Bone morphogenetic protein-9 inhibits lymphatic vessel formation via activin receptor-like kinase 1 during development and cancer progression

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Lymphatic vessels (LVs) play critical roles in the maintenance of fluid homeostasis and in pathological conditions, including cancer metastasis. Although mutations in *ALK1*, a member of the transforming growth factor (TGF)-β/bone morphogenetic protein (BMP) receptor family, have been linked to hereditary hemorrhagic telangiectasia, a human vascular disease, the roles of activin receptor-like kinase 1 (ALK-1) signals in LV formation largely remain to be elucidated. We show that ALK-1 signals inhibit LV formation, and LVs were enlarged in multiple organs in *Alk1*-depleted mice. These inhibitory effects of ALK-1 signaling were mediated by BMP-9, which decreased the number of cultured lymphatic endothelial cells. *Bmp9*-deficient mouse embryos consistently exhibited enlarged dermal LVs. BMP-9 also inhibited LV formation during inflammation and tumorigenesis. BMP-9 downregulated the expression of the transcription factor prospero-related homeobox 1, which is necessary to maintain lymphatic endothelial cell identity. Furthermore, silencing prospero-related homeobox 1 expression inhibited lymphatic endothelial cell proliferation. Our findings reveal a unique molecular basis for the physiological and pathological roles of BMP-9/ALK-1 signals in LV formation.

lymphangiogenesis | angiogenesis | blood vascular endothelial cells

Fluid homeostasis in vertebrates is maintained by two tubular networks, blood vessels (BVs) and lymphatic vessels (LVs), both of which are formed by endothelial cells and surrounding mural cells (1). The major role of LVs is to drain interstitial fluid that leaks from blood capillaries and return it to the BVs.

An insufficiency or obstruction in the lymphatic system results in lymphedema, which is characterized by disabling swelling in the affected tissues. LVs also provide a major pathway for tumor metastasis in many types of cancer, and regional lymph node metastasis has been correlated with cancer progression. Understanding the molecular mechanisms that govern lymphangiogenesis is crucial because of the importance of LVs in both normal and pathological conditions (1).

During embryogenesis, a subset of blood vascular endothelial cells (BECs) in cardinal veins begins to express the homeobox transcription factor Prox1. These prospero-related homeobox 1 (Prox1)-expressing cells differentiate into lymphatic endothelial cells (LECs) (2), which sprout from veins and migrate toward vascular endothelial growth factor (VEGF)-C-expressing mesenchymal cells (3). In *Prox1*-null mice, the sprouting of lymphatic endothelial progenitors from the veins appears unaffected at embryonic day (E)10.5; however, their migration is arrested at around E11.5–12.0, leading to a complete absence of lymphatic vasculature. This finding suggests that Prox1 is necessary to specify LEC phenotypes in a subset of venous endothelial cells (4). Furthermore, as a homeobox transcription factor, Prox1 is known to upregulate the expression of LEC markers and downregulate BEC markers in mature endothelial cells (5, 6). These findings suggest that Prox1 regulates the differentiation program of embryonic BECs to LECs by functioning as a binary transcriptional switch, turning the BEC program off and the LEC program on. Several lines of evidence have suggested that Prox1 is necessary not only for the differentiation of LECs but also for the maintenance of LEC identity (5–7). When the *Prox1* gene is ablated postnatally, the expression of LEC markers decreases, resulting in the dedifferentiative reprogramming of LECs to BECs.

Postnatal lymphangiogenesis is regulated by the differentiation of CD11b+ macrophages into LECs (8) and proliferation of existing LECs (9). LEC proliferation is regulated by various growth factors and cytokines, such as VEGF-C/D, VEGF-A, fibroblast growth factor 2, hepatocyte growth factor, insulin-like growth factor 1, and angiopoietin 1 (reviewed in ref. 10).

Significance

Because lymphatic vessels (LVs) play critical roles not only in physiological processes such as maintenance of fluid homeostasis but also in pathological conditions including cancer metastasis, identification of factors that control LV formation is crucial. Signals mediated by activin receptor-like kinase 1 (ALK-1), a receptor for bone morphogenetic protein 9 (BMP-9), have been implicated in the formation of blood vessels because of its linkage to a human vascular disease. However, their roles in LV formation largely remain to be elucidated. Here, we show that BMP-9/ALK-1 signals inhibit LV formation in physiological and pathological conditions. Furthermore, we elucidated its molecular mechanisms in detail, using both in vivo and in vitro systems. These findings will help develop therapeutic strategies for LV-related diseases such as lymphedema and cancer.

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Although many factors have been identified as prolymphangiogenic factors, there have been few reports on endogenous antilymphangiogenic factors. In addition to interferon-γ (11), we previously reported that TGF-β is a negative regulator of lymphangiogenesis (12). Although both these inhibitors decrease Prox1 expression, the molecular mechanisms and physiological relevance of their actions remain to be understood.

The TGF-β superfamily consists of more than 30 structurally related members, including TGF-βs, activin, and bone morphogenetic proteins (BMPs; reviewed in ref. 13). The BMP family consists of four subfamilies, including BMP-9, one of the TGF-β superfamily ligands, which has been implicated in angiogenesis (reviewed in ref. 14). Activin receptor-like kinase 1 (ALK-1) is a specific type I receptor for BMP-9. *ALK1*, its coreceptor endoglin (*ENG*), and the downstream signal-transmitting molecule *SMAD4* have been identified as causal genes for the genetic vascular disorder known as hereditary hemorrhagic telangiectasia (HHT) (15–17). *Alk1*-deficient mice exhibit abnormal vascular phenotypes reminiscent of those of HHT patients (18–20).

Some groups have reported that BMP-9/ALK-1 signals negatively regulate angiogenesis in retina and in some in vitro experiments (21–23). However, Cunha et al. reported that treatment with ALK-1-Fc inhibited tumor angiogenesis (24). We also showed that BMP-9 induced the proliferation and migration of endothelial cells (25). Therefore, BMP-9 has a dual effect on angiogenesis that is dependent on context, the mechanisms of which still need to be elucidated.

Compared with their role in angiogenesis, the role of BMP-9/ALK-1 signals during lymphangiogenesis has not yet been clarified. Although Niessen et al. reported that ALK-1-Fc treatment perturbs the postnatal lymphangiogenesis in retina, tail, and ear skin (26), the precise mechanisms underlying this inhibition remain to be elucidated. In this study, we demonstrate that BMP-9/ALK-1 signals negatively regulate the formation of LVs in both physiological and pathological conditions by inhibiting the proliferation of LECs. We observed that this inhibition is caused by BMP-9-mediated decreases in Prox1 expression, resulting in the suppressed expression of cyclin family members. Furthermore, BMP-9 induces the dedifferentiation of LECs to BECs through a possible reduction in Prox1 expression, which is necessary to maintain LEC identity.

Results

Lymphatic Vessels Were Enlarged in Multiple Organs in *Alk1*-depleted Mice. Although *Alk1*-inducible knockout (KO) mice reportedly exhibit arteriovenous malformations in the lungs, gastrointestinal tract, uterus, and wounded skin (20), the effects of *Alk1* deletion on the lymphatic vasculature have not been investigated. To study the physiological roles of ALK-1-mediated signals in the formation of LVs, we first investigated the phenotypes of the LVs of multiple organs in *Alk1*-depleted mice [*ROSA26*-Cre^{ERT2} (tamoxifen-inducible expression system of Cre recombinase in the *ROSA26* locus); *ALK1*$^{floxed/floxed}$] (Fig. 1). The densities of LVs in the corneal limbus (Fig. 1 *A* and *D*), intestine (Fig. 1 *B* and *E*), and diaphragm (Fig. 1 *C* and *F*) of *Alk1*-depleted mice were higher than those of wild-type mice. The diameters of LVs were also significantly increased in the intestine (Fig. 1 *G* and *I*) and corneal limbus (Fig. 1 *H* and *J*) of *Alk1*-depleted mice compared with wild-type mice, suggesting enhanced proliferation of the LECs. Furthermore, although the structure of peripheral LVs in the ear skin of control mice was stable after birth at postnatal day (P)8, the number of filopodia in the LVs of *Alk1*-depleted mice increased with the appearance of typical tip cell-like structures, suggesting that these LVs were actively growing because of the loss of ALK-1 signals (Fig. 1 *K* and *L*). These results suggest that the signals mediated by ALK-1 physiologically maintain the structure of LVs by inhibiting excessive LV formation. We note that enlarged LVs were not observed in the tail dermis of *Alk1*-depleted mice, and their honeycomb structure was severely disorganized (*SI Appendix*, Fig. S1), which is consistent with a previous report (26). These results also imply that the roles of ALK-1 signals are context- and tissue-dependent.

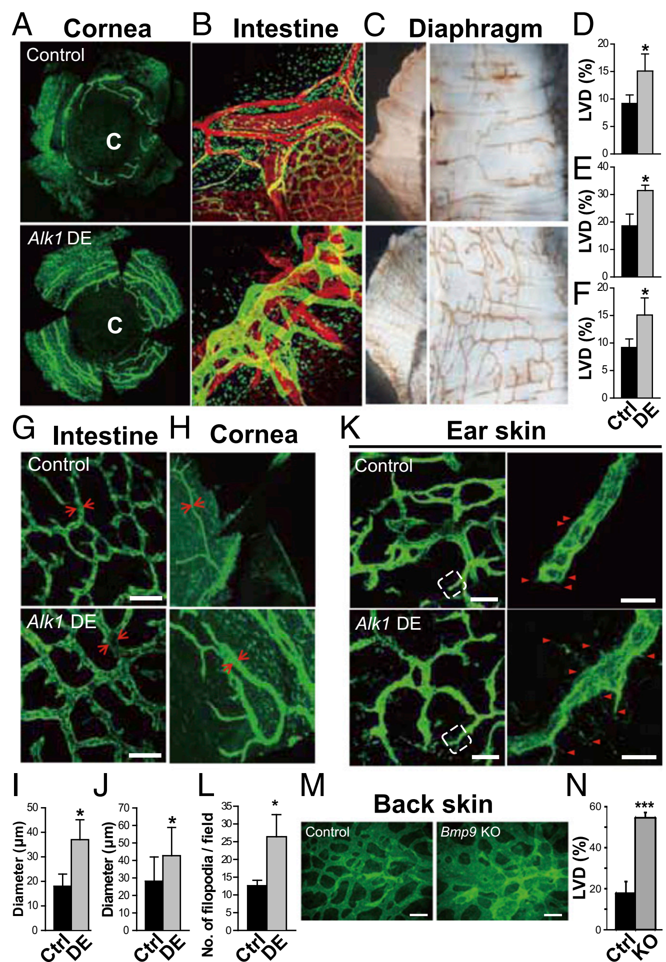

Fig. 1. Physiological roles of BMP-9/ALK-1 in the formation of LVs. (*A*–*L*) Phenotypes of the LVs from multiple organs in *Alk1*-depleted mice (DE) and control mice (Ctrl). C, center of the cornea. (*A*–*F*) LYVE-1+ lymphatic (green) and platelet endothelial cell adhesion molecule-1+ blood (red) vascular structures and graphs of the quantified density of LVs (LVD) in the cornea (*A* and *D*), intestine (*B* and *E*), and diaphragm (*C* and *F*) are shown. (*G*–*J*) LYVE-1+ lymphatic vascular structures (green) and graphs of the measured diameters of LVs in the intestine (*G* and *I*) and cornea (*H* and *J*). Arrows indicate representative lymphatics with measured diameters. Scale bars, 200 μm. (*K*) Structures of LYVE-1+ LVs in the postnatal ear skin of *Alk1*-depleted mice and control littermates in higher-magnification insets from left photos (dotted white line). (*Left*) Scale bars, 200 μm. Arrowheads indicate filopodia in the sprouting cells. (*Right*) Scale bars, 20 μm. (*L*) The number of filopodia in the LVs per field. (*M* and *N*) Phenotypes of dermal LVs in *Bmp9* KO mice. (*M*) Morphology of LV formation in the back skin tissues of *Bmp9* KO and control heterozygous mice at E15.5. Scale bars, 100 μm. (*N*) Quantification of the VEGFR3+ LV area in *Bmp9* KO mice (*n* = 4) relative to heterozygous mice (*n* = 5).

Deletion of *Bmp9* Expression Induced Dilation of the Lymphatic Vasculature in Mice. Several lines of evidence have suggested that BMP-9 and BMP-10 are the physiological ligands for ALK-1 (27-29). To examine whether the loss of *Bmp9* expression exhibits phenotypes similar to those observed in *Alk1*-depleted mice, we analyzed the structure of LVs in *Bmp9* KO mice. *Bmp9* KO mice were viable and fertile without gross abnormalities (29). We investigated the embryonic phenotypes in dermal lymphatics by performing whole-mount fluorescence immunostaining of embryonic back skin, using an antibody to VEGFR3. VEGFR3-positive LVs were actively formed at E15.5. The lymphatics of

Bmp9 KO mice were larger than those of control (*Bmp9* $^{+/-}$) mice (Fig. 1 *M* and *N*). This enlargement of LVs was caused by increase in the number of LECs in *Bmp9* KO LVs because the density of cell nuclei in *Bmp9* KO LVs was not significantly different from that in control LVs (*SI Appendix*, Fig. S2). These results suggest that BMP-9 negatively regulates LV formation during embryogenesis.

BMP-9 Decreased the Number of Lymphatic Endothelial Cells. The formation of LVs in embryonic skin is mainly caused by the proliferation of existing dermal LECs. We used primarily cultured human dermal LECs (HDLECs) to examine whether the inhibitory effects of BMP-9 on embryonic dermal lymphangiogenesis were mediated by its direct effects on LECs. When HDLECs were cultured in the presence of 0.5% (vol/vol) serum and treated with 1 ng/mL BMP-9 for 48 h, the number of HDLECs was significantly lower than that of the control (Fig. 2 *A* and *B*). Next, we examined the effects of BMP-9 on the proliferation and apoptosis of HDLECs. When HDLECs were treated with BMP-9, the number of HDLECs that were positive for MIB-1, a mitotic index protein, decreased (*SI Appendix*, Fig. S3*A*; Fig. 2*C*), suggesting that BMP-9 reduces the size of a subpopulation of proliferating HDLECs. We also performed a TUNEL assay using HDLECs treated with BMP-9 and found that BMP-9 significantly increased the subpopulation of HDLECs that underwent apoptosis (*SI Appendix*, Fig. S3*B*; Fig. 2*D*). Next, we investigated whether BMP-9 also had negative effects on other types of LECs. The number of human lung lymphatic microvascular endothelial cells (HMVEC-LLy) was also decreased on stimulation by BMP-9 (*SI Appendix*, Fig. S4*A*). These results suggest that BMP-9 inhibits lymphangiogenesis by decreasing the number of multiple types of LECs.

BMP-9 Reduced the Number of HDLECs Through ALK-1. Although Niessen et al. reported that the induction of *SMAD6* expression by BMP-9 required ALK-1 in human microvascular dermal neonatal LECs, they did not describe whether BMP-9 changed the number of LECs (26). Therefore, we attempted to examine which type I receptor mediates the BMP-9 signals that reduce the number of HDLECs. BMP family members transduce their signals through receptor complexes that phosphorylate intracellular Smad proteins (14). We used semiquantitative RT-PCR analysis to study the expression profiles of TGF-β superfamily signaling components (*SI Appendix*, Fig. S5). To compare the expression of these signaling components in LECs and BECs, we used HDLECs and human dermal BECs (HDBECs) prepared from the same donor. HDLECs and HDBECs expressed transcripts for most components of the TGF-β superfamily signaling pathways, suggesting they are capable of transducing the signals mediated by TGF-βs, activins, and BMP members. To our interest, among the BMP-specific type I receptors (i.e., *ALK-1*, *ALK-2*, *ALK-3*, and *ALK-6*), only *ALK-1* and *ALK-2*, both of which are reportedly activated by BMP-9 (22), were expressed in HDLECs and HDBECs. In accordance with these results, we observed that the addition of BMP-9 induced the phosphorylation of Smad1/5 at equivalent levels in HDLECs and HDBECs (*SI Appendix*, Fig. S4*B*). Phosphorylation of Smad1/5 by BMP-9 was also observed in HMVEC-LLy (*SI Appendix*, Fig. S4*C*), suggesting that BMP-9 activates BMP-specific intracellular Smad signals.

David et al. have shown that BMP-9 is present at a high level in serum, as the growth inhibitory effects of serum on BECs were canceled by an anti-BMP-9 neutralizing antibody (21). When an ALK-1-Fc decoy receptor was added to HDLEC culture in the presence of 5% (vol/vol) serum, the number of HDLECs grew significantly (Fig. 3*A*), suggesting that BMP-9 and/or BMP-10 in serum decreases the number of HDLECs.

We next studied the physiological type I receptor through which BMP-9 signals elicit inhibitory effects on LEC proliferation. Previous reports have shown that BMP-9 binds both ALK-1 and ALK-2, both of which are expressed in HDLECs (*SI Appendix*, Fig. S5) (22). However, the receptor that is physiologically relevant to BMP-9-mediated signaling in HDLECs remains to be elucidated. When *ALK-1* expression was knocked down by siRNAs in HDLECs, the BMP-9-induced expression of *ID-1* was abrogated (*SI Appendix*, Fig. S6*A*). Furthermore, silencing *ALK-1* expression also canceled the BMP-9-induced reduction of the number of HDLECs (Fig. 3*B*). In contrast, knocking down *ALK-2* expression did not alter the expression of BMP-9 target genes or the number of HDLECs. These results suggest that ALK-1, but not ALK-2, is necessary for BMP-9-mediated signals in HDLECs. *ALK-2* expression was also induced by BMP-9 via ALK-1 (*SI Appendix*, Fig. S6*A*).

Furthermore, when a constitutively active *ALK-1* mutant (ca*ALK-1*) was adenovirally transduced into HDLECs (*SI Appendix*, Fig. S6*B*), the number of HDLECs fell in the absence of BMP-9 (Fig. 3*C*), suggesting that the activation of ALK-1 signals was sufficient to mimic the inhibitory effects of BMP-9 on the number of HDLECs. These results suggest that BMP-9 signals through ALK-1 to decrease the number of HDLECs.

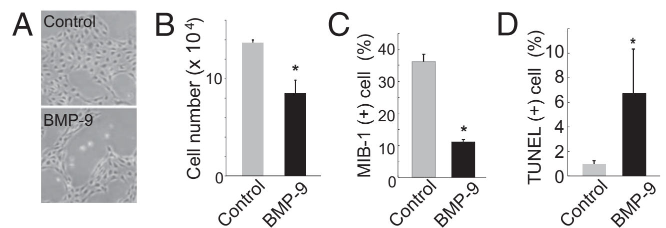

Fig. 2. Effects of BMP-9 on the number of lymphatic endothelial cells. (*A*) Morphological differences in HDLECs treated with BMP-9 for 24 h relative to control cells. (*B*) Number of HDLECs after 48 hours of treatment with BMP-9 compared with controls. (*C* and *D*) Quantification of MIB-1+ proliferating (*C*) and TUNEL+ apoptotic (*D*) HDLECs treated with BMP-9 vs. control.

BMP-9 Directly Downregulated *PROX1* Expression via ALK-1. To screen for factors that are involved in the BMP-9-induced inhibition of HDLEC proliferation, we performed cDNA microarray analyses to investigate the genome-wide effects of BMP-9 on the HDLEC transcriptome profile (*SI Appendix*, Tables S1 and S2). Gene ontology analysis revealed that the top five gene ontology annotation clusters that corresponded to the early-response genes for BMP-9 treatment (4 h) included annotations associated with vascular development, transcriptional regulation, and BMP signaling (*SI Appendix*, Fig. S7*A* and Table S3). In contrast, the top five clusters that corresponded to the late-response genes for BMP-9 treatment (24 h) were associated with cell surface proteins, cytoskeletal regulation, cell cycle, and cell death (*SI Appendix*, Fig. S7*B* and Table S4). This result suggested that the BMP-9-induced modification of transcriptional programs that were involved in vascular development was in an early phase and subsequently caused phenotypic changes in HDLECs. Therefore, we hypothesized that BMP-9/ALK-1 signals directly modulate the expression of transcription factors that regulate the expression of cell-cycle-related factors. We identified *PROX1* as one of the most downregulated genes of the early-response genes that reacted to BMP-9 treatment (*SI Appendix*, Fig. S7*C* and Table S1). We confirmed this finding at the RNA and protein levels, using quantitative RT-PCR analysis (Fig. 4*A*) and Western blotting (Fig. 4*B*), respectively.

We next examined how BMP-9 directly downregulated *PROX1* expression. Because the BMP-9-induced decrease in the expression of *PROX1* and *SMAD7*, a direct target of BMP/Smad signals, was observed within 1 h of treatment (*SI Appendix*, Fig. S8*A*) and was not altered by the addition of cycloheximide (*SI Appendix*, Fig. S8*B*), *PROX1* appeared to be directly regulated by BMP-9 without de novo protein synthesis. We also examined whether BMP-9 induced a decrease in *PROX1* expression through ALK-1. When the expression of *ALK-1*, but not *ALK-2*, was knocked down by specific siRNAs, BMP-9 failed to reduce

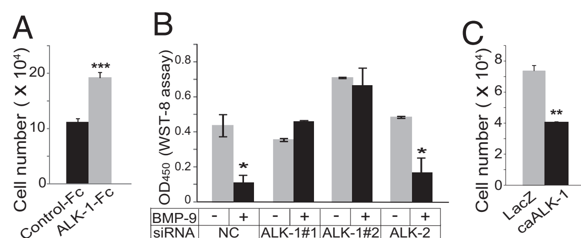

Fig. 3. Effects of ALK-1-mediated signals on the number of lymphatic endothelial cells. (*A*) Number of HDLECs after treatment with ALK-1-Fc for 48 h relative to control. (*B*) Number of HDLECs after treatment with siRNA for ALK-1 or ALK-2 for 48 h in the presence or absence of BMP-9 relative to control. NC, negative control. (*C*) Number of HDLECs infected with Ads encoding ca*ALK-1* or *LacZ* for 48 h.

PROX1 expression (*SI Appendix*, Fig. S8*C*). Furthermore, the adenoviral expression of ca*ALK-1* in HDLECs decreased *PROX1* expression (*SI Appendix*, Fig. S8*D*). These results suggest that ALK-1 mediates the BMP-9-induced downregulation of *PROX1* expression.

Silencing *PROX1* Expression Reduced the Number of HDLECs. To examine the causal relationship between BMP-9-induced decreases in cell number and reduced *PROX1* expression, we knocked down *PROX1* expression in HDLECs using siRNA and found that the cell number was decreased in the same manner as that observed with respect to BMP-9 (*SI Appendix*, Fig. S9). Furthermore, when two different Prox1-specific siRNAs that reduced *PROX1* expression to different extents were used, the levels of *PROX1* expression were correlated with the number of HDLECs (Fig. 4*C*). These results suggest that the effect of BMP-9 on the number of HDLECs results at least partly from the decreased expression of *PROX1*.

Decreased Expression of Cyclin Family Members by BMP-9 and Silencing of *PROX1* Expression Were Involved in the BMP-9-induced Decrease in HDLEC Number. Finding that treatment with BMP-9 and knockdown of *PROX1* expression decreased the number of HDLECs prompted us to identify the factors whose expression is regulated by BMP-9 and Prox1. We performed cDNA microarray analysis using HDLECs transfected with control siRNA (siNC) or Prox1 siRNA (siProx1) and compared results using siProx1-treated or siNC-treated samples with results obtained from BMP-9-treated samples (*SI Appendix*, Tables S1, S2, and S5). We found that a number of genes were concomitantly decreased in both siProx1-treated samples and samples that had been treated with BMP-9 for 24 h. Consistent with the late effects of BMP-9 on cell-cycle-related genes in gene ontology analysis, we observed that some cyclin family genes were significantly suppressed by both the 24-h BMP-9 treatment and siProx1 treatment (Fig. 4 *D* and *E*).

Cyclin family members have been shown to regulate the cell cycle. In particular, the cyclin E2 gene (*CYCLINE2*, or *CCNE2*) has been reported as a target gene of Prox1 (5). To examine whether CCNE2 was a causative factor involved in the BMP-9-induced decrease in HDLEC number, we silenced *CCNE2* expression in HDLECs, using siRNA. When *CCNE2* expression decreased, the HDLEC number decreased in a dose-dependent manner (Fig. 4*F*). These results suggest that the reduction of Prox1 expression by BMP-9 leads to an alteration in cell-cycle-related gene expression, which in turn results in a decrease in the number of HDLECs.

BMP-9 Treatment and Prox1 Knockdown Each Induced the Reprogramming of LECs to BECs. Several lines of evidence have suggested that endogenous Prox1 expression is necessary to maintain LEC identity (5–7). Johnson et al. reported that the loss of *Prox1* expression not only reduced the expression of various LEC markers but also increased the expression of BEC markers (7). Furthermore, we reported that BMP-9 increases the expression of various BEC markers in various types of BECs (25). Taken together with the present finding that BMP-9 decreases Prox1 expression in LECs, we hypothesized that BMP-9 induces HDLECs to undergo phenotypic changes from the LEC phenotype to the BEC phenotype.

To test this possibility, we performed gene set enrichment analysis, using the list of BEC- and LEC-specific genes reported by Petrova et al. (5). This analysis demonstrated that the expression levels of most LEC-type genes were downregulated [false discovery rate (FDR) q < 0.001] and that many BEC-type genes were up-regulated as a result of BMP-9 treatment (FDR q = 0.002) (Fig. 5 *A* and *B*; *SI Appendix*, Table S6). Furthermore, BMP-9 more clearly suppressed and elevated the expression of Prox1-dependent LEC- and BEC-specific genes, respectively (Fig. 5 *B* and *C*), suggesting that the effects of BMP-9 on BEC- and LEC-specific genes may be largely attributable to the suppression of Prox1. Sixty-two of 167 LEC-type genes were downregulated, and 27 of 135 BEC-type genes were upregulated by the BMP-9 treatment, especially in 24-h observations (*SI Appendix*, Table S6). Representative genes that express well-known LEC markers (e.g., *VEGFR3*, *ANGPT2*, *PODOPLANIN*, *NEUROPILIN-2*, and *LYVE-1*) were downregulated (Fig. 5*D*), whereas those expressing well-known BEC markers (e.g., *ENG*, *TIE-2*, *VEGFR2*, and *NEUROPILLIN-1*) were upregulated (Fig. 5*E*). These results suggest that BMP-9 not only decreases the number of LECs but also induces the dedifferentiation of LECs to their ancestral BECs.

BMP-9 Inhibited Lymphangiogenesis in a Mouse Model of Chronic Aseptic Peritonitis. Up to this point, we have shown that BMP-9/ALK-1 signals inhibit the proliferation of in vitro–cultured LECs and the in vivo formation of LVs under physiological conditions. Because lymphangiogenesis is observed and plays important roles in inflammation and cancer (1), we examined whether our findings were observed under such pathological conditions.

We used a mouse model of chronic aseptic peritonitis to examine whether BMP-9 inhibits inflammatory lymphangiogenesis (30). To examine the effects of BMP-9, adenoviruses (Ad) encoding *lacZ*

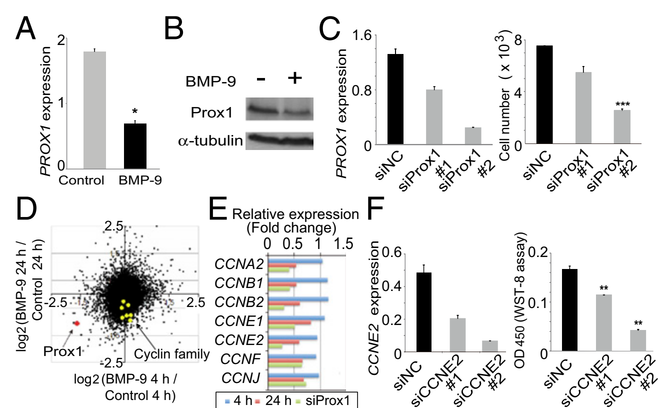

Fig. 4. Identification of Prox1 as a target of BMP-9/ALK-1 signaling and elucidation of its roles in regulating the number of HDLECs. (*A* and *B*) Prox1 mRNA (*A*) and protein (*B*) expression in HDLECs treated with BMP-9 for 4 h and 6 h, respectively. (*C*) *PROX1* expression (*Left*) and HDLEC cell number (*Right*) after sham siRNA knockdown and Prox1 siRNA knockdown. (*D*) Scatter plot depicting the fold change of expression for every gene transcript in HDLECs treated with BMP-9 for 4 h and 24 h. The expression of *PROX1* (red dot) and cyclin family members (yellow dots) are indicated. (*E*) Relative expression of several cyclin family members (yellow dots in *D*) among HDLECs treated with BMP-9 for 4 h and 24 h and treated with siRNA against Prox1. (*F*) Analysis of *CYCLINE2* (*CCNE2*) expression (*Left*) and HDLEC cell number (*Right*) after sham siRNA knockdown (siNC) and CCNE2 siRNA knockdown (siCCNE2).

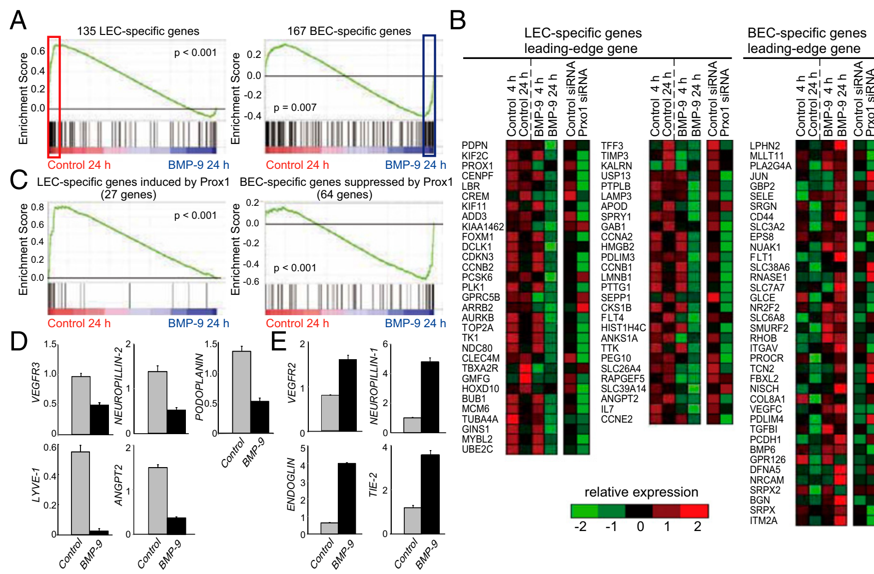

Fig. 5. Effects of BMP-9 on the maintenance of lymphatic endothelial cell identity. (*A*) Gene set enrichment analysis of HDLECs treated with and without BMP-9 using the LEC-specific (*Left*) and BEC-specific (*Right*) gene sets. (*B*) A heat map representing the relative expression change in selected LEC-specific genes (*Left* and *center*) and selected BEC-specific genes (*Right*) in HDLECs treated with BMP-9 for 4 h and 24 h and treated with Prox1 siRNA. (*C*) Gene set enrichment analysis of HDLECs treated with and without BMP-9, using sets of LEC-specific (*Left*) and BEC-specific (*Right*) genes whose expression is regulated by Prox1. (*D* and *E*) The expression of representative LEC (*D*) and BEC (*E*) markers in HDLECs treated with BMP-9 for 24 h vs. control.

(control) and *BMP9* were intraperitoneally administered with thioglycollate medium (a proinflammatory agent) to immune-competent BALB/c mice. By day 16, inflammatory plaques consisting mainly of macrophages had formed on the peritoneal surface of the diaphragm. Diaphragms from killed mice were subjected to immunostaining for LYVE-1, a LEC marker. The area of LYVE-1-positive tissue was significantly diminished in the diaphragms of Ad-*BMP9*-injected mice compared with the control diaphragms of mice injected with Ad encoding *lacZ* (Fig. 6*A*). This result suggests that BMP-9 inhibits inflammatory lymphangiogenesis in vivo.

BMP-9 Inhibited Tumor Lymphangiogenesis in Models of Mouse Breast Cancer Allografts. We used a mouse breast cancer allograft model that employs 4T1 cells to study the roles of BMP-9 in tumor lymphangiogenesis. We established murine 4T1 breast carcinoma cells that stably expressed *GFP* (control) or *BMP9* transgenes. We confirmed that expression of the *BMP9* transgene did not affect the rate of 4T1 cell proliferation in vitro (*SI Appendix*, Fig. S10). Both of these cell lines were s.c. inoculated into immunocompromised BALB/c nude mice to obtain tumors. As we previously reported in a mouse xenograft model of human pancreatic BxPC3 cancer, expression of BMP-9 increased the density of blood vessels (*SI Appendix*, Fig. S11), suggesting that BMP-9 induced tumor angiogenesis in this breast cancer allograft model. In contrast, as shown in Fig. 6*B*, tumor LVs that stained positive for LYVE-1 were significantly decreased by treatment with BMP-9. These results suggest that BMP-9 inhibits lymphangiogenesis in tumors.

Discussion

In the present study, we have demonstrated that BMP-9/ALK-1 signaling inhibits lymphangiogenesis under both physiological and pathological conditions. We previously reported that BMP-9/ALK-1 signals induced the proliferation of BECs by inducing the expression of *VEGFR2* and *TIE-2*, both of which induce BEC proliferation (25). It is of interest to note that this induction of *VEGFR2* and *TIE-2* by BMP-9 was observed in LECs (Fig. 5*E*). These results suggest that BMP-9/ALK-1 signals induce the expression of common target genes that activate the BEC program in both BECs and LECs. However, BMP-9 elicited opposite effects in LECs. We reasoned that these differential effects of BMP-9 on BECs and LECs were caused by the expression of Prox1 in LECs. BMP-9 downregulated Prox1 expression (Fig. 4; *SI Appendix*, Fig. S12), which led to the inhibition of LEC proliferation (Fig. 4; *SI Appendix*, Fig. S12) and the dedifferentiation of LECs to BECs (Fig. 5; *SI Appendix*, Fig. S12). In summary, the inhibitory effects of BMP-9/ALK-1 signaling to inactivate the LEC program won vs activation of the BEC program (*SI Appendix*, Fig. S12). In agreement with this model, the inhibitory effects of BMP-9 on LECs were partially canceled when HDLECs were treated with BMP-9 in combination with VEGF-A (a ligand of VEGFR2) (*SI Appendix*, Fig. S13).

Although we showed that BMP-9 decreases the number of LECs through the downregulation of Prox1 expression, the BMP-9-induced reduction in the expression of other proliferation-related factors, including *C-MYC*, and antiapoptotic factors, including *BCLXL*/*BCL2L1*, was independent of Prox1; silencing *PROX1* expression did not alter their expression (*SI Appendix*, Tables S1 and S5). These results suggest that BMP-9 decreases the LEC population through Prox1-dependent and Prox1-independent mechanisms (*SI Appendix*, Fig. S12).

Niessen et al. studied the roles of ALK-1 signaling in the formation of LVs by systemic injection of neonatal mice with an ALK-1-Fc decoy receptor or anti-ALK-1 neutralizing antibody to inhibit ALK-1 signaling, which resulted in defective lymphatic development in multiple organs (26). Although they observed

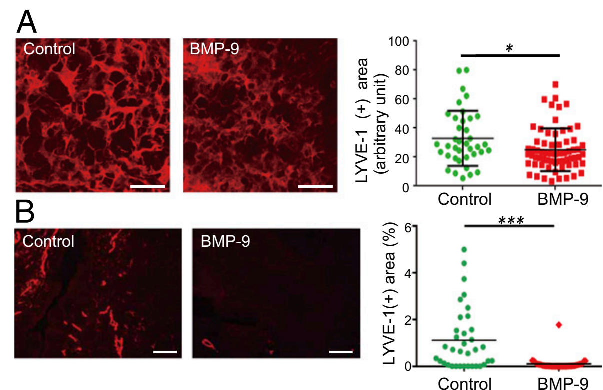

Fig. 6. Roles of BMP-9 in inflammatory and tumor lymphangiogenesis. (*A*, *left*) Images of the thioglycollate-induced formation of LVs in the peritoneal surface of murine diaphragms that were adenovirally injected with *lacZ* (Control) or *BMP9*. Scale bars, 100 μm. (*A*, *right*) Quantification of the relative area of newly formed LVs in the diaphragm. (*B*, *left*) Images of the formation of LVs in tumors derived from allografted murine breast carcinomas expressing *GFP* (control) or *BMP9*. (*B*, *right*) Quantification of the relative area of LVs in these tumors. Scale bars, 100 μm.

that the blockade of ALK-1 signals led to the disorganization of lymphatic structures, they did not describe whether LVs were enlarged. The variance from our finding that deletion of the ALK-1 gene results in the enlargement of LVs in multiple organs (Fig. 1) may have been caused by differences in the method used to inhibit ALK-1 signaling. Furthermore, this variance may also have been caused by the contribution of BMP-10, which is another physiological ligand of ALK-1 (29). Although *Bmp10*-deficient mice exhibit severe defects in cardiac tissues, no phenotypes in vascular systems have been described in *Bmp10*-deficient mice (27), suggesting that the physiological roles of BMP-10 may be limited to the cardiac development. When BMP-10 was added to the culture of HDLECs, their number was decreased with the decrease in the Prox1 expression (*SI Appendix*, Fig. S14), suggesting that BMP-10 is capable of negatively regulating the formation of LVs in a similar manner as BMP-9. Because the expression of BMP-10 is restricted to heart and developmentally regulated, the contribution of BMP-10 in combination with BMP-9 to embryonic and postnatal lymphangiogenesis needs to be studied, using *Bmp10*-deficient mice.

TGF-β activates the ALK-5 type I receptor, which induces the phosphorylation of Smad2/3 and their nuclear translocation with Smad4 and inhibits the proliferation of vascular endothelial cells in vitro (13). In lens epithelium, nuclear accumulation of Smad4 has been implicated in the regulation of Prox1 expression (31). In addition, we previously reported that TGF-β also inhibited the proliferation of HDLECs in vitro and lymphangiogenesis in vivo (12). Notably, TGF-β also downregulates *PROX1* expression in HDLECs. It is of interest whether or not this decrease in *PROX1* expression is involved in the TGF-β-induced inhibition of lymphangiogenesis. Furthermore, the molecular mechanisms of how two distinct signaling pathways that are mediated by Smad1/5/8 and Smad2/3 regulate the *PROX1* expression need to be elucidated in the future.

BMP-9/ALK-1 signals have been implicated in various human diseases. Mutations in the *ALK1* gene cause HHT2 (16). LV dysfunction has not been reported in HHT patients. Because HHT is an autosomal dominant disorder, the mutation of one copy of the *ALK1* gene may not be enough to cause the lymphatic phenotypes observed in the present study. Furthermore, we observed that the formation of LVs decreased in BMP-9-expressing tumors, whereas the formation of BVs increased. The expression of BMP-9 in various types of tumors has been reported previously. In ovarian tumors, BMP-9 expression was shown to be elevated, which led to the promotion of tumor cell proliferation in an autocrine fashion (32). In contrast, BMP-9 expression was reportedly decreased or absent in prostate cancer (33). Because BMP-9 induces apoptosis in prostate carcinoma cells, the effects of BMP-9 appear to be context-dependent. It will be of great interest to study whether LV density differs among tumor specimens that express different levels of BMP-9. Recently, BMP-9/ALK-1 signals have been attracting attention as a target of cancer therapy, as the inhibition of ALK-1 signaling interferes with tumor angiogenesis, which may inhibit tumor growth (24, 34). Because the formation of LVs in tumors is associated with lymph node metastases in cancer, it is of interest to know whether blocking ALK-1 signals might influence tumor lymphangiogenesis.

Materials and Methods

HDLECs and HMVEC-LLy were purchased from Lonza and characterized (*SI Appendix*, Fig. S15). The 4T1 murine breast carcinoma cell line was obtained from the American Type Culture Collection. 293FT cells were obtained from Invitrogen. More information is provided in *SI Appendix*, *Materials and Methods* and *SI Appendix*, Table S7.

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