox-1* were detected equally in the LECs of *Spred-1/2* DKO

FIG. 5. Suppression of capillary formation of HULECs by Spred-1 overexpression. (A) Sendai-GFP or Sendai-Flag-Spred-1 virus-infected HULECs were incubated with or without 400 ng/ml VEGF-C for 5 and 15 min. Cell extracts were immunoblotted with anti-P-ERK1/2, anti-ERK2, anti-phospho-Akt (P-Akt), anti-Akt, and anti-Flag Abs. (B) Capillary formation assay. Infected HULECs were seeded on precoated 24-well plates with Matrigel and incubated at 37°C for 24 h.

and WT mouse embryos (Fig. 6B and data not shown). These data suggest that Spred-1 and Spred-2 are physiological negative regulators of VEGF-C/VEGFR-3 signaling.

To further investigate the functions of Spreds on VEGF-C/VEGFR-3 signaling in vitro, we generated 293T cells that stably expressed human VEGFR-3 (293T/hVEGFR-3 cells). The overexpression of Spred-1 efficiently suppressed VEGF-C-induced ERK activation in 293T/hVEGFR-3 cells (Fig. 7A). We could not determine the effect of Spred-1 on Akt activation, since Akt was already highly activated in 293T cells before stimulation (data not shown). An *Elk-1* reporter assay indicated that Spred-1 and Spred-2 showed similar suppressive effects on VEGF-C-induced ERK activation (Fig. 7B). Interestingly, Spred-1 and Spred-2 suppressed VEGFR-3-mediated ERK activation more strongly than Sprouty4 did (Fig. 7B),

while Sprouty4 suppressed VEGF-A-mediated ERK activation as efficiently as Spreds did (Fig. 7C).

To define the effect of the loss of Spred-1/2 expression on VEGFR-3 signaling, we stably expressed hVEGFR-3 in MEFs derived from WT and *Spred-1/2* DKO embryos. The expression levels of hVEGFR-3 were similar in WT and *Spred-1/2* DKO MEFs (Fig. 8A). VEGF-C induced much stronger ERK and Akt activation in *Spred-1/2* DKO MEFs than in WT MEFs (Fig. 8A). Recently, it has been shown that PDGF-BB potently induces the growth of lymphatic vessels in vivo (4). Thus, we examined PDGF-BB-induced ERK activation using WT and *Spred-1/2*-deficient MEFs (Fig. 8B). Only a slight enhancement of PDGF-BB-induced ERK activation was observed by *Spred-1/2* deletion. Moreover, only a slight enhancement of VEGF-A-induced ERK activation was observed by *Spred-1/2* deletion (Fig. 8C). These data suggest that the effect of *Spred-1/2* deletion was more specific to VEGF-C than to PDGF-BB and VEGF-A. Consequently, the growth and migration of *Spred-1/2* DKO MEFs were strongly enhanced by VEGF-C compared with WT MEFs (Fig. 8D and E). VEGF-C was not a strong mitogen in WT MEFs, while the proliferation of *Spred-1/2* DKO MEFs was enhanced with VEGF-C (Fig. 8D). Similarly, the migration of *Spred-1/2* DKO MEFs in response to VEGF-C was more evident than that of WT MEFs (Fig. 8E). These data indicate that Spred-1 and Spred-2 are important negative regulators of the VEGF-C/VEGFR-3 pathway.

DISCUSSION

Our present study indicates that Spred-1 and Spred-2 are critical for the separation of lymphatic vessels from the parental vein, because the *Spred-1/2* DKO phenotype resembles that of *Syk*-, *SLP-76*-, and *PLCγ2*-deficient mice and differs from that of *angiopoietin-2*-, *neuropilin-2*-, or *podoplanin*-deficient mice (2, 21). Although *Syk*, *SLP-76*, *PLCγ2*, and *Spred-1/2* are all involved in tyrosine kinase signal transduction, the molecular link between VEGFR-3 and *Syk*/*SLP-76*/*PLCγ* has not been clarified. These molecules, however, appeared to be essential for lymphatic vessel separation from the parental vein. Further study will provide a clearer understanding of the mechanism of this system. One simple possibility is that *Syk*, *SLP-76*, and *PLCγ2* are the downstream effectors of VEGFR-3, while Spred-1 and Spred-2 are negative regulators for VEGFR-3. The expression of *Syk* has been demonstrated in endothelial cells (31). For the precise separation of lymphatic vessels from the parental vein, VEGF-C/VEGFR-3 signals might be correctly regulated. Positive signals of VEGFR-3 promote LEC budding; thereafter, those signals might be down-regulated by Spreds at the separation from BECs.

There is a more complex possibility that Spreds and *Syk*/

FIG. 4. Functions of *Spred-1* and *Spred-2* in LECs. (A) The efficacy of Sendai virus infection of embryonic cells (left) and mRNA levels of Spred-1 overexpression (right) are shown. (B and C) Images and numbers of LYVE-1-positive LEC colonies grown on OP9 cell monolayers. In panel B, Spred-1 (right) or GFP (left) was expressed in WT endothelial cells by the Sendai virus. In panel C, cells from *Spred-1/2* DKO embryos (right) or WT embryos (left) were seeded on OP9 cells. The bars represent the relative numbers of packed round colonies expressed as percentages of control cultures. The graphs represent the mean and SEM ($n = 3$). (D) Cells from *Spred-1*^{-/-} *Spred-2*^{-/-} embryos or WT embryos were infected with Sendai-*Spred-1* or Sendai-GFP and then seeded on OP9 cells. The bars represent the relative numbers of packed round colonies expressed as percentages of control cultures infected with GFP. The graphs represent the means $\pm$ SEM ($n = 3$). *, $P < 0.05$.

FIG. 6. Enhanced VEGF-C-mediated ERK activation in *Spred-1/2*-deficient embryos. (A) Immunohistochemical detection of P-ERK in response to 400 ng/ml VEGF-C in control and *Spred-1/2* DKO embryos. The embryos were incubated in the presence [VEGF-C (+)] (bottom) or absence [VEGF-C (-)] (top) of VEGF-C for 30 min. The arrows indicate LECs. (B) Immunohistochemical detection of VEGFR-3 in control and *Spred-1/2* DKO embryos. The arrows indicate VEGFR-3-positive LECs. Scale bars, 50 μ m.

SLP-76/PLC γ 2 function independently. Syk/SLP-76/PLC γ 2 has been shown to function in hematopoietic cells, which may play unidentified roles in the separation of lymphatic vessels from blood veins. There are also apparent abnormalities in

Spred-1^{-/-} or *Spred-2*^{-/-} hematopoietic cells, as described previously (11, 19, 20). Thus, we could not completely rule out the possibility that the presence of blood cells in the lymphatics may be related to the hematopoietic differentiation defects

FIG. 7. Suppression of VEGF-C-induced ERK activation by Spreds. (A) 293T cells stably expressing hVEGFR-3 were transfected with empty or Myc-Spred-1 plasmids. After being stimulated with VEGF-C for the indicated periods, cell extracts were immunoblotted with the indicated Abs. (B and C) Flag-tagged *Spred* or *Sprouty* plasmids were cotransfected into 293T cells with the *Elk-1* reporter. *VEGFR-3* (B) or *VEGFR-2* (C) plasmids were also cotransfected. Cells were treated with (+) or without (-) 400 ng/ml VEGF-C (B) and 25 ng/ml VEGF-A (C) for 6 h and then analyzed by the luciferase assay. The results presented are for one representative experiment assayed in triplicate. The error bars represent SEM.

FIG. 8. Enhancement of VEGF-C signaling by *Sprad-1/2* deficiency in MEFs. (A and B) WT and *Sprad-1/2* DKO MEFs stably expressing hVEGFR-3 were stimulated with VEGF-C (A) or PDGF-BB (B). Cell extracts were immunoblotted with the indicated Abs. (C) WT and *Sprad-1/2* DKO MEFs stably expressing hVEGFR-2 were stimulated with VEGF-A. Cell extracts were immunoblotted with the indicated Abs. (D) The rate of cell growth in the presence of VEGF-C was evaluated by MTT assay. The data represent means $\pm$ SEM of triplicate measurements. *, $P < 0.05$. (E) Wound-healing assay. WT and *Sprad-1/2* DKO MEFs stably expressing hVEGFR-3 were scraped with a sharp edge to make a cell-free area (0 h). Cells were cultured in the presence of mitomycin C and VEGF-C for the indicated periods and photographed.

observed in single knockouts. One possibility is that LECs share similar phenotypes with hematopoietic cells more extensively than BECs do. Another possibility is that proper VEGF-C signals are critical for the differentiation of LECs from hemangioblasts. In addition, we cannot rule out the possibility that *Sprad-1/2* may also regulate other late tyrosine kinase signals, such as those of angiopoietin-2 and neuropilin-2, since all *Sprad-1/2* DKO embryos die by E15.5, whereas a substantial number of *Syk*-, *SLP-76*-, and *PLC γ 2*-deficient mice survive until birth (1, 30). Further study is necessary to define these possibilities.

The VEGF-C receptor, VEGFR-3, is present on all endothelial cells during development, but in the adult it becomes restricted to LECs and certain fenestrated blood vascular endothelial cells (12). Knockout and developmental studies suggest that VEGFR-3 signaling is essential, not only for lymphangiogenesis, but also for the development of blood vessels during the embryonic stage (7). However, VEGF-C is critical for lymphatic vessel development, but not for blood vessel development (13). Although Spreds efficiently suppressed VEGFR-3 signaling, blood vessel development in *Sprad-1/2* DKO embryos was almost normal (Fig. 1I and 2H and J). Thus, VEGFR-3 signals in BECs could be regulated by other molecules, such as Sproutys. We have shown that *Sprouty2* and *Sprouty4* efficiently suppressed VEGF-A/VEGFR-2-mediated

ERK activation, but not EGF/EGFR-mediated ERK activation (Fig. 7C) (23). *Sprouty4* suppressed VEGF-A-mediated ERK activation as efficiently as Spreds did (Fig. 7C), but the effect of *Sprouty4* on VEGF-C-mediated ERK activation was not as strong as that of Spreds (Fig. 7B). Furthermore, our preliminary results suggest that *Sprouty2/Sprouty4* DKO embryos are lethal and exhibit abnormalities on blood vessels rather than lymphatic vessels (27; K. Taniguchi, unpublished data). In addition, *Sprouty2* and *Sprouty4* were more strongly expressed in BECs than in LECs (Fig. 3B). We suspect that VEGF-A/VEGFR-1/2 signals are mainly regulated by Sproutys, while VEGF-C/VEGFR-3 signals are mainly modulated by Spreds. The functional redundancy and specificity of Sproutys and Spreds on endothelial cells need to be investigated further.

The roles of *Sprouty* and *Spred* proteins during gastrulation in *Xenopus tropicalis* were compared (24). *Spred* proteins preferentially inhibit the Ras/ERK cascade that directs mesoderm formation, whereas *Sprouty* proteins block the Ca^{2+} and protein kinase C δ signals required for morphogenetic movement during gastrulation. Thus, the expression of *Sprouty* and *Spred* genes at specific times during gastrulation might redirect fibroblast growth factor signals toward mesoderm formation or morphogenesis, respectively. Our results also suggest that the expression of *Sprouty* and *Spred* genes at specific times might regulate angiogenesis and lymphangiogenesis in mammalian

development. An investigation into the molecular mechanism of VEGFR signal regulation by Sproutys and Spreds will improve our understanding of how tyrosine kinases regulate the development of blood and lymphatic vessels.

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