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

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ABSTRACT

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

**Objective:** To delineate the role of BMP2 signaling on 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 (ES) cell-derived embryoid bodies (EBs) 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, result attenuated expression of PROX1 during development.

**Conclusions:** Our data identify BMP2 as a key negative regulator for the emergence of the lymphatic lineage during vertebrate development.

**Keywords:** Lymphangiogenesis, BMP2, PROX1, miRNA, vertebrate development, developmental biology, lymphatic capillary, signaling pathways, SMAD, microRNA

**Nonstandard Abbreviations and Acronyms:**
- **BEC** Blood Vascular Endothelial Cell
- **BMP** Bone Morphogenetic Protein
- **BRE** BMP Response Element
- **CV** Cardinal Vein
- **DA** Dorsal Aorta
- **fli1a** friend leukemia integration 1 a
- **kdrl** kinase insert domain receptor like
- **LEC** Lymphatic vascular Endothelial Cell
- **PROX1** Prospero homeobox protein 1
- **TD** Thoracic Duct
- **VEGF-C** Vascular Endothelial Growth Factor-C
INTRODUCTION

The lymphatic system is essential for maintenance of interstitial fluid balance, uptake of lipids, and provides the major conduit for immune cells. The dysfunction of the lymphatic system leads to tissue fluid accumulation and edema, a condition commonly known as lymphedema, illustrating the critical function of lymphatic system in fluid homeostasis. For instance, inherent mutations can predispose patients to primary lymphedema. Similarly, removal of lymph node during cancer treatment or parasite infection such as lymphatic filariasis can result secondary lymphedema. In addition to its role in fluid homeostasis, the lymphatic system plays a critical role in the onset and progression of diverse pathological conditions, including tumor metastasis and obesity. While a number of genes required for lymphatic development have been identified, the generation of novel and effective therapies to improve patient outcomes awaits a more comprehensive understanding of the coordination among endogenous agonistic and antagonistic factors governing lymphatic fate.

During development, lymphatic vessels emerge around 3 days post-fertilization (dpf) in zebrafish E10 in mice, and embryonic week 6 to 7 in humans, and, only after the formation of blood vessels and initiation of blood circulation. Lineage tracing in mice embryos and in vivo time lapse analyses in zebrafish demonstrated that the lymphatic vessels sprout from the veins, as previously proposed by Florence Sabin. In zebrafish, LECs originate from the presumptive parachordal vessel and migrate out to form the thoracic duct (TD) between the dorsal aorta (DA) and cardinal vein (CV). At 5dpf, an elaborate and extensive lymphatic network is present in zebrafish. In mice, a subset of venous endothelial cells (ECs) in the CV becomes committed to the lymphatic lineage and begin to express Sox18 and Proxl, which subsequently migrate out of the CV following the gradient formed by Vascular Endothelial Growth Factor-C (VEGF-C). At least two distinct mechanisms have been suggested to explain the initial segregation of LECs from the blood endothelial cells (BECs), and formation of the jugular lymph sacs, the first lymphatic organ to emerge during development. Similarly, additional lymph sacs are formed along the anterior-posterior axis, which contribute to the primary capillary plexuses. Around E15.5, lymphatic vessels undergo morphogenetic changes to form mature lymphatic structures such as capillaries, pre-collectors, and collecting vessels. Although several key factors promoting lymphatic development have been identified including PROX1, SOX18, VEGF-C, and VEGFR3, which appear to have evolutionarily conserved function, those that inhibit the differentiation of lymphatic vessels remain largely unknown.

Bone Morphogenetic Protein (BMP) molecules, which are members of the Transforming Growth Factor beta (TGF-β) super-family, is a potential candidate signaling pathway that may negatively regulate lymphatic development. During development, diverse components of BMP signaling, including BMP2, influence vascular development in vertebrates, and dysregulation of BMP signaling through genetic mutations has been implicated in diverse human pathological conditions including hereditary hemorrhagic telangiectasia (HHT) and primary pulmonary arterial hypertension (PAH). Mechanistically, BMP ligands transduce their activity via various signaling cascades. Upon 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. In addition to the SMAD-dependent pathway, BMP ligands can transduce signaling via MAP kinase pathways. During vascular development, BMP signaling appears to possess a context-dependent function to regulate diverse aspects of endothelial cell behaviors. For instance, BMP2 functions as a venous specific pro-angiogenic cue in zebrafish and is essential for the stalk cell fate in the mouse hindbrain plexus, while BMP9 appears to function as a circulating vascular quiescence factor.

In this report, 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 \textit{miR-31} and \textit{miR-181a}, to negatively regulate PROX1 expression, in a SMAD-dependent, but not ERK1/2-dependent manner. While several factors have been described to negatively modulate lymphangiogenesis postnatally including endostatin, TGF-\(\beta\), and IFN-\(\gamma\), to our knowledge, these data are the first \textit{in vivo} demonstration of a negative modulator for lymphatic fate during vertebrate development.

\section*{METHODS}

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

\textbf{Zebrafish husbandry, heatshock conditions, drug treatment and MO injection.} Zebrafish (\textit{Danio Rerio}) embryos were raised as previously described\textsuperscript{46}. The following transgenic fish lines were utilized: \textit{Tg(fli1a:EGFP)y1}\textsuperscript{47}, \textit{Tg(fli1a.ep:DsRedEx)um13}\textsuperscript{48}, \textit{Tg(kdrl:GFP)}\textsuperscript{845, 49}, \textit{Tg(hsp70:bmp2b)y44}\textsuperscript{50}, \textit{Tg(Bre:nmCherry)nc24} (this study) and \textit{TgBAC(prox1:KalT4-UAS:uncTagRFP)}\textsuperscript{nim5} (this study). Micro-injections of MO were carried out as previously described\textsuperscript{29}. Conditions for heatshock or drug treatments and sequence of MOs can be found in Online Supplement. Detailed description on the construction of \textit{Tg(Bre:nmCherry)nc24} and \textit{TgBAC(prox1:KalT4-UAS:uncTagRFP)}\textsuperscript{nim5} transgenic lines can be found in Online Supplement.

\textbf{Cell culture and embryoid body differentiation.} HMVEC-dLy (Lonza, Cat#CC-2810) were grown on 0.1% gelatin-coated plates with EGM2-MV (Lonza) media and used at passages 3-7. HMVEC-dLy were stimulated with 50ng/mL BMP2 (Sigma H4791) or 5ug/mL actinomycin D (Sigma A1410). siRNA silencing was performed with \textit{SMAD4} (Qiagen, SI00076020) and Control Allstar siRNAs (Qiagen) and Lipofectamine 2000 (Invitrogen).

ESCs were cultured on irradiated-MEF feeder cells in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 15% Knock-out serum replacement (KO-SR, Invitrogen), LIF-conditioned media, 0.1mM non-essential amino acid (MEM-NEAA, Invitrogen), 2mM L-glutamine (Invitrogen), 1mM sodium pyruvate (Invitrogen), 1% penicillin and streptomycin (Invitrogen) and 0.1mM 2-mercaptoethanol (Sigma). When ESCs reached 70-80% confluency, cultures were dissociated with 0.25% trypsin-EDTA (Invitrogen) and were cultured on gelatin-coated tissue culture dishes in order to adapt to feeder-free conditions. During this adaptation stage, ESCs were grown in Iscove’s modified Dulbecco’s medium (IMDM, Invitrogen) supplemented with 15% KO-SR, LIF-conditioned medium, 2mM L-glutamine, 1% penicillin and streptomycin and 0.1mM 1-thioglycerol (Sigma). After 2 days in feeder-free culture, mESC colonies were dissociated and hanging drops containing 540 ESCs in 15\(\mu\)l differentiation medium were prepared. Embryoid bodies (EBs) formed within 2 days of hanging drop cultures in differentiation medium containing IMDM, 15% fetal bovine serum (Gemini), 50\(\mu\)g/ml ascorbic acid (Sigma) and 2mM L-glutamine. To differentiate embryoid bodies (EBs), 2dpc EBs were plated on gelatin-coated on 8-well chamber slides (Lab-Tek) or 12-well dishes at a density of 6.25 EBs/cm\(^2\) in complete differentiation medium without LIF. To manipulate EBs, BMP2, VEGF-C (R&D System, 2176-VC), Noggin (R&D System, 6057-NG), and DMH1 (Sigma, D8946) were used. At 5dpc and 7dpc, EBs were treated with 0.1%BSA or growth factors (VEGF-C 200ng/mL, BMP2 100ng/mL, Noggin 250ng/mL, DMH1 0.1\(\mu\)M). At 8dpc, EBs were analyzed by immunocytochemistry (ICC) or flow cytometry with CD31 and LYVE1 antibodies. IHC samples were imaged by confocal microscopy (Leica SP5), equally processed by Adobe Photoshop, and quantified by Image J (NIH). For quantification, images were acquired at 2-3 random fields of the periphery of at least 4 EBs per condition per experiment. Each experiment was performed at least 3 times. The ratio of lymphatic vessel area to total vascular area was determined by differential immunofluorescence stains (LYVE1/CD31).
Zebrafish imaging and reconstruction.

4dpf Tg(fli1a.ep:DsRedEx)^un13^ Tg(kdrl:GFP)^843^ or Tg(kdrl:GFP)^843^;TgBAC(prox1:KalT4-UAS:uncTagRFP)^nim5^ double transgenic embryos were dechorionated, anesthetized with tricaine and embedded in 1% agarose/PBS on a glass bottom Petri dish (MatTek). Trunk regions immediately dorsal to the yolk extension were imaged with a Nikon T-E Eclipse confocal microscope with Hamamatsu camera (C9100-50). Volocity software (Perkin Elmer) was used to generate 3-D optical projections.

Quantification of lymphatic development in zebrafish embryos.

To quantify lymphatic defects in developing transgenic embryos, the percentage thoracic duct formation was quantified in 10 consecutive segments in Tg(fli1a.ep:DsRedEx)^un13^; Tg(kdrl:EGFP)^843^ or Tg(kdrl:GFP)^843^;TgBAC(prox1:KalT4-UAS:uncTagRFP)^nim5^ double transgenic embryos. At least 10 embryos per genotype/condition were analyzed from at least three independent clutches.

Statistical analyses.

All data were analyzed using the 2-tailed Student’s t test assuming unequal variance. A P value < 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 was required for venous angiogenesis in the developing zebrafish. Interestingly, Bmp2b appears to increase the number of venous endothelial cells without affecting cell proliferation. Considering that the lymphatic endothelial cells (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 endothelial cells at the expense of presumptive LECs.

To examine this possibility, we first over-expressed Bmp2b in zebrafish embryos with Tg(fli1a:DsRed); Tg(kdrl:EGFP) double transgenic background and examined the effects on lymphatic development. Since kdrl is expressed only in blood endothelial cells (BECs) while fli1a is expressed in both LECs and BECs, emerging LECs can be easily distinguished (Online Figure I). We found that Bmp2b over-expressing embryos completely lack any detectable lymphatic structures (Figures 1A and 1B). For instance, while wild-type embryos have a clearly separated thoracic duct between dorsal aorta and caudal vein, it is largely absent in embryos with excessive Bmp2b expression (Figures 1A and 1B). To further confirm the lymphatic defects in Bmp2b over-expressing embryos, we utilized another transgenic line, TgBAC(prox1a:KalT4-UAS:uncTagRFP)^nim5^ transgenic line (referred as Tg(prox1a:RFP) hereafter). 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 was reminiscent of the previously reported expression of endogenous prox1a (Online Figure II).

In wild-type embryos, consistent with previous reports, prox1a:RFP^+,fli1a:EGFP^+ cells first emerged along the horizontal myoseptum as a string of individual LECs approximately at 50hpf. Subsequently, RFP^+ LECs migrated dorsally and ventrally along arterial intersegmental vessels to form lymphatic structures (Online Figures IIB and IIC). Upon Bmp2b over-expression, we found that RFP expression within lymphatic vessels was selectively diminished, while the expression is maintained in other organs, indicating that Bmp2b may regulate the expression of prox1a in a tissue specific manner.
(Online Figure II). Consequently, at 60hpf, LECs were completely absent Bmp2b over-expressing embryos, which led to failure of forming the TD at 4dpf as we observed in Tg(fli1a:RFP); Tg(kdrl:EGFP) double transgenic background (Figures 1C and 1D). Conversely, inhibition of Bmp2b signaling appears 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 (Figures 1E and 1F) and increased the number of lymphatic endothelial cells 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 anti-lymphangiogenic effects of BMP2 signaling.**

Since Bmp signaling activates two main downstream effectors, SMADs and ERK1/2, in zebrafish development54,55, 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 (Figures 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 utilized a modified mouse embryoid bodies (EBs) lymphatic differentiation model (Figure 3A). We found that differentiation of LECs spontaneously occur within EBs as previously reported (Figure 3B)57,58. In this model, VEGF-C treatment starting at 5 days post-plating which is equivalent to E8.5 when the endothelial lineage emerges59, substantially increased the number of LECs (Figure 3B), consistent with previous reports57,60. 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 co-treated EBs with BMP2 and VEGFC. Co-treatment with BMP2 completely abrogated VEGF-C-mediated LEC induction in the EB periphery, and substantially decreased the number of CD31+/LYVE1+ LECs (Figures 3D-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 the BMP2 signaling is a highly conserved negative modulator of lymphatic development throughout vertebrates, and its anti-lymphangiogenic effect is mediated by SMAD activity.

**Reduced level of BMP signaling activity in lymphatic endothelial cells.**

Since over-expression 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:nnmCherry)nc24, which expresses nuclear mCherry under the regulation of minimal BMP Response Element (BRE) from mouse Id2 gene53 (Online Figure IV). We reasoned that Id2 promoter would more faithfully represent Bmp2b signaling in the zebrafish vasculature since we previously found that id2 is the most highly induced Id family member in Tg(hsp70l:bmp2b)fr13 after heat-shock induction51.

In developing zebrafish, mCherry expression faithfully recapitulated the endogenous Bmp2b activity (Online Figure IV) and overlapped with previously reported BMP signaling activity during development51. For instance, mCherry co-localized with p-SMAD-1/5/8 (Online Figure IV). In addition, mCherry expression was elevated in Bmp2b over-expressing 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 3dpf, and the thoracic duct (TD) at 4dpf zebrafish embryos (Figures 4A to 4C), suggesting that 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 endothelial cells within the developing cardinal vein. 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) transgenic mouse embryos, where BMP signaling activity can be measured by the expression level of EGFP, we found EGFP expression was similarly reduced in LECs compared to BECs within cardinal veins (Figures 4E and 4F). Taken together, our data allude 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 which demonstrated the lack of BMP2 transcript in LECs.62

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 wildtype and Bmp2b over-expressing 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 Prox1,12,63, was significantly reduced, suggesting that Bmp2b signaling may attenuate lymphatic development by antagonizes prox1a expression during early stages of lymphangiogenesis in zebrafish. Similarly, BMP2 stimulation significantly reduced basal PROX1 expression level in fully differentiated human dermal microvascular lymphatic endothelial cells (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, two 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 down-regulation by BMP2 (Figure 5C). Considering that lymphatic development is highly sensitive to small fluctuations in the level of Prox1 expression,64,65 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 post-transcriptional level. Since BMP signaling can influence the processing of miRNAs, it is tempting to speculate that the anti-lymphangiogenic effect of BMP2 signaling may be mediated by a miRNA-dependent mechanism. To explore this possibility, changes in miRNA expression upon BMP2 stimulation in hLECs was evaluated using a miRNA PCR Array. An elevated level of BMP2 signaling significantly altered the expression level of multiple miRNAs (Figure 6A).
Interestingly, two of the up-regulated 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 over-expressing 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 anti-lymphangiogenic effects of BMP2 signaling. Consistent with previous reports that miR-31 and miR-181a post-transcriptionally suppress PROX1 mRNA level in humans and mice, we found functionally conserved miR-31 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 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 a drastic decrease in the level of prox1a expression (Online Figure VI), further support 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 rhot2 (in case of miR-31) and ptpn2a (in case of miR-181a) were similarly down-regulated in embryos over-expressing Bmp2b (Online Figure VI).

To further investigate how BMP2 signaling increases the level of these miRNAs, we first examined the role of SMAD activity. Knock-down of SMAD4 by siRNA completely abrogated the BMP2-mediated up-regulation of miR-31 and miR-181a (Figure 6C). Unexpectedly, we observed that the miR-31 and miR-181a expression levels following BMP2 treatment in the SMAD4 knockdown were decreased compared to vehicle treated, suggesting the presence of potential negative feedback regulation. In addition, treatment with Actinomycin D to inhibit transcription completely abolished the effects of BMP2 signaling on the levels of miR-31 and miR-181a in hLECs (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 anti-lymphangiogenic effects of Bmp2b signaling, therefore, at least partially restores 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 over-expressing zebrafish embryos (Figure 7A). A significant portion of MO injected embryos contained at least partially formed thoracic duct (up to 70% of embryos), and a sizable portion of embryos generated complete thoracic duct (between 10 to 25 %) (Figures 7A and 7B). Therefore, miR-31 and miR-181a are likely to be the essential to mediate anti-lymphangiogenic 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 find that lymphatic progenitors are largely devoid of BMP signaling activity. Since it has been reported that several Bmp ligands are expressed in endothelial cells or their vicinity during zebrafish development including Bmp2b and Bmp4, it remains to be identified which specific Bmp ligands function as the major regulator for the lymphatic fate.

In our study, we find that BMP2 signaling strongly inhibits the expression of PROX1, the master regulator for the lymphatic fate, in a SMAD- and miRNA-dependent manner. Taken together, our data suggest that BMP2 signaling may modulate fate specification within endothelial cells, similar to its role in

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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 appear to be important mediators for anti-lymphangiogenic effects of BMP2 signaling. Using miRNA PCR arrays, we have identified several miRNAs are up-regulated by BMP2 signaling, including miR-31 and miR-181a, in a SMAD-dependent manner (Figure 6C). Considering Actinomycin D treatment completely inhibited the BMP2-induced up-regulation of miR-31 and miR-181a expression, it is likely that active BMP2 signaling influences transcription of these miRNAs. However, we could not identify consensus SMAD Binding Element (SBE) 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 of variant of SBEs. Alternatively, activated SMAD may bind to the unknown enhancer elements that influence the transcription of miR-31 and miR-181a since SMADs can bind to enhancer located outside of promoter region. Further analyses on the promoter region of miRNAs that are up-regulated by BMP2 stimulation are warranted to delineate precise molecular mechanisms on the SMAD-mediated transcriptional regulation of these miRNAs. In Bmp2b over-expressing zebrafish embryos, we found that expression of prox1a:RFP is selectively down-regulated in developing LECs without affecting the expression in other tissues. The tissue specific enrichment of miR-31 and miR-181a, which appear to be essential to mediate anti-lymphangiogenic effects of Bmp2b signaling, in BECs, may contribute to creating the tissue specific response of Bmp2b over-expression in developing zebrafish embryos.

Since each miRNA can bind wide variety of target mRNAs, additional targets of miR-31 and miR-181a may augment the anti-lymphatic effects of BMP2 signaling in coordination with PROX1. Although we cannot formally exclude this possibility, we propose that PROX1 is likely to be the primary targets for these miRNAs during lymphatic development. First, PROX1 is the master transcription factor for the lymphatic fate. By regulating PROX1 expression, which is auto-regulatory, and continuously required to maintain the lymphatic fate, a modest decrease of its expression in early lymphangiogenesis can be amplified to manifest severe lymphatic defects. Second, it is the only lymphatic marker whose expression is down-regulated in response to BMP2 signaling. Other lymphatic markers, including LYVE1 and VEGFR3 were not altered upon BMP2 stimulation in hLECs or zebrafish embryos.

It is interesting that let-7 family members are among the miRNAs that are down-regulated by excessive BMP2 signaling (Figure 6A) considering a previous report that let-7 negatively regulates expression of TGF-βR1 to decrease TGF-β signaling, which are known to inhibit lymphatic regeneration. Therefore, down-regulation 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 to BECs, the level of BMP2 signaling within LECs appear to be substantially attenuated. Since diverse modifiers of BMP signaling are expressed within EC, it is tempting to speculate that endogenous antagonist of BMP2 may be preferentially localized and/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 other BMP ligands may play an additional role in this process, our model is consistent with previous finding that LECs do not express high level of Bmp2 transcript. Considering developmental cues that negatively regulate the emergence of LECs are poorly understood, our findings in this report could help us to formulate more comprehensive idea on lymphatic development and to develop theoretical framework to develop therapeutic manipulation of lymphangiogenesis in development and diseases.

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DISCLOSURE
None.

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FIGURE LEGENDS

Figure 1: Bmp2b signaling may function as an anti-lymphangiogenic cue in zebrafish embryos. (A) Transverse section from the trunk region of 4dpf wildtype and Tg(hsp70l:bmp2b)fr13 embryos in Tg(kdr:EGFP)842;Tg(fli1a.ep.DsRedEx)um15 background. Over-expression of Bmp2b abrogates thoracic duct (arrowhead in wild-type) formation. (B) Quantification of percent TD formation measured over 10 segments. Compared to wild-type embryos (n=50), the formation of thoracic duct is substantially reduced in embryos with an elevated level of Bmp2b activity by heatshock treatment at either 26hpf (n=39) or 50hpf (n=36). (C) Lateral view of wild-type and Bmp2b over-expressing embryos at 4dpf. Thoracic duct (arrow) is absent in Tg(hsp70l:bmp2b)fr13 embryo. Embryos shown have TgBAC(prox1:KalT4-UAS:uncTagRFP)um5;Tg(kdr:GFP)842 background. (D) Quantification of percent TD formation in wildtype and Tg(hsp70l:bmp2b)fr13 embryos measured over 10 segments. (n=36 for wild-type embryos, and 32 for Tg(hsp70l:bmp2b)fr13 embryos). (E) The number of ventral sprouts from the thoracic duct (asterisks) increased in 1μM DMH1-treated embryos compared to DMSO-treated control embryos. (F) Quantification on the numbers of ventral sprouts from the TD in control and DMH1-treated embryos (n=23 for DMSO-treated control embryos, and 25 for DMH1-treated embryos). Compared with control group, DMH1-treated embryos had a significant increase in the number of ventral sprouts (p<0.001). (G) Quantification of flow sorted LECs prox1a:RFP, fl1i:EGFP LECs relative to all fl1:EGFP+ ECs in DMSO- or DMH1-treated embryos (N=3). Abbreviations: DA: dorsal aorta, CV: cardinal vein, TD: thoracic duct. Scale bar is (A):25μm, (C):50μm, and (E):100μm.

Figure 2: Anti-lymphangiogenic effects of Bmp2b signaling are mediated by SMAD proteins. (A) 4dpf Tg(hsp70l:bmp2b)fr13;TgBAC(prox1:KalT4-UAS:uncTagRFP)um5 embryos treated with DMSO, 1μM DMH1 (Smad1/5/8 inhibitor), or 10μM U0126 (Erk1/2 inhibitor). Arrow points the thoracic duct in DMH1-treated embryos. Asterisks mark lack of thoracic duct. (B) Quantification of percent thoracic duct formation in DMSO, DMH1, or U0126-treated Tg(hsp70l:bmp2b)fr13 embryos measured over 10 segments (n=30 for DMSO treatment, 37 for DMH1-treatment, and 32 for U0126 treatment). The percentage of Tg(hsp70l:bmp2b)fr13 embryos with lymphatic structure development increased significantly between DMSO and DMH1-treated embryos (25% and 94%, respectively, p<0.001), while U0126-treated embryos were unaffected (21%). Abbreviations: DA: dorsal aorta, CV: cardinal vein, TD: thoracic duct. Scale bar is 50μm.

Figure 3: BMP2 attenuates LEC differentiation from cultured mouse embryoid bodies. (A) Mouse embryoid bodies (EBs) were prepared by hanging drop then grown in two dimensional culture to induce lymphatic differentiation. At EB day 8, the expression of Vegfr2 and Prox1 increased together (arrow), indicating that LECs emerge within the EBs. (B) Differentiation of lymphatic endothelial cells (CD31+/LYVE1+) in mouse embryoid bodies was increased in the presence of VEGF-C. (C) Differentiated lymphatic endothelial cells within mouse embryoid bodies express hallmark of lymphatic lineage specific genes, including LYVE1 (red) and Podoplanin (blue). (D) Representative micrographs of the periphery of embryoid bodies upon treatment with VEGF-C, BMP2, VEGF-C and BMP2, or VEGF-C, BMP2, and DMH1. (E) Quantification of (D) 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 (B):50μm, (C):50μm, and (D):100μm.

Figure 4: Different levels of BMP signaling activity in BECs and LECs. (A) Emerging parachordal lymphangioblasts (red arrows), the lymphatic progenitors in zebrafish, lack BRE:mCherry expression at 60hpf, while ECs within intersegmental vessels (yellow arrowheads) are strongly positive for BRE:mCherry. (B) LECs are largely devoid of BMP signaling activity. Arrows point mCherry/EGFP+ LECs, and arrowheads point mCherry+/EGFP+ BECs. (C) Quantification on the numbers of EGFP+ and EGFP+/mCherry+ ECs within the dorsal aorta, cardinal vein, and thoracic duct (N=3, 10 embryos per experiment). (D) Transverse section of E11.5 mouse embryos stained with PROX1 and P-SMAD1/5/8.
antibodies. Two sections adjacent to each other are shown. P-SMAD1/5/8 staining is largely absent in developing lymph sac (LS), while strongly shown (arrowheads) in cardinal vein (CV). (E) Transverse section of E10.5 and E11.5 Tg(BRE:EGFP) mouse embryo through the jugular region. PROX1 (red) and PECAM (blue) are visualized by antibody staining. Areas containing the wall of cardinal vein are shown. Arrows point LECs within the vein wall, and arrowheads point to delaminated LECs. (F) Quantification on the numbers of EGFP+ and EGFP+/PROX1+ ECs in CV and TD (N=4). Abbreviations: DA: dorsal aorta, CV: cardinal vein, TD: thoracic duct, PL: parachordal lymphangioblast, ISV: intersegmental vessel. Scale bar is (A):50 μm, (B):50 μm, (C): 20 μm, and (E):20 μm.

Figure 5: Bmp2b signaling selectively represses the expression of prox1a in zebrafish embryos. (A) Expression of prox1a mRNA substantially decreased upon Bmp2b stimulation. Quantitative RT-PCR on selected markers for LECs and BECs from fli1a:EGFP+ ECs isolated from 72hpf wildtype and Tg(hsp70l:bmp2b)fr13 embryos following heat-shock at 50hpf. (B) The expression level of PROX1 was substantially down-regulated upon BMP2 stimulation in hLECs starting at 60 minutes (N=4). (C) Inhibition of SMAD4 relieves the suppression of PROX1 by BMP2 signaling.

Figure 6: BMP2/Bmp2b signaling induces expression of miR-31 and miR-181a in zebrafish embryos and hLECs. (A) miRNA PCR array expression analysis of cultured HDMVEC-Ly (hLECs) cells after treatment with 50ng/mL BMP2 for 1 hr. Several miRNAs with significant expression changes were found including miR-31 and miR-181a (N=4). Only miRNAs whose expression level exhibited statistically significant changes (P<0.05) in response to BMP2 stimulation are shown. (B) Time course of miR-31 and miR-181a expression from fli1a:EGFP+ ECs isolated at indicated times from wildtype and Tg(hsp70l:bmp2b)fr13 embryos following heat-shock at 26hpf (N=4). (C) siRNA knockdown of SMAD4 in LECs prevented BMP2 mediated induction of miR-31 and miR-181a. Additionally, miR-31 and miR-181a expression levels following BMP2 treatment in the SMAD4 knockdown were decreased compared to vehicle treated, suggesting potential negative feedback regulation (N=4). (D) Pre-treatment of hLECs with 5 μg/mL Actinomycin D to block transcription completely inhibited BMP2-induced expression of miR-31 and miR-181a, suggesting BMP2-induced up-regulation of miR-31 and miR-181a may require transcriptional activation of these miRNAs (N=4).

Figure 7: BMP2 signaling represses the expression of PROX1 via miRNA dependent mechanism. (A) Confocal projection of Tg(hsp70l: bmp2b)fr13 embryos injected with 5ng control, miR-31, or miR-181a MOs, followed by heatshock at 26hpf. Inhibition of miR-31 or miR-181a can partially rescue the lymphatic defects induced by Bmp2b over-expression. Arrows point rescued thoracic duct in MO-injected embryos. (B) Quantification of percent thoracic duct formation in control, miR-31, or miR-181a MOs injected wild-type and Tg(hsp70l: bmp2b)fr13 embryos measured over 10 segments (n>40 for all conditions). Thoracic duct formation was largely unaffected by MO knockdown of miR-31, or miR-181a in wildtype embryos. However, MO-mediated knockdown of miR-31 or miR-181a in the Tg(hsp70l: bmp2b)fr13 background resulted in 50-70% of the embryos forming at least a partial thoracic duct and between 20-25% forming a complete thoracic duct compared to 25% and 0% in control MO injected, respectively (p< .001 for both groups). Scale bar is 50μm. (C) During development, active BMP2 signaling promotes the expression of miR-31/miR-181a in BECs (blue), therefore, aids BECs to maintain their fate as venous endothelial cells. However, in presumptive LECs (green), the activity of BMP2 signaling is attenuated by unknown mechanism, thereby releasing miRNA-mediated repression of PROX1. Abbreviations: DA: dorsal aorta, CV: cardinal vein, TD: thoracic duct.
NOVELTY AND SIGNIFICANCE

What Is Known?

- Lymphatic dysfunction can result in debilitating lymphedema.
- Lymphatic vasculature arises from pre-existing venous system driven by prospero homeobox protein 1 (PROX1) expression and promoted through several growth factors including vascular endothelial cell growth factor C (VEGFC).
- 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 on 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 due to genetic defects, disease, or after surgical lymph node removal for cancer treatment, patients are at risk of developing lymphedema or disturbed immune responses. Additionally, in cancer metastasis, the lymphatic system is often utilized 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 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 microRNAs 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.
Figure 2

A. Tg(kdrl:EGFP,prox1a:Kal4T-UAS:RFP)

DMSO
DA
CV

DMH1
DA
TD

U0126
CV

B. Percent Embryos @ 4dpf

- Complete TD
- 30-90%
- 0-30%
- No TD
Figure 7

A. Tg(kdr:EGFP) Tg(prox1a:Kal4T-UAS:RFP)

CTRL MO
DA CV

miR-31 MO
DA TD CV

miR-181a-1 MO
DA TD CV

B. Wildtype Embryos at 4dpf

0% 10% 20% 30% 40% 50% 60% 70% 80% 90% 100%

CTRL MO miR-31 MO miR-181a MO

Tg(hsp70:bmp2b) Embryos at 4dpf

No TD 0-30% 30-90% Complete TD

C. BMP2

Cardinal Vein

Lymphatic EC

Venous EC

BMP Activity ↓

miR-31/miR-181a ↓

PROX1 ↑ Lymphatic Fate

BMP Activity ↑

miR-31/miR-181a ↑

PROX1 ↓ Venous Fate

?
Bone Morphogenetic Protein 2 Signaling Negatively Modulates Lymphatic Development in Vertebrate Embryos
William P Dunworth, Jose Cardona-Costa, Esra Cagavi, Jun-dae Kim, Johanna C Fischer, Stryder Meadows, Yeqi Wang, Ondine Cleaver, Yibing Qyang, Elke A Ober and Suk-Won Jin

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Supplemental Experimental Procedures

Zebrafish husbandry, heatshock treatment, drug treatment and MO injection

Tg(hsp70l:bmp2b)fr13 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'-TTAGCTCAAAACTCACCAGACAGCGT-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 fltlα:EGFP+ cells from zebrafish, heads were removed to eliminate fltlα:EGFP+ neural crest cells and the trunk regions were isolated from wildtype and Tg(hsp70l:bmp2b)fr13 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 *fli1a:EGFP*+ cells were isolated for downstream applications. For analysis of *fli1a:EGFP*+/*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 10⁴ cells/well transfected using Lipofectamine 2000 with 0.1μg pmiRGlo (Promega E1330) containing zebrafish *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 Tg(Bre:nmCherry)*nc24* and TgBAC(prox1:KalT4-UAS:uncTagRFP)*nim5*

The minimal elements of the murine *Id2* promoter (-3000bp to -2734 fused with -302 to +80 proximal promoter) 53 were synthesized with Gateway cloning sites into pUC57 vector (GeneSynthesis). Multi-site Gateway cloning (Invitrogen) was performed to generate *Tg(BRE:nmCherry)* transgenic construct. *Tg(Bre:nmCherry)*nc24 and *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 *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)6/n embryos in Tg(kdrl::EGFP)n843; Tg(fli1a.ep::DsRedEx)n12 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 250 μm.
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 100$\mu$m. (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 2.5$\mu$M 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): 250$\mu$m, (C): 100$\mu$m, and (D): 250$\mu$m.
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)s843 zebrafish 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

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