Bone Morphogenetic Protein 2 Signaling Negatively Modulates Lymphatic Development in Vertebrate Embryos

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Rationale: The emergence of lymphatic endothelial cells (LECs) seems to be highly regulated during development. Although several factors that promote the differentiation of LECs in embryonic development have been identified, those that negatively regulate this process are largely unknown.

Objective: Our aim was to delineate the role of bone morphogenetic protein (BMP) 2 signaling in lymphatic development.

Methods and Results: BMP2 signaling negatively regulates the formation of LECs. Developing LECs lack any detectable BMP signaling activity in both zebrafish and mouse embryos, and excess BMP2 signaling in zebrafish embryos and mouse embryonic stem cell-derived embryoid bodies substantially decrease the emergence of LECs. Mechanistically, BMP2 signaling induces expression of miR-31 and miR-181a in a SMAD-dependent mechanism, which in turn results in attenuated expression of prospero homeobox protein 1 during development.

Conclusions: Our data identify BMP2 as a key negative regulator for the emergence of the lymphatic lineage during vertebrate development. (Circ Res. 2014;114:56-66.)

Key Words: BMP2 protein • developmental biology • lymphatic vessels • microRNAs
BMP2, influence vascular development in vertebrates,\textsuperscript{28–32} and development, diverse components of BMP signaling, including may negatively regulate lymphatic development. During development, different components of BMP signaling, including BMP2, influence vascular development in vertebrates,\textsuperscript{28–32} and dysregulation of BMP signaling through genetic mutations has been implicated in diverse human pathological conditions, including hereditary hemorrhagic telangiectasia and primary pulmonary arterial hypertension.\textsuperscript{33–35} Mechanistically, BMP ligands transduce their activity via various signaling cascades. On ligand binding, activated type I BMP receptors phosphorylate Smad transcription factors SMAD1, SMAD5, and SMAD8, which along with the receptor-independent SMAD protein, SMAD4, translocate to the nucleus and regulate the expression of the target genes, such as the inhibitor of DNA-binding (Id) gene family.\textsuperscript{36} In addition to the SMAD-dependent pathway, BMP ligands can transduce signaling via MAP kinase pathways.\textsuperscript{37} During vascular development, BMP signaling seems to possess a context-dependent function to regulate diverse aspects of EC behaviors. For instance, BMP2 functions as a venous-specific proangiogenic cue in zebrafish\textsuperscript{38} and is essential for the stalk cell fate in the mouse hindbrain plexus,\textsuperscript{31} whereas BMP9 seems to function as a circulatory vascular quiescence factor.\textsuperscript{39–40}

In this article, we present compelling evidence indicating a novel role of BMP2 signaling as a negative modulator for the lymphatic fate. We show that BMP2 signaling inhibits the differentiation of LECs in zebrafish embryos and mouse embryoid bodies (EBs). Mechanistically, we demonstrate that BMP2 signaling promotes the expression of miRNAs, including miR-31 and miR-181a, to negatively regulate PROX1 expression, in a SMAD-dependent but not ERK1/2-dependent manner. Although several factors have been described to negatively modulate lymphangiogenesis postnatally, including endostatin, TGF-β, and IFN-γ,\textsuperscript{31–34} these data, to our knowledge, are the first in vivo demonstrations of a negative modulator for lymphatic fate during vertebrate development.

### Methods

An expanded Material and Methods section can be found in the Online Data Supplement.

### Zebrafish Husbandry, Heatshock Conditions, Drug Treatment, and MO Injection

Zebrafish (\textit{Danio rerio}) embryos were raised as previously described.\textsuperscript{47} The following transgenic fish lines were used: Tg(flh1a:EGFP)\textsuperscript{28}; Tg(flh1a:ep:DsRedEx)\textsuperscript{29}; Tg(kdrl:GFP)\textsuperscript{30}; Tg(kdsp70l:bmp2b)\textsuperscript{15}; Tg(Bre::nmCherry)\textsuperscript{24} (this study); and TgBAC(prox1:Kdrl-TUS:uncTagRFP)\textsuperscript{16} (this study). Microinjections of morpholino anti-sense oligonucleotide (MO) were performed as previously described.\textsuperscript{30} Conditions for heatshock or drug treatments and sequence of MOs can be found in the Online Data Supplement. Detailed description of the construction of Tg(Bre::nmCherry)\textsuperscript{24} and TgBAC(prox1:Kdrl-TUS:uncTagRFP)\textsuperscript{16} transgenic lines can be found in the Online Data Supplement.

### Cell Culture and Embryoid Body Differentiation

HMVEC-dLy (Cat\#CC-2810; Lonza) were grown on 0.1% gelatin-coated plates with EGM2-MV (Lonza) media and used at passages 3 to 7. HMVEC-dLy were stimulated with 50 ng/mL BMP2 (Sigma H4791) or 5 μg/mL actinomycin D (Sigma A1410). siRNA silencing was performed with SMARTsi (SI00076020; Qiagen) and Control Allstar siRNAs (Qiagen) and Lipofectamine 2000 (Invitrogen).

Embryonic stem cells were cultured on irradiated mouse embryonic fibroblast feeder cells in Dulbecco modified Eagle medium (Invitrogen) supplemented with 15% knockout serum replacement (Invitrogen), leukemia inhibitory factor–conditioned media, 0.1 mmol/L nonessential amino acid (MEM-NEAA; Invitrogen), 2 mmol/L L-glutamine (Invitrogen), 1 mmol/L sodium pyruvate (Invitrogen), 1% penicillin and streptomycin (Invitrogen), and 0.1 mmol/L 2-mercaptoethanol (Sigma). When embryonic stem cells reached 70% to 80% confluency, cultures were dissociated with 0.25% Trypsin-EDTA (Invitrogen) and then transferred to gelatin-coated tissue culture dishes to adapt to feeder-free conditions. During this adaptation stage, embryonic stem cells were grown in Iscove modified Dulbecco medium (Invitrogen) supplemented with 15% knockout serum replacement, leukemia inhibitory factor–conditioned medium, 2 mmol/L L-glutamine, 1% penicillin and streptomycin, and 0.1 mmol/L 1-thio glycerol (Sigma). After 2 days in feeder-free culture, mouse embryonic stem cell colonies were dissociated, and hanging drops containing 540 embryonic stem cells in 15 μL differentiation medium were prepared. EBs formed within 2 days of hanging drop cultures in differentiation medium containing Iscove modified Dulbecco medium, 15% fetal bovine serum (Genniun), 50 μg/mL ascorbic acid (Sigma), and 2 mmol/L L-glutamine. To differentiate EBs, 2 days postcoitum (dpc) EBs were plated on gelatin-coated 8-well chamber slides (Laboratory-Tek) or 12-well dishes at a density of 6.25 EB/cm\textsuperscript{2} in complete differentiation media without leukemia inhibitory factor. To manipulate EBs, BMP2, VEGF-C (2176-VC; R&D System), Noggin (6057-NG; R&D System), and DHH1 (D8946; Sigma) were used. At 5 and 7 dpc, EBs were treated with 0.1% bovine serum albumin or growth factors (200 ng/mL VEGF-C, 100 ng/mL BMP2, 250 ng/mL Noggin, 0.1 μmol/L DHH1). At 8 dpc, EBs were analyzed by immunocytochemistry or flow cytometry with CD31 and LYVE1 antibodies. Immunohistochemistry samples were imaged by confocal microscopy (Leica SP5), equally processed by Adobe Photoshop, and quantified by Image J (National Institutes of Health). For quantification, images were acquired at 2 to 3 random fields of the periphery of ≥4 EBs per condition per experiment. Each experiment was performed ≥3 times. The ratio of lymphatic vessel area to total vascular area was determined by differential immunofluorescence stains (LYVE1/CD31).

### Zebrafish Imaging and Reconstruction

At 4 dpf, Tg(flh1a:ep:DsRedEx)\textsuperscript{30}; Tg(kdrl:GFP)\textsuperscript{15} or Tg(kdsp70l:bmp2b)\textsuperscript{15}; TgBAC(prox1:Kdrl-TUS:uncTagRFP)\textsuperscript{16} double-transgenic embryos were dechorionated, anesthetized with tricaine, and embedded in 1% agarose/phosphate-buffered saline on a glass-bottom Petri dish (MatTek). Trunk regions immediately dorsal to the yolk extension were imaged with a Nikon T-E Eclipse confocal

### Nonstandard Abbreviations and Acronyms

\begin{table}
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\hline
BEC & blood endothelial cell \\
BMP & bone morphogenetic protein \\
CV & cardinal vein \\
EB & embryoid body \\
flh1a & friend leukemia integration 1a \\
kdr1 & kinase insert domain receptor like \\
LEC & lymphatic endothelial cell \\
MO & morpholino anti-sense oligonucleotide \\
PROX1 & prospero homeobox protein 1 \\
TD & thoracic duct \\
VEGF-C & vascular endothelial growth factor-C \\
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microscope with Hamamatsu camera (C9100-50). Velocity software (Perkin Elmer) was used to generate 3-dimensional optical projections.

Quantification of Lymphatic Development in Zebrafish Embryos
To quantify lymphatic defects in developing transgenic embryos, the percentage of TD formation was quantified in 10 consecutive segments in Tg(fli1a.ep;DsRedEx)um13;Tg(kdrl:EGFP)s843 or Tg(kdrl:GFP)s84;TgBAC(prox1a:KalT4-UAS:uncTagRFP)jum5 double-transgenic embryos. At least 10 embryos per genotype/condition were analyzed from ≥3 independent clutches.

Statistical Analyses
All data were analyzed using the 2-tailed Student t test assuming unequal variance. P<0.05 was considered significant.

Results
BMP2 Signaling Inhibits Zebrafish Lymphatic Development
We have previously demonstrated that Bmp2b (an ortholog for mammalian BMP2) signaling components are enriched in the caudal vein plexus and surrounding mesenchyme and are required for venous angiogenesis in the developing zebrafish.30,32 Interestingly, Bmp2b seems to increase the number of venous ECs without affecting cell proliferation.30,32 Considering that LECs have a venous origin during development, it is tempting to speculate that an excessive level of Bmp2b signaling may increase the number of venous ECs at the expense of presumptive LECs.

To examine this possibility, we first overexpressed Bmp2b in zebrafish embryos with Tg(fli1a:DsRed);Tg(kdrl:EGFP) double transgenic background and examined the effects on lymphatic development. Because kdrl (kinase insert domain receptor-like) is expressed only in BECs, whereas fli1a (friend leukemia integration 1a) is expressed in both LECs and BECs, emerging LECs can be easily distinguished (Online Figure I).12,17 We found that Bmp2b-overexpressing embryos completely lack any detectable lymphatic structures (Figure 1A and 1B). For instance, although wild-type embryos have a clearly separated TD between dorsal aorta and caudal vein, it is largely absent in embryos with excessive Bmp2b expression (Figure 1A and 1B). To further confirm the lymphatic defects in Bmp2b-overexpressing embryos, we used another transgenic line, TgBAC(prox1a:KalT4-UAS:uncTagRFP)jum5.
hereafter referred to as Tg(prox1a:RFP). In Tg(prox1a:RFP), strong RFP expression can be detected in developing lens, heart, liver, neural tube, and somites, in addition to lymphatic vessels, which is reminiscent of the previously reported expression of endogenous prox1a (Online Figure II).

In wild-type embryos, consistent with previous reports,12 prox1a:RFP+ and flila:EGFP+ cells first emerged along the horizontal myoseptum as a string of individual LECs approximately at 50 hours postfertilization. Subsequently, RFP+ LECs migrated dorsally and ventrally along arterial intersegmental vessels to form lymphatic structures (Online Figure IIB and IIC). On Bmp2b overexpression, we found that RFP expression within lymphatic vessels was selectively diminished, whereas the expression was maintained in other organs, indicating that Bmp2b may regulate the expression of prox1a in a tissue-specific manner (Online Figure II). Consequently, at 60 hours postfertilization, LECs were completely absent in Bmp2b-overexpressing embryos, which led to failure of forming the TD at 4 dpf as observed in Tg(flila:RFP); Tg(kdrl:EGFP) double-transgenic background (Figure 1C and 1D). Conversely, inhibition of Bmp2b signaling seems to promote lymphatic growth. Treatment with DMH1, a potent chemical inhibitor for Bmp type I receptors, induced ectopic lymphangiogenesis and led to precocious and excessive ventral sprouting of the TD (Figure 1E and 1F) and increased the number of LECs in an otherwise phenotypically wild-type zebrafish embryos (Figure 1G), further supporting the idea that Bmp2b signaling may negatively regulate lymphatic development in zebrafish.

**SMAD, but Not ERK1/2, Mediates Antilymphangiogenic Effects of BMP2 Signaling**

Because Bmp signaling activates 2 main downstream effectors, SMADs and ERK1/2, in zebrafish development,55,56 we next examined which downstream effector mediates the effects of Bmp2b signaling during lymphatic development. Inhibition of Erk1/2 activity by U0126 did not alleviate the lymphatic defects in embryos with excessive Bmp2b activity. However, inhibition of SMAD activity by addition of DMH1 substantially restored the lymphatic defects in Tg(hsp70l:bmp2b)fr13 embryos (Figure 2A and 2B), indicating that SMAD activity is essential to mediate Bmp2b signaling during lymphatic development.

To examine the effect of BMP2 signaling on mammalian lymphatic specification, we used a modified mouse EB lymphatic differentiation model (Figure 3A). We found that differentiation of LECs spontaneously occurs within EBs as previously reported (Figure 3B).55,56 In this model, VEGF-C treatment starting at 5 days postplating, which is equivalent to E8.5 when the endothelial lineage emerges,57 substantially increased the number of LECs (Figure 3B), consistent with previous reports.55,56 The CD31+/LYVE1+ cells derived from mouse EBs also express podoplanin, indicating that these cells are fully differentiated LECs (Figure 3C). To examine whether Bmp2 inhibited lymphangiogenesis in this model, we cotreated EBs with BMP2 and VEGF-C. Cotreatment with BMP2 completely abrogated VEGF-C–mediated LEC induction in the EB periphery and substantially decreased the number of CD31+/LYVE1+ LECs (Figure 3D and 3E). The inhibitory effect of BMP2 stimulation was completely blocked when EBs were treated with DMH1, further corroborating that BMP2-mediated inhibition of lymphangiogenesis requires SMAD activity. Moreover, we found that inhibiting BMP signaling through the addition of the endogenous BMP inhibitor Noggin to mouse EBs resulted in increased LEC specification, further illustrating that BMP2 signaling may function to inhibit lymphatic development (Online Figure III). Taken together, these data suggest that BMP2 signaling is a highly conserved negative modulator of lymphatic development throughout vertebrates, and its antilymphangiogenic effect is mediated by SMAD activity.

**Reduced Level of BMP Signaling Activity in LECs**

Because overexpression of Bmp2b adversely affected lymphatic development, we hypothesized that Bmp2b signaling may need to be suppressed in developing LECs. To further examine whether developing LECs lack BMP2 signaling activity, we generated a transgenic zebrafish, Tg(Bre:mmCherry)n5, that expresses nuclear mCherry under the regulation of minimal BMP response element (BRE) from mouse ld2 gene59 (Online Figure IV). We reasoned that ld2 promoter would more faithfully represent Bmp2b signaling in the zebrafish vasculature because we previously found that ld2 is the most highly induced Id family member in Tg(hsp70l:bmp2b)fr13 after heatshock induction.32

In developing zebrafish, mCherry expression faithfully recapitulated the endogenous Bmp2b activity (Online Figure IV) and overlapped with previously reported BMP signaling activity during development.60 For instance, mCherry colocalized with p-SMAD-1/5/8 (Online Figure IV). In addition, mCherry expression was elevated in Bmp2b-overexpressing embryos and was decreased in embryos with reduced Bmp2b signaling (Online Figure IV), suggesting that BRE:mCherry expression can be used as a surrogate measure for Bmp2b...
signaling activity. During development, mCherry expression was widely detected at gastrula stages. At later stages, mCherry could be widely detected in diverse tissues and cell types, including all fli1a+ BECs within the arteries and veins (Online Figure IV). Unexpectedly, mCherry expression was completely absent in LECs at 3 dpf and the TD at 4 dpf zebrafish embryos (Figure 4A–4C), suggesting the lack of Bmp2b signaling activity in developing LECs.

To examine whether BMP signaling activity within emerging LECs is similarly modulated during mammalian development, we first examined the status of Bmp2b signaling within PROX1+ LECs in mouse embryos. At E11.5, pSMAD1/5/8 staining can be detected in the majority of ECs within the developing CV. In contrast, in nearby LECs, pSMAD1/5/8 staining was largely absent, supporting our idea that BMP2 signaling activity is attenuated in LECs during development (Figure 4D). Similarly, in Tg(BRE:EGFP)36 transgenic mouse embryos, where BMP signaling activity can be measured by the expression level of EGFP, we found that EGFP expression was similarly reduced in LECs compared with BECs within CVs (Figure 4E and 4F). Taken together, our data allude to an intriguing possibility that BMP2 signaling may need to be suppressed to allow the emergence of LECs. This notion is also consistent with previous findings that demonstrated the lack of BMP2 transcript in LECs.61

BMP2 Signaling Negatively Regulates PROX1 Transcripts in a SMAD-Dependent Manner
To uncover the molecular mechanisms responsible for BMP2-mediated inhibition of lymphangiogenesis, we isolated fli1a:EGFP+ ECs from wild-type and Bmp2b-overexpressing zebrafish embryos in Tg(fli1a:EGFP)y1 background and examined the expression levels of endothelial-specific transcripts critical for active angiogenesis and lymphangiogenesis (Figure 5A). Among these markers, only the expression of prox1a, the zebrafish ortholog of mammalian Prox1,12,62 was significantly reduced, suggesting that Bmp2b signaling may attenuate lymphatic development by antagonizing prox1a expression during early stages of lymphangiogenesis in zebrafish. Similarly, BMP2 stimulation significantly reduced basal PROX1 expression level in fully differentiated human dermal microvascular LECs (hLECs), demonstrating evolutionary conserved effects of BMP2 signaling on PROX1 activity (Figure 5B).

To determine the downstream effectors that mediate the inhibitory effects of BMP2 on the level of PROX1 expression, we examined the function of SMAD and ERK, 2 main effectors of BMP2, in this process. Consistent with our finding that Smad inhibition restored Bmp2b-induced lymphatic defects in zebrafish embryos (Figure 2), siRNA knockdown of SMAD4 in hLECs prevented PROX1 downregulation by BMP2
Considering that lymphatic development is highly sensitive to small fluctuations in the level of Prox1 expression, a reduction in Prox1 expression by BMP2 signaling is likely to substantially impede lymphatic development.

**miR-31 and miR-181a Function Downstream of BMP2 Signaling to Modulate PROX1 Expression**

Considering that the expression of sox18, which is known to induce transcription of prox1a during lymphatic development, was largely unchanged (Figure 5A), it is possible that Bmp2b signaling may modulate the level of prox1a transcript at the posttranscriptional level. Because BMP signaling can influence the processing of miRNAs, it is tempting to speculate that the antilymphangiogenic effect of BMP2 signaling may be mediated by a miRNA-dependent mechanism.

To explore this possibility, changes in mRNA expression on BMP2 stimulation in hLECs were evaluated using miRNA polymerase chain reaction arrays. An elevated level of BMP2 signaling significantly altered the expression level of multiple miRNAs (Figure 6A).

Interestingly, 2 of the upregulated miRNAs, miR-31 and miR-181a, have been previously reported to bind the Proxl 3′-UTR, the master transcription factor to initiate and maintain lymphatic fate, leading to transcript degradation and translational inhibition and subsequent maintenance of BEC identity. Similarly, ECs isolated from Bmp2b-overexpressing zebrafish embryos revealed increased transcript levels of miR-31 and miR-181a (Figure 6B). In zebrafish and mice, we found that both miR-31 and miR-181a are expressed in the BECs during lymphatic specification (Online Figure V), suggesting the role of these miRNAs in mediating antilymphangiogenic effects of BMP2 signaling. Consistent with previous reports that miR-31 and miR-181a post transcriptionally suppress Proxl mRNA level in humans and mice, we found functionally conserved miR-31-binding and miR-181a-binding sites in zebrafish prox1a 3′-UTR (Online Figure V). To further test that zebrafish miR-31 and miR-181a could target the zebrafish prox1a 3′-UTR, a luciferase reporter containing the full-length of zebrafish prox1a 3′-UTR was generated. We found that both miR-31 and miR-181a mimics recognized the full-length of zebrafish prox1a 3′-UTR, leading to a significant decrease in luciferase activity (Online Figure VI). In addition, injection of miRNA mimics to single-cell-stage zebrafish embryos resulted in a drastic decrease in the level of prox1a expression (Online Figure VI), further supporting our idea that miR-31...
and miR-181a may mediate Bmp2b signaling and attenuate prox1a expression. Similar to prox1a, other predicted targets of miR-31 and miR-181a, such as rho2 (in case of miR-31) and ptpn2a (in case of miR-181a), were similarly downregulated in embryos overexpressing Bmp2b (Online Figure VI).

To further investigate how BMP2 signaling increases the level of these miRNAs, we first examined the role of SMAD activity. Knockdown of SMAD4 by siRNA completely abrogated the BMP2-mediated upregulation of miR-31 and miR-181a (Figure 6C). Unexpectedly, we observed that miR-31 and miR-181a expression levels after BMP2 treatment in SMAD4 knockdown were decreased compared with vehicle treatment, suggesting the presence of potential negative feedback regulation. In addition, treatment with actinomycin D to inhibit transcription completely inhibited BMP2-induced expression of miR-31 and miR-181a (Figure 6D). Therefore, it is likely that BMP2 signaling promotes transcription of miR-31 and miR-181a in a SMAD-dependent manner.

Next, we examined whether inhibition of miR-31/miR-181a would abrogate antilymphangiogenic effects of Bmp2b signaling and, therefore, at least partially restore the lymphatic defects caused by excessive Bmp2b signaling. We found that blocking the activity of either miR-31 or miR-181a by MOs (validated in Online Figure VII) alleviated lymphatic defects in Bmp2b-overexpressing zebrafish embryos (Figure 7A). A significant portion of MO-injected embryos contained at least partially formed TD (≈70% of embryos), and a sizable portion of embryos generated complete TD (between 10% and 25%; Figure 7A and 7B). Therefore, miR-31 and miR-181a are likely to be essential to mediate antilymphangiogenic effects of Bmp2b/BMP2 signaling during lymphatic development.

Discussion

Our data present compelling evidence indicating that BMP signaling functions as a negative regulator for lymphatic fate during vertebrate development. We found that lymphatic progenitors are largely devoid of BMP signaling activity. Because it has been reported that several Bmp ligands are expressed in ECs or their vicinity during zebrafish development, including Bmp2b and Bmp4,52 it remains to be identified which specific Bmp ligands function as the major regulator for the lymphatic fate.

In our study, we found that BMP2 signaling strongly inhibits the expression of PROX1, the master regulator for the lymphatic fate, in a SMAD-dependent and miRNA-dependent manner. Taken together, our data suggest that BMP2 signaling may modulate fate specification within ECs, similar to its role in regulating blood vessel development.
role in other cell types, including osteoblasts, neurons, cardiomyocytes, and hepatocytes. Combined with our previous reports on the role of BMP2 signaling in promoting venous angiogenesis, our results illustrate the context-dependent role of BMP2 signaling during vascular development.

During lymphatic development, miRNAs seem to be important mediators for antilymphangiogenic effects of BMP2 signaling. Using miRNA polymerase chain reaction arrays, we identified that several miRNAs are upregulated by BMP2 signaling, including miR-31 and miR-181a, in a SMAD-dependent manner (Figure 6C). Considering that actinomycin D treatment completely inhibited the BMP2-induced upregulation of miR-31 and miR-181a, it is likely that active BMP2 signaling influences transcription of these miRNAs. However, we could not identify consensus SMAD-binding element within the promoter region of miR-31 and miR-181a loci. It is possible that BMP2/SMAD-dependent transcriptional activation of miR-31 and miR-181a is mediated by novel and yet-unidentified variants of SMAD-binding elements. Alternatively, activated SMAD may bind to the unknown enhancer elements that influence the transcription of miR-31 and miR-181a because SMADs can bind to enhancer located outside of promoter region. Further analyses of the promoter region of miRNAs that are upregulated by BMP2 stimulation are warranted to delineate precise molecular mechanisms on SMAD-mediated transcriptional regulation of PROX1 via miRNA-dependent mechanism. A, Confocal projection of Tg(hsp70:bmp2b) embryos injected with 5 ng control, miR-31, or miR-181a MOs, followed by heatshock at 26 hours postfertilization. Inhibition of miR-31 or miR-181a can partially rescue the lymphatic defects induced by BMP2 overexpression. Arrows point rescued thoracic duct (TD) in MO-injected embryos. B, Quantification of percent TD formation in control, miR-31, or miR-181a MO-injected wild-type and Tg(hsp70:bmp2b) embryos measured over 10 segments (n>40 for all conditions). TD formation was largely unaffected by MO knockdown of miR-31 or miR-181a in wild-type embryos. However, MO-mediated knockdown of miR-31 or miR-181a in the Tg(hsp70:bmp2b) background resulted in 50% to 70% of embryos forming at least a partial TD and between 20% to 25% forming a complete TD compared with 25% and 0% in control and MO-injected, respectively (P<0.001 for both groups). Scale bar, 50 μm. C, During development, active BMP2 signaling promotes the expression of miR-31/miR-181a in blood endothelial cells (blue) and, therefore, aids BECs to maintain their fate as venous endothelial cells. However, in presumptive lymphatic endothelial cells (green), the activity of BMP2 signaling is attenuated by unknown mechanism, thereby releasing miRNA-mediated repression of PROX1. CV indicates cardinal vein; and DA, dorsal aorta.
these miRNAs. In Bmp2b-overexpressing zebrafish embryos, we found that expression of prox1a:RFP is selectively downregulated in developing LECs without affecting the expression in other tissues. The tissue-specific enrichment of miR-31 and miR-181a, which seem to be essential to mediate anti-lymphangiogenic effects of Bmp2b signaling in BECs, may contribute to creating the tissue-specific response of Bmp2b overexpression in developing zebrafish embryos.

Because each miRNA can bind a wide variety of target mRNAs, additional targets of miR-31 and miR-181a may augment the antilymphogenic effects of Bmp2b signaling in LECs, which may contribute to creating the tissue-specific response of Bmp2b overexpression in developing zebrafish embryos. Therefore, downregulation of let-7 miRNA by BMP2 signaling activity and the formation of LECs during lymphatic development. Because diverse modifiers of BMP signaling are expressed throughout lymphatic defects. Second, it is the only lymphatic marker whose expression is downregulated in response to BMP2 signaling. Other lymphatic markers, including LYVE1 and VEGFR3, were not altered on BMP2 stimulation in hLECs concerning a previous report that stated that let-7 negatively regulates expression of TGF-βR1 to decrease TGF-β signaling, which is known to inhibit lymphatic regeneration. Therefore, downregulation of let-7 miRNA by BMP2 signaling is likely to elevate the level of TGF-β signaling, which in turn further inhibits lymphatic development and provides an additional mechanism for BMP2 signaling to modulate lymphatic development, independent of miR-31/181a and PROX1-mediated regulation.

Our analyses suggest a strong negative correlation between BMP2 signaling activity and the formation of LECs during development. Compared with BECs, the level of BMP2 signaling within LECs seems to be substantially attenuated. Because diverse modifiers of BMP signaling are expressed within ECs, it is tempting to speculate that endogenous antagonist of BMP2 may be preferentially localized or expressed near the presumptive LECs to allow them to adopt the lymphatic fate (Figure 7C). Although it is not clear how activity of BMP2 signaling is attenuated in LECs, or how other BMP ligands may play an additional role in this process, our model is consistent with previous finding that LECs do not express high levels of Bmp2 transcript. Considering that developmental cues that negatively regulate the emergence of LECs are poorly understood, our findings in this report could help us to formulate more comprehensive ideas regarding lymphatic development and to construct a theoretical framework to develop therapeutic manipulation of lymphangiogenesis in development and diseases.

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Disclosures

None.

References


What Is Known?

• Lymphatic dysfunction can result in debilitating lymphedema.
• Lymphatic vasculature arises from the preexisting venous system driven by prospero homeobox protein 1 (PROX1) expression and is promoted through several growth factors, including vascular endothelial cell growth factor-C (VEGF-C).
• Bone morphogenetic protein (BMP) signaling is a critical regulator of angiogenesis, mural cell recruitment, and cardiomyocyte specification.

What New Information Does This Article Contribute?

• BMP2 signaling negatively impacts specification of lymphatic endothelial cells in zebrafish and mouse embryonic stem cells.
• BMP2 signaling decreases the level of PROX1 transcript via SMAD-dependent and miRNA-mediated mechanisms.

The lymphatic vascular system mirrors the blood vascular system and performs several essential physiological functions, including removing excess fluids from peripheral tissues and serving as the main conduit for trafficking immune cells in the body. Subsequently, when the lymphatic system fails to function properly because of genetic defects, disease, or after surgical lymph node removal for cancer treatment, patients are at risk for development of lymphedema or disturbed immune responses. Additionally, in cancer metastasis, the lymphatic system is often used by cancerous cells to spread to distal tissues. Therefore, in both lymphedema and cancer, developing new therapies that can modulate lymphatic vessel growth and function are imperative to improve patient outcomes. However, to date, no pharmacological therapies are available to alleviate symptoms associated with lymphatic dysfunction. In this study, we have identified BMP2 signaling as a novel negative regulator of lymphatic specification during vertebrate development. BMP2 signaling inhibits the expression of PROX1, required to retain lymphatic identity, by inducing the expression of miRNAs targeting PROX1 transcripts. Manipulation of BMP2 signaling alone or in combination with other lymphangiogenic factors may provide more effective therapeutic avenues to regulate lymphatic vessel growth and function.
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Supplemental Experimental Procedures

Zebrafish husbandry, heatshock treatment, drug treatment and MO injection

*Tg(hsp70: bmp2b)* \(fr^{13}\) embryos were heat-shocked at 25 or 50hpf for 25min at 42°C. Each embryo was genotyped by PCR to determine the presence of the transgene. Drug treatments with 1μm DMH1 (Sigma, D8946) or 10μm U0126 (Sigma U120) were performed at 25hpf. Briefly, embryos were injected at the one-cell stage with 5ng of control, *miR-31*, or *miR-181a-1* MO (Gene Tools). The Standard Control (Gene Tools), *miR-31* and *miR-181a-1* MO sequences are 5'-CCTCTTACCTCAGTTACAATTTATA-3', 5'-CAGCTATGCCAACATCTTGCCATCT-3', and 5'-TTAGCTCAAAACTCACCAGCGTT-3' respectively. Confirmation of knockdown was measured by RT-PCR (See below)

Immunohistochemistry and *in situ* Hybridization

Hybridization of *miRNA-181a* (Exiqon, Cat# 18066-05), *miR-31* (Exiqon, Cat# 35184-05(zebrafish), 39153-05(mouse), and scrambled-miR (Exiqon, 99004-05) DIG-labeled probes was detected with NBT/BCIP colorimetric reaction (Roche). For co-localization, fluorescent immunohistochemistry was performed after completion of the colorimetric reaction followed by confocal microscopy with detection of NBT/BCIP in the near infrared.

Zebrafish embryos were stained with P-Smad 1/5/8 antibodies (Cell Signaling, 9516) as previously described 31. ID1:BRE-EGFP (Monteiro et al., 2008) mouse embryos were dissected and fixed overnight in 4%PFA/PBS. Embryos were embedded in OCT and 8-10μm cryosections were obtained as previously described 52. Sections were rehydrated with PBS, quenched with NH₄Cl, permeabilized with 0.1% Triton-X 100/PBS, blocked with 5% goat serum/PBS, and incubated overnight with PROX1 (Millipore, AB5475) antibodies. The sections were then washed with PBS, incubated with secondary antibodies (Jackson Immunoresearch) for 2hr at room temperature, washed, and then mounted with Prolong Gold (Invitrogen) for imaging.

RT-PCR

For mRNA studies, RNA was isolated from cells or tissues using RNeasy mini kit (Qiagen) as per manufactures instructions and cDNA was amplified through reverse transcription (Applied Biosystems). For miRNA RT-PCR, total RNA was isolated with miRCURY isolation kit (Exiqon) and cDNA amplification was carried out with Universal cDNA synthesis kit (Exiqon). PCR was performed with iQ SYBR master mix (BioRad), measured by BioRad C1000 thermocycler. For mRNA transcripts, primer sequences can be found in Supplemental Table 1. For human cell samples, mature *miR-31* and *miR-181a* transcripts were quantified with LNA primers (Exiqon, Cat# 204236, 204566) and normalized to U6 (Exiqon, Cat# 203907). For zebrafish samples, *miR-31* was detected with a custom made LNA primers made by Exiqon (dre-miR-31Forward: 5’-GGCAAGAUGUUGGCAUAGGCUG-3’ and Universal Reverse primer). *miR-181a* was detected with Exiqon Cat# 204566. Samples were normalized to 18s. The miRNA PCR array was purchased from Exiqon (Cat#203822). All RT-PCR data was analyzed using comparative quantitation (DDCT).

Flow Cytometry

For isolation of *flil1a:EGFP*⁺ cells from zebrafish, heads were removed to eliminate *flil1a:EGFP*⁺ neural crest cells and the trunk regions were isolated from wildtype and *Tg(hsp70l: bmp2b)* \(fr^{13}\) embryos at
indicated times and disassociated with Liberase (Roche), washed with and filtered to obtain a single cell suspension. Samples were processed with BD FACS Aria and \textit{fli1a:EGFP}^+ cells were isolated for downstream applications. For analysis of \textit{fli1a:EGFP}^+;\textit{prox1a:RFP}^+ cells, samples were prepared as above but were processed with BD LSRII and analyzed with FloJo software.

**Luciferase Assay**

HeLa cells were plated in 96-well dishes (Nunc 136101) at 1 x 10^4 cells/well transfected using Lipofectamine 2000 with 0.1\(\mu\)g pmiRGlo (Promega E1330) containing zebrafish \textit{prox1a} 3`UTR and 30nm of miRNA mimics (Ambion, miR Neg Ctrl-4464058, miR-31 4464014, miR-181a 4464066. Cells were harvested 48 hours post-transfection and luminescent signals were detected using the Dual-Glo Luciferase Assay System (Promega) and quantified with Bio-Tek Synergy HT microplate reader.

**Construction of \textit{Tg(Bre:nmCherry)}^{nc24} and \textit{TgBAC(prox1:KalT4-UAS:uncTagRFP)}^{nim5}**

The minimal elements of the murine \textit{Id2} promoter (-3000bp to -2734 fused with -302 to +80 proximal promoter) \cite{53} were synthesized with Gateway cloning sites into pUC57 vector (GeneSynthesis). Multisite Gateway cloning (Invitrogen) was performed to generate \textit{Tg(BRE:nmCherry)} transgenic construct. \textit{Tg(Bre:nmCherry)}^{nc24} and \textit{Tg(Bre:nmCherry)}^{nc25} transgenic lines were further confirmed by analyzing their response to Bmp2b stimulation or down-regulation (Further characterization in Dunworth et al submitted). Construction of \textit{TgBAC(prox1:KalT4-UAS:uncTagRFP)}^{nim5} will be described in elsewhere.
Online Figure I: Different levels of BMP signaling activity in BECs and LECs
Trunk region of 4dpf wildtype and Tg(hsp70l;bmp2b)<sup>6r13</sup> embryos in Tg(kdrl:EGFP)<sup>a843</sup>; Tg(fli1a.ep:DsRedEx)<sup>μm13</sup> background, shown in lateral view. LECs are shown in red since they lack EGFP expression. Abbreviations: DA: dorsal aorta, CV: cardinal vein, TD: thoracic duct, LISV: lymphatic intersegmental vessel, aISV: arterial intersegmental vessel, and vISV: arterial intersegmental vessel. Scale bar is 25μm.
Online Figure II: Bmp2b signaling selectively attenuates expression of RFP in lymphatic vessels in TgBAC(prox1a:KalT4-UAS:uncTagRFP)nim5 embryos

(A) Confocal projections of 4dpf wild-type (top) or Tg(hsp70l:bmp2b)fr13 (bottom) embryos in TgBAC(prox1a:KalT4-UAS:uncTagRFP)nim5;Tg(kdrl:GFP)s843 background. Bmp2b over-expression selectively abrogated the RFP transgene expression in lymphatic vessels (LY) without affecting expression within other tissues, indicating tissue specific attenuation of prox1a expression by Bmp2b signaling. The expression of RFP is similarly observed in the lens (L), hindbrain (H), liver (LV) and neural tube (NT), somites, and lymphatic vessels (LY) of wildtype (top) and Tg(hsp70l:bmp2b)fr13 (bottom) embryos. (B) Three dimensional reconstruction of confocal stacks taken from the trunk region of 3dpf TgBAC(prox1a:KalT4-UAS:uncTagRFP)nim5;Tg(kdrl:GFP)s843 embryos. Dorsoventral (D-V), anterioposterio (A-P), and mediolateral (M-L) axes are shown yellow dashed lines. (C) Lateral view of Three dimensional reconstruction of confocal stacks taken from 3dpf TgBAC(prox1a:KalT4-UAS:uncTagRFP)nim5;Tg(kdrl:GFP)s843 embryos. Newly emerging LECs are labeled as yellow (white arrows), which preferentially locate at the base of the venous intersegmental vessels (vISVs) at this stage. Scale bar is 250μm.
Online Figure III: Noggin enhances LEC differentiation from cultured mouse embryoid bodies

(A) Representative micrographs of the periphery of embryoid bodies upon treatment with Vehicle (0.1%BSA), VEGF-C, and Noggin. (B) Quantification of (A) measuring the ratio of lymphatic vessel area (LYVE1+/CD31+) to total vasculature (CD31+) at the EB periphery (N=3, 4-6 EBs analyzed per condition per experiment). Scale bar is 100μm.
Online Figure IV: Tg(BRE:nmCherry)nc24 recapitulates endogenous BMP signaling activity

(A) Sequence comparison of human, mouse, and zebrafish Id2 promoter. In all species, evolutionarily conserved BMP response element (red shaded region) and SMAD binding sites are present (green shaded region). (B) Confocal images of 4hpf (left) and 17hpf (right) Tg(Bre:nmCherry)nc24 zebrafish embryos. Scale bar is 100μm. (C) Co-localization of p-SMAD1/5/8 with nmCherry expression in developing zebrafish embryos. Confocal images of 18 somites (18.5hpf) and 24hpf Tg(Bre:nmCherry)nc24 zebrafish embryos stained with pSMAD1/5/8 antibody. Arrowheads point cells co-positive for BRE:mCherry and pSMAD1/5/8 staining at 18.5hpf. (D) 48hpf wild-type (top), Bmp2b over-expressing (middle), and 2.5μM DMH1-treated embryos in Tg(Bre:nmCherry)nc24 transgenic background. Expression of nmCherry is dependent on the level of BMP2 signaling strength, up-regulated in Bmp2b over-expressing embryos but severely attenuated when treated with DMH1. Scale bar is (B): 250μm, (C): 100μm, and (D): 250μm.
Online Figure V: *miR-31* and *miR-181a* are expressed within endothelial cells

(A) In situ hybridization expression analysis of *miR-31* and *miR-181* in E10.5 and E11.5 mouse embryos. Vessels (arrows) and their vicinity strongly express *miR-31* and *miR-181*. (B) In situ hybridization expression analysis of *miR-31* and *miR-181a* in 36hpf Tg(kdrl:GFP) embryos. Both *miR-31* and *miR-181a* were detected within the dorsal aorta, cardinal vein, and intersegmental vessels. Arrowheads point expression of these miRNAs within intersegmental vessels. Abbreviations: DA: dorsal aorta, CV: cardinal vein and ISV: intersegmental vessel. Scale bar is (A):20μm and (B):50μm.
Online Figure VI: *miR-31* and *miR-181a* regulate *prox1* transcript in zebrafish embryos

(A) Regulation of *prox1a* expression by *miR-31* and *miR-181a*, shown by luciferase assay. Full length zebrafish *prox1a* 3’-UTR in pmiR-Glo vector was transfected into HeLa cells in the presence of absence of zebrafish *miR-31* and *miR-181a* mimics and miRNA targeting was assessed by normalized Firefly:Renilla luciferase activity. Both *miR-31* and *miR-181a* mimics recognized the *prox1a* 3’-UTR and reduced luciferase activity (N=4). (B) Injection of *miR-31* or *miR-181a* mimics caused drastic decrease in the level of *prox1a* expression in 48hpf zebrafish embryos. (C) Other predicted targets (identified by Target scan) of *miR-31* and *miR-181a* are also down-regulated by Bmp2b over-expression in zebrafish embryos.
Online Figure VII: Validation of MOs against miR-31 and miR-181a

MO-targeted down-regulation of miR-31 and miR-181a resulted in efficient knockdown of the targets. Samples were collected at 26hpf after introduction of 5ng of MO at the one-cell stage (N=4).
### Supplemental Table

**Online Table I: List of primers used in this research**

<table>
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<tr>
<th>Target</th>
<th>Forward Primer (5’-3’)</th>
<th>Reverse Primer (5’-3’)</th>
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