Alk3/Alk3b and Smad5 Mediate BMP Signaling during Lymphatic Development in Zebrafish

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Lymphatic vessels are essential to regulate interstitial fluid homeostasis and diverse immune responses. A number of crucial factors, such as VEGFC, SOX18, PROX1, FOX2C, and GJC2, have been implicated in differentiation and/or maintenance of lymphatic endothelial cells (LECs). In humans, dysregulation of these genes is known to cause lymphedema, a debilitating condition which adversely impacts the quality of life of affected individuals. However, there are no currently available pharmacological treatments for lymphedema, necessitating identification of additional factors modulating lymphatic development and function which can be targeted for therapy. In this report, we investigate the function of genes associated with Bone Morphogenetic Protein (BMP) signaling in lymphatic development using zebrafish embryos. The knock-down of BMP type II receptors, Bmpr2a and Bmpr2b, and type I receptors, Alk3 and Alk3b, as well as SMAD5, an essential cellular mediator of BMP signaling, led to distinct lymphatic defects in developing zebrafish. Therefore, it appears that each constituent of the BMP signaling pathway may have a unique function during lymphatic development. Taken together, our data demonstrate that BMP signaling is essential for normal lymphatic vessel development in zebrafish.

INTRODUCTION

Bone morphogenetic proteins (BMPs) are growth factors that belong to the tumor growth factor (TGF) superfamily and have been shown to regulate multiple biological processes of development and morphogenesis (David et al., 2009; Kawabata et al., 1998; Kondo, 2007; Sieber et al., 2009). Numerous BMP ligands, type I receptors (BMPRI/ALK), and type II receptors (BMPRII) have been identified in diverse experimental model systems and in the human genome (Beets et al., 2013; Guo and Wu, 2012). In general, BMP ligands signal throughout hetero-tetrameric receptor complexes, which consist of two BMPRIs and two BMPRIIs. Upon activation, the BMP signaling complex undergoes Clathrin and Dab2 dependent internalization (Kim et al., 2012) and induces phosphorylation of receptor regulated SMADs (R-SMAD), such as SMAD1, 5, or 8 (9 in zebrafish), within the early endosomes (Hartung et al., 2006). Once phosphorylated, R-SMADs recruit a common mediator SMAD (Co-SMAD), SMAD4, and translocate to the nucleus to promote transcription of BMP targets such as ID1 and SMAD6 (Guo and Wu, 2012).

During early embryogenesis, the activities of BMP signaling are important for dorso-ventral axis formation and the establishment of mesoderm-derived cell lineages (Kondo, 2007). Lack of functional BMP signaling in embryos leads to severe dorsalization, therefore, adversely affecting specification of ventral and mesodermal cell fates (Kondo, 2007; Little and Mullins, 2008; Sieber et al., 2009). During organogenesis, BMP signaling regulates morphogenesis of diverse mesoderm-derived organs. For instance, a reduced level of BMP signaling causes defects in the formation of heart primordial cells and cardiac valves (Abdelwahid et al., 2001; Chocron et al., 2007; Eisenberg and Markwald, 1995). In addition, increasing evidence suggests that BMP signaling also regulates morphogenesis of vascular networks by modulating behaviors of endothelial cells in vertebrates (Schmitt et al., 2013; Wiley and Jin, 2011; Wiley et al., 2011). In endothelial cells, BMP signaling can elicit opposite responses, depending on the type of ligand; while BMP2 and BMP6 promote angiogenesis (Finkenzeller et al., 2012; Wiley et al., 2011), BMP9 and BMP10 are known to induce quiescence of endothelial cells (Lamirée et al., 2012; Moya et al., 2012). More recently, BMP and TGFβ signaling have been shown to regulate lymphatic endothelial cells (LECs) (Dunworth et al., 2013; Kinashi et al., 2013; Levet et al., 2013; Yoshimatsu et al., 2013), which are specialized endothelial cells derived from endothelial cells within blood vessels. However, since BMP signaling can elicit drastically distinct outcomes in the same tissue dependent on the context (Kim et al., 2012; Wiley et al., 2011) and there is a high degree of redundancy within the signaling pathways (Guo and Wu, 2012; Little and Mullins, 2009), it is possible that BMP signaling differently regulates LECs in a context-dependent manner. For instance, BMP ligands may bind to distinct Type I receptors (i.e. Alk2 vs Alk3), or preferentially activate distinct R-SMADs (i.e. SMAD1 vs SMAD5) to...
either activate or repress lymphatic development.

Therefore, we examined the function of individual BMP signaling components during lymphatic development using zebrafish as a model system. We found that many genes which function within the BMP signaling pathway, including bmpr2, bmpr1, bmpr1b, and smad5 are essential to promote lymphatic development in zebrafish. In contrast, the functions of alk2, smad1, and smad9 appear to be dispensable for early lymphatic development. Therefore, it appears that BMP signaling may promote lymphatic development via BMPR1/Alk3 and SMAD5 in zebrafish. Combined with our previous analyses on the role of BMP2 signaling in lymphatic development (Dunworth et al., 2013), our data presented here illustrate the complex and context-dependent nature of BMP signaling during lymphatic development.

MATERIALS AND METHODS

Zebrafish husbandry and microinjection

Zebrafish (Danio rerio) Tg(fli1a:nEGFP) y5; Tg(kdrl:mCherry) sm43 transgenic embryos and adults were raised and maintained under IACUC guidelines of Yale university. Sequences of morpholino anti-sense oligonucleotides (MO; Gene Tools, LLC) can be found in Table 1. MOs were injected at 1-2 cell stage as desired concentrations. The efficacy of the MO was validated by semi-quantitative reverse transcriptase (RT) PCR.

Morphological analysis and quantification of lymphatic phenotype

Zebrafish embryos were anesthetized, plated and oriented laterally on a glass bottom dish at 4dpf. Image acquisition from zebrafish embryos was achieved using a Nikon confocal microscope and merged Z-stack images by MBF ImageJ program. The number of LECs in developing thoracic ducts of zebrafish embryos was individually counted from the trunk region spanning 7 somites, from somite boundary 8 or 9 to 15, on Z-stacked confocal images. Experiments were performed in triplicate. Quantification graphs were generated by PRISM program. Results were evaluated by two-tailed and/or unpaired Student’s t test and each error bar represents the standard error of the mean (SEM).

RESULTS

BMPRII/BMPR2 is the main type II receptor for BMP ligands, although BMP ligands can bind to ActRII and ActRIIB (Beets et al., 2013; Wakefield and Hill, 2013; Wiley and Jin, 2011). In the zebrafish genome, two orthologs of human BMPR2, bmpr2a and bmpr2b, exist and are highly expressed in developing venous endothelial cells (Wiley et al., 2011). To define the function of individual Bmpr2s during lymphatic development, we first attenuated the level of Bmpr2a and Bmpr2b activities by anti-sense morpholino oligonucleotide (MO)-mediated knock-down in Tg(fli1a:nEGFP); Tg(kdrl:mCherry) transgenic zebrafish embryos. This double transgenic line allows us to visualize individual lymphatic endothelial cells (LECs), therefore, allowing us to precisely quantify the number of developing LEC in the thoracic duct (TD) at 4dpf (Fig. 1). We found that Bmpr2 is critical for lymphatic development. Therefore, it appears that BMP signaling may promote lymphatic development through a complex and context-dependent nature of BMP signaling during lymphatic development.

Table 1. Morpholinos (MO) used in this paper

| Zebrafish gene | Sequence | MO type | References |
|---------------|----------|---------|------------|
| bmp2a         | 5'-CTCTTAACCGTTCAATTTATA-3' | Negative control | This study (gene tools) |
| bmpr2b        | 5'-GATTACCGAAATACCTTGC-3' | Splicing blocking | Wiley et al. (2011) |
| ak1/acr1l     | 5'-GATGACTGACGATGACGACT-3' | Translation blocking | Roman et al. (2002) |
| alk2/acr1l    | 5'-GTCGTTGTCATGACGACT-3' | Translation blocking | Bauer et al. (2001) |
| alk3/bmpr1aa  | 5'-GACGCATTGTCAAATTTGTCG-3' | Translation blocking | Little et al. (2009) |
| alk3/bmpr1ab  | 5'-GTCGTTGTCATGACGACT-3' | Translation blocking | Little et al. (2009) |
| smad1         | 5'-TAACAATTTAGCCACGCTACCTGG-3' | Splicing blocking | McReynolds et al. (2007) |
| smad5         | 5'-ATCGTGAACACCTCCTGGACTTCT-3' | Splicing blocking | This study |
| smad9         | 5'-AGTCTGGACTGTCACCTTGG-3' | Splicing blocking | This study |

BMP Signaling in Zebrafish Lymphatic Vessel Development

Jun-Dae Kim & Jongmin Kim

http://molcells.org Mol. Cells 271
MOs against alk1 (7 ng/embryo), alk2 (5.4 ng/embryo), alk3 (14 ng/embryo), or alk3b (3.6 ng/embryo) at high concentrations cause gastrulation defects, and other morphological abnormalities such as cardiac edema (data not shown). Therefore, to bypass the earlier requirement of Alks, we titrated the concentration of MO to determine the dose which does not affect early development and heart formation. At lower concentrations, MOs against alk1, alk2, alk3, and alk3b (3.6 ng/embryo) at high concentrations did not cause any discernible abnormalities in axis formation, cardiac morphogenesis, blood vessel development, or lymphatic vessel formation (3.6 ng/embryo for alk1, 2.7 ng/embryo for alk2 MOs, 7 ng/embryo for alk3, and 1.8 ng/embryo for alk3b) (data not shown and Fig. 2). Thus, a partial reduction of each Alk appears to have negligible effects on lymphatic vessel development (Fig. 2). Since it is possible that BMPRIs have redundant roles during lymphatic development in zebrafish, we analyzed the lymphatic phenotype of embryos which were injected with a combination of MOs targeting two Alks. In case any two Alk receptors may function redundantly, injecting suboptimal doses of MO targeting both Alks together may create a synthetic phenotype, which cannot be observed when a single MO was injected with a suboptimal dose. When we injected alk2 and alk3 together at sub-optimal doses (1.4 ng/embryo for alk2 MO and 3.8 ng/embryo for alk3) (Figs. 3A, 3B, and 3E), or alk2 and alk3b together (1.4 ng/embryo for alk2 MO and 0.9 ng/embryo for alk3b) (Figs. 3A, 3C, and 3E), lymphatic vessels in the injected embryos were comparable to control embryos, suggesting that Alk2 does not have any redundant role with Alk3 or Alk3b in lymphatic development. In contrast, co-injection of alk3 and alk3b MOs with a sup-optimal dosage caused a substantial loss of LECs in the developing TD at 4dpf (Figs. 3A, 3D, and 3E), suggesting that Alk3 and Alk3b may redundantly regulate lymphatic development in zebrafish.

Upon activation, the BMP receptor complex induces phosphorylation of SMAD1, 5, 8 (9 in zebrafish), which is collectively known as R-SMAD (R-Smad in zebrafish). Phosphorylated R-SMADs translocate into the nucleus with Co-SMAD, and function as a transcription factor to specific target genes (Kawabata et al., 1998; Wiley and Jin, 2011). It is known that each Smad might function differently in hematopoiesis of zebrafish (McReynolds et al., 1999). For example, Smad1 and Smad5 appear to have an opposite effect in hematopoiesis of zebrafish (McReynolds et al., 2007). While the number of blood cells is increased in Smad1-deficient embryos, hematopoiesis is substantially decreased in Smad5-deficient embryos (McReynolds et al., 2007). Therefore, we speculate that each Smad might function differently in LECs of zebrafish. To examine this notion, we first designed splice-blocking MOs to inhibit the endogenous smad mRNAs processing which allows us to bypass the early requirement of these genes (MO efficacy was validated by semi-quantitative RT-PCR in Figs. 4A and 4B). Injection of splicing MO against each smad did not cause any morphological defects such as abnormalities in axis formation, cardiac function, or formation of blood vessels (data not shown). However, the number of LECs within the TD in smad5 MO-injected embryos was drastically decreased (3.64 ± 0.70) compared to control embryos (8.04 ± 0.624). In contrast, we did not find any obvious decrease in the number of LECs in smad1 or smad9 MO-injected embryos (Figs. 4C-4G). Therefore, Smad5 appears to be the most critical downstream mediator for BMP signaling in developing LECs in zebrafish.
DISCUSSION

Our data demonstrate that BMP signaling is essential for developing lymphangiogenesis in zebrafish. Among BMP Type I receptors, combined function of Alk3 and Alk3b appear to be essential for lymphatic development, while Alk2 appears to be largely dispensable for this process. In addition, Smad5, but not Smad1 or Smad9, is required to mediate BMP signaling within LECs. Our findings are consistent with recent findings which suggest the importance of BMP signaling in LECs (Dunworth et al., 2013; Farnsworth et al., 2011; Levet et al., 2013; Yoshimatsu et al., 2013). For instance, the binding of BMP9 to ALK1 receptors inhibits lymphangiogenesis and regulates lymphatic valve formation and maturation of LECs (Levet et al., 2013; Yoshimatsu et al., 2013). In addition, our recent data shows that the over-expression of Bmp2b at 48hpf in zebrafish decreases the number of LECs, illustrating the anti-lymphangiogenic activity of BMP2 signaling in lymphatic vessel formation (Dunworth et al., 2013). Therefore, as in the case of blood vessels (Kim et al., 2012; Lamvée et al., 2012; Moya et al., 2012; Wiley et al., 2011), it appears that BMP signaling may modulate development and/or maintenance of lymphatic vessels in a context-dependent manner. Considering the complex regulation of BMP signaling in other systems (Collery and Link, 2011; David et al., 2009; Farnsworth et al., 2011; Hartung et al., 2006; Miyazono et al., 2010), it is seemingly possible that distinct BMP ligands, of which distribution is spatiotemporally regulated, may exert pro- or anti-lymphangiogenic effects during development. Our analyses identify Smad5 as the most important downstream mediator of BMP signaling within lymphatic endothelial cells. Considering that the majority of BMP signaling eventually converges at SMAD1, 5, 8/9, delineating how SMAD5 can distinguish activation by pro-lymphangiogenic BMP ligands (i.e. BMP9) and anti-lymphangiogenic BMP ligands (i.e. BMP2) may help us to better understand highly complex effects of BMP signaling in LECs.

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BMP Signaling in Zebrafish Lymphatic Vessel Development
Jun-Dae Kim & Jongmin Kim

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