Published in final edited form as:
Arterioscler Thromb Vasc Biol. 2010 December ; 30(12): 2553–2561. doi:10.1161/ATVBAHA.110.214999.

Functional analyses of the non-receptor kinase Bmx in VEGF-induced lymphangiogenesis

Dennis Jones[1,2], **Zhe Xu**[1], **Haifeng Zhang**[1], **Yun He**[1], **Martin S. Kluger**[1], **Hong Chen**[3], and **Wang Min**[1,*]

[1]Interdepartmental Program in Vascular Biology and Therapeutics, Yale University School of Medicine, 10 Amistad St., New Haven, CT 06520

[2]Department of Immunobiology, Yale University School of Medicine, 10 Amistad St., New Haven, CT 06520

[3]Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, OK 73104

Abstract

Objective—To investigate the novel hypothesis that Bone Marrow kinase on the X chromosome (Bmx), an established inflammatory mediator of pathological angiogenesis, promotes lymphangiogenesis.

Methods and Results—We have recently demonstrated a critical role for Bmx in inflammatory angiogenesis. However, the role of Bmx in lymphangiogenesis has not been investigated. Here we show that in WT mice, Bmx is upregulated in lymphatic vessels in response to vascular endothelial growth factor (VEGF). In comparison to WT mice, Bmx deficient mice (Bmx-KO) mount weaker lymphangiogenic responses to VEGF-A and VEGF-C in two mouse models. In vitro, Bmx is expressed in cultured human dermal microvascular lymphatic endothelial cells (HLEC). Furthermore, pharmacological inhibition and siRNA mediated silencing of Bmx reduces VEGF-A and VEGF-Cinduced signaling and LEC tube formation. Mechanistically, we demonstrate that Bmx differentially regulates VEGFR-2 and VEGFR-3 receptor signaling pathways: Bmx associates with and directly regulates VEGFR-2 activation while Bmx associates with VEGFR-3 and regulates downstream signaling without an effect on the receptor autophosphosphorylation.

Conclusion—Our in vivo and in vitro results provide the first insight into the mechanism by which Bmx mediates VEGF-dependent lymphangiogenic signaling.

Keywords

Bmx; VEGF; VEGFR-2; VEGFR-3; lymphangiogenesis; vascular biology

*Corresponding authors: Dr. Wang Min, Interdepartmental Program in Vascular Biology and Therapeutics, Department of Pathology, Yale University School of Medicine, 10 Amistad St., New Haven, CT 06520. Tel: 203-785-6047; Fax: 203-737-2293; wang.min@yale.edu.

Introduction

In normal physiology, the open-ended lymphatic vascular system drains extravasated interstitial fluid from peripheral tissue and returns it to the blood via the thoracic duct 1. In addition, the lymphatics absorb dietary fat in the intestine, and assist in immune surveillance by allowing antigen-presenting cells (APCs) to migrate through lymphatic vessels to reach lymph nodes for antigen presentation to T and B-lymphocytes 2, 3. In pathology, lymphatics play a substantial role in conditions such as chronic inflammation 4, tumor growth and metastasis 5–7. However, despite the growing interest in lymphatic biology, the molecular mediators of lymphatic vessel function have remained poorly characterized. In ontogeny, the prevalent theory is that lymphatic vessels originate from a subset of venous endothelial cells in the anterior cardinal vein that express the homeobox transcription factor Prox-1 around embryonic (E) 9.5 of mouse development, and subsequently commit to the lymphatic endothelial cells (LEC) lineage 8. These cells sprout to form the primary lymph sacs. Peripheral lymphatic vessels form by centrifugal sprouting from the primary lymph sacs and form a network, followed by maturation of large collecting lymphatic vessels. Identification of lymphatic endothelial specific markers present in developing lymphatic vessels, such as Prox-1, podoplanin 9, and lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) 10 has advanced the study of lymphatic cells during the past decade. While these markers distinguish lymphatic endothelium from blood endothelium in the adult, some proteins are common to both blood and lymphatic endothelium, such as surface receptors VEGFR-2 and VEGFR-3 11. Interestingly, VEGFR-3 is expressed on the blood and lymphatic endothelium during development 12, and is restricted to lymphatics in the adult 13. In addition to known roles for the originally identified ligands for (VEGF-C and VEGF-D) 14, 15, lymphatic growth is also stimulated by VEGF-A in several experimental systems, and the activation of its receptor, VEGFR-2, seems to be required for LEC organization into functional capillaries 16–18. Furthermore, VEGF-A/C-VEGFR-2/3 pathways have also been shown to be involved in the pathologic formation of lymphatic vessels 19, 20. However, intracellular signaling mediators remain poorly characterized.

Bone marrow tyrosine kinase in chromosome X (Bmx; also called endothelial/epithelial tyrosine kinase [Etk]) is a member of the Tec family of non-receptor tyrosine kinases. Members of the Tec kinase family (Bmx, Btk, Itk, Rlk, and Tec) constitute the second largest family of non-receptor tyrosine kinases. They share common structural domains including a pleckstrin homology (PH) domain, a Tec homology (TH) domain, a Src-homolog domain-3 (SH3), an SH2 domain and a kinase domain 21 Despite some redundancy, specific roles for each member of this family have been identified using genetically deficient mice. Bmx-KO are viable and do not display any obvious developmental defects 22. However, upon pathological insult using an ischemia hindlimb model, Bmx-KO mice had reduced arteriogenesis and angiogenesis that led to decreased clinical recovery, limb perfusion, and ischemic reserve capacity relative to control mice 23, 24. The role of Bmx in lymphatic endothelium is unknown. In the present study, we show that Bmx is expressed in mouse lymphatic endothelial cells in vivo and in lymphatic cells isolated from human skin (HLEC). By inhibiting Bmx in HLEC, we reveal that Bmx is involved in lymphangiogenic responses induced by VEGF-A and VEGF-C. More importantly, our results from Bmx-deficient mice (Bmx-KO) elucidate a role of Bmx in lymphangiogenesis in two mouse models. Our data suggest that Bmx directly contributes to lymphatic remodeling in vivo by regulating VEGF-A/C-dependent signaling pathways.

Methods

The detailed materials and methods are provided in the online supplement. These methods include mouse cornea lymphangiogenesis assay, histology and immunohistochemistry,

lymphatic EC culture, tube formation assay and immunoblotting for Bmx-VEGFR2/3 signaling. These methods have been previously used for angiogenesis assays 23, 25.

Results

Bmx is upregulated in lymphatic vessels by VEGF and contributes to VEGF-induced lymphangiogenesis

Although Bmx is specifically expressed in aortas/arteries in normal physiology, we have previously shown that Bmx is highly induced in capillaries of ischemic tissues and is critical for inflammatory angiogenesis 23. One of many factors produced during chronic inflammation is VEGF. VEGF has been shown to induce lymphangiogenesis in several experimental systems 16–18. We used Bmx deficient mice to determine the role of Bmx in VEGF-induced lymphangiogenesis by systemically delivery of adenovirus expressing VEGF-A. Bmx was expressed at a low level in lymphatic vessels of unstimulated WT lymph nodes (LNs). However, Bmx was highly induced by VEGF in LYVE-1-positive lymphatic vessels (Fig. 1A). Expression of Bmx was luminal while expression of LYVE-1 was on the abluminal side of the vessel, as has previously been described for LYVE-1 26. As controls, Bmx was not detected in Bmx-KO mice (Supplemental Fig. 1a) or mice receiving LacZ adenovirus (not shown). Expression of Bmx showed a similar pattern in PECAM-1-positive blood vessels (Supplemental Fig. 1b). Quantitative analyses indicated that VEGF-induced lymphangiogenesis in LN was significantly reduced in Bmx-KO mice, relative to WT mice (Fig. 1B with quantification in Fig. 1C). Similar results were obtained for the role of Bmx in VEGF-induced LN angiogenesis (Supplemental Fig. 1b with quantification in Fig. 1c). Taken together, these data suggest that Bmx is inducible in lymphatic vessels and contributes to VEGF-A-induced lymph node lymphangiogenesis.

VEGF-induced cornea lymphangiogenesis is attenuated in Bmx-KO mice

The mammalian cornea is one of only a few avascular tissues. Pathologic corneal lymphangiogenesis has been reported to occur in response to various growth factors, including two members of the VEGF family of proteins (VEGF-A and VEGF-C) 27, 28. Therefore, we sought to determine the role of Bmx in this model of lymphangiogenesis by implantation of Hydron pellet containing VEGF-A or VEGF-C recombinant proteins into the cornea of WT and Bmx KO mice. Compared to saline controls, both growth factors stimulated growth of new blood vessels. Neovascularization induced by VEGF-A and VEGF-C was detected around day 7 (Fig. 2A with quantification in Fig. 2B). Sprouting from the preexisting corneal limbus was observed and was visualized by stereomicroscopy. VEGF-C induced a stronger lymphangiogenic response relative to VEGF-A (Fig. 2C with quantification in Fig. 2D), as identified by LYVE-1 staining. Interestingly, lymphangiogenesis could be detected on day 7 and peaked on day 14 post-implantation in WT mice when newly generated blood vessels started to regress (not shown). These LYVE-1-positive vessels were larger (diameter) vessels compared to newly developed LYVE-1-negative PECAM-1-positive blood vessels. Most importantly, VEGF-A and VEGF-C-induced lymphatic vessels in WT mice were longer, showed more sprouting, and covered more area than those induced in the Bmx-KO mice (Fig. 2C with quantification in 2D). These results support a role for Bmx in lymphangiogenesis in VEGF-A and VEGF-C induced cornea lymphangiogenesis.

Bmx mediates VEGF signaling and function in human lymphatic endothelial cells

To determine the mechanism by which Bmx mediates VEGF-induced lymphangiogenesis, we first examined Bmx expression in purified human lymphatic endothelial cells (HLEC) obtained from two independent sources (see Supplemental Materials and Methods). For characterization, cells were stained with Prox-1 and podoplanin, nuclear and transmembrane

markers for HLEC, respectively. Earlier passage cells were >95% positive for LEC markers. These markers were not expressed by human umbilical vein EC (HUVEC, not shown), nor detected using IgG controls (Supplemental Fig. IIa). However, HUVEC and HLEC were positive for the blood vascular lineage markers PECAM-1 (data not shown). All experiments were performed in early passages (passages 3–6) and cells remained Prox-1 positive. Next, we examined if Bmx is expressed in HLEC by immunofluorescence microscopy and Western blot with anti-Bmx antibody. Bmx was expressed in the cytoplasm of HLEC obtained from both independent sources (Clonetics cells shown in Supplemental Fig. IIa). Western blot showed that Bmx (75 kDa) expression was detected in HLEC and HUVEC (Supplemental Fig. IIb). We also examined expression of VEGF receptors that are important for lymphangiogenesis. As expected, HUVEC expressed both VEGFR-1 and VEGFR-2. However, HLEC expressed VEGFR-3 and VEGFR-2, but not VEGFR-1, as has been reported for LECs 29 (Supplemental Fig. IIb). The specificity of the Bmx antibody was confirmed by Western blot using control and Bmx knockdown HLEC (Supplemental Fig. IIc). Interestingly, we did not detect Bmx induction in HLEC by VEGF at both short and long treatments (Supplemental Fig. IIc,d, respectively). This is in contrast to the observed in vivo induction of Bmx by VEGF, suggesting that Bmx upregulation by VEGF is a secondary effect by unknown factor(s). The specificity of anti-VEGFR antibodies was determined by Western blot using cell lysates expressing each individual VEGF receptor (Supplemental Fig. IIe). To our knowledge, this is the first report to show Bmx expression in purified human lymphatic endothelial cells.

To determine the function of Bmx in HLEC, we first used an siRNA knockdown approach to determine the role of Bmx in VEGF signaling in lymphatic EC. Immunoblots revealed a >80% decrease of Bmx expression by each individual siRNA (Supplemental Fig. IIIa) or by a combination of the three siRNAs (Fig. 3A). Bmx knockdown was also confirmed by decreased mRNA transcript and did not cause an interferon response compared to parental cells as determined by quantitative PCR for the interferon-responsive genes oligo-adenylate synthetase and RNA-dependent protein kinase (Supplemental Fig. IIIb). To investigate a functional role for Bmx, we then determined the effect of Bmx knockdown on the formation of tube-like structures by HLEC in a Matrigel assay in which endothelial cells interconnect to form tube like structures - a process known to involve adhesive and migratory events as well as changes in cell morphology. HLEC tube formation was enhanced by the presence of VEGF-A or VEGF-C, with the greater enhancement using VEGF-C. Knockdown of Bmx in HLEC led to a significant reduction in the ability of HLEC to form tube-like structures in a Matrigel in response to VEGF-C (Fig. 3A with quantification in 3B). Using a second approach to assess function of Bmx in HLEC, we used an inhibitor of Bmx, LFM-A13. This drug has been shown to block Bmx signaling in HUVEC at a dose of 30 μmol/L 30. Hence, we used this concentration to pretreat HLECs for 1 hr before the Matrigel assay. Similar to the Bmx siRNA, LFM-A13 significantly reduced VEGF-C induced tube formation (Fig. 3C) compared to mock (DMSO), with quantification in (Fig. 3D).

To investigate the signaling mechanisms to explain our in vivo and in vitro lymphangiogenesis observations, we examined the effects of Bmx inhibition on the VEGF signaling. VEGF-A and VEGF-C induced phosphorylation of PLC-γ and Akt, two well-characterized (lymph)angiogenic pathways, as determined by phospho-specific antibodies. Interestingly, VEGF-A and VEGF-C differentially activated these two pathways. VEGF-A preferentially induced phosphorylation of PLC-γ whereas VEGF-C strongly activated Akt (Fig. 3E). Importantly, Bmx knockdown was able to reduce both VEGF-A and VEGF-C responses in HLEC (Fig. 3E). To determine if Bmx kinase activity is necessary for its function in VEGF signaling, we used LFM-A13. Similar to the Bmx siRNA, LFM-A13 significantly inhibited VEGF-A and VEGF-C-induced signaling compared to mock (DMSO) (Fig. 3F). Taken together, this data provides evidence that Bmx protein and its kinase

activity are necessary for VEGF-A/C lymphangiogenic signaling and tube formation in lymphatic EC.

Bmx differentially regulates VEGFR-2 and VEGFR-3-dependent signaling in human lymphatic endothelial cells

Differential activation of PLC-γ and Akt by VEGF-A and VEGF-C in HLEC prompted us to examine activation of upstream receptors. VEGF-A activates VEGFR-2 whereas VEGF-C activates both VEGFR-2 and VEGFR-3. However, a mutant form of VEGF-C (C156S, CS) binds to and activates VEGFR-3 only but not VEGFR-2 31, 32. We first examined the VEGF-receptor signaling in HLEC, which express both VEGFR-2 and VEGFR-3. Similar to HUVEC (Supplemental Fig. IVa), VEGF-A strongly induced phosphorylation of VEGFR-2 (Fig. 4A). As expected, VEGF-C, but not VEGF-CS mutant, weakly induced phosphorylation of VEGFR-2. However, VEGF-C and VEGF-CS (but not VEGF-A) induced phosphorylation of VEGFR-3 as determined by a phospho-specific antibody (pY1063/68, phosphorylation sites within the kinase activation loop essential for VEGFR-3 kinase activity 33–36) (Fig. 4A). We then determined the role of Bmx in VEGFR-2 and VEGFR-3 activation using Bmx siRNA and inhibitor. Bmx siRNA caused a significant decrease in phosphorylation of VEGFR-2 induced by VEGF-A and VEGF-C in HLEC. In contrast, Bmx siRNA did not alter or slightly increased phosphorylation of VEGFR-3 in response to VEGF-C and VEGF-CS (Fig. 4A). Similar results were obtained from the Bmx inhibitor (Fig. 4B). These data indicate that Bmx and its kinase activity are required for the activation of VEGFR-2, but not of VEGFR-3.

Since VEGF-C could utilize both VEGFR-3 homodimer and VEGFR-2/3 heterodimer to transduce the downstream signaling 36, it was not clear whether or not Bmx had any role in the VEGFR-3-dependent pathway. To determine if Bmx plays any role in VEGFR-3-dependent signaling, we examined the effects of Bmx siRNA on VEGFR-3-specific signaling. VEGF-CS, like VEGF-C, strongly induced activation of VEGFR3 downstream Akt signaling (Fig. 4C). Silencing of Bmx revealed complete inhibition of VEGF-CS induced Akt activation (Fig. 4C). Taken together, these results suggest that Bmx regulates VEGFR-3-dependent signaling downstream of VEGFR-3. However, Bmx may function at a level of the receptor complex in the VEGFR-2 signaling, consistent with our previous observations that VEGFR-2 and Bmx reciprocally activate each other 24.

Bmx via multiple domains strongly associates with VEGFR-2 in HLEC

The results above prompted us to examine if Bmx associates with VEGFR-2 in lymphatic EC. To this end, HLEC were untreated or treated with VEGF-A. Association of Bmx with VEGFR-2 was determined b y a co-immunoprecipitation assay with anti-Bmx followed by Western blot with anti-VEGFR-2. Association of Bmx with VEGFR-2 was detected in resting HLEC and VEGF-A had no effects on the association (Fig. 5A). Bmx contains several structural domains (PH, TH, SH3, SH2, and kinase domains, Fig. 5B). To determine the domain of Bmx responsible for interaction with VEGFR-2, we used FLAG-tagged expression constructs encoding different Bmx structural domains 37. As shown in Fig. 5B, Bmx-K contains the kinase domain only. Bmx-SH3/2 contains the src homology 2 and src homology 3 domains. Bmx-PTH contains the pleckstrin homology and Tec homology domains. 293T cells were co-transfected with FLAG-tagged Bmx mutants and VEGFR-2 constructs, and interaction of proteins with VEGFR-2 was performed by co-immunoprecipitation with anti-FLAG followed by Western blot with anti-VEGFR-2. Results showed that multiple domains of Bmx are important for its association with VEGFR-2 (Fig. 5C).

In a co-immunoprecipitation assay, we could not detect Bmx-VEGFR-3 complex either in resting or VEGF-C-treated HLEC (not shown). Since the level of endogenous VEGFR-3 is significantly less compared to VEGFR-2 (see Supplemental Fig. II), we overexpressed VEGFR-3 and Bmx in 293T cells. VEGFR-3 was found to associate with Bmx. We then mapped the critical domains of Bmx for VEGFR-3 binding. Co-transfection of FLAG-tagged Bmx mutants and VEGFR-3 construct show that the SH3/2 domain of Bmx is essential for association with VEGFR-3 (Fig. 5D). Taken together, these results suggest that Bmx and VEGFR-3 may form a signaling complex to mediate VEGFR-3 signaling downstream of VEGFR-3 phosphorylation (Fig. 5E).

Discussion

In the present study, we identified Bmx as a novel mediator of inflammatory lymphangiogenesis. We show that Bmx expression in lymphatic vessels can be induced by VEGF, and Bmx-KO mice exhibit reduced VEGF-induced lymphangiogenesis relative to WT mice using in vivo mouse models. Recently, two reports have described upregulation of Bmx in tumor growth 38, 39, consistent with the role of Bmx in cell growth. Expression of Bmx is appears to be transient as Bmx levels at two weeks after VEGF stimulation in vivo were similar to levels found pre-stimulation. This is consistent with the idea of Bmx being important for growth, but the not maintenance of lymphatics. In vitro, Bmx is expressed in human lymphatic EC and plays an important role in the formation of tube-like structures. Mechanistically, we demonstrate that Bmx differentially regulates VEGFR-2 and VEGFR-3 signaling pathways (Fig. 5E, A model for Bmx function in VEGF-A/C signaling): VEGF-A activates VEGFR-2 whereas VEGF-C activates both VEGFR-2 and VEGFR-3. In human lymphatic EC, VEGF-A preferentially induces phosphorylation of PLC-γ. In contrast, VEGF-C preferentially activates Akt. Interestingly, Akt phosphorylation appears to correlate with the lymphangiogenic activity of VEGF-C (Fig. 3). Bmx associates with and directly regulates VEGFR-2 activation; however, Bmx mediates VEGFR-3 downstream signaling without an effect on the receptor autophosphorylation. Therefore, Bmx is important in both VEGF-A (via VEGFR-2) and VEGF-C (via both VEGFR-2 and VEGFR-3) mediated lymphangiogenesis. Previous studies of Bmx functions have been restricted to the blood endothelium. Our current study is the first demonstration for a role of Bmx in lymphatic endothelium.

Vascular endothelial growth factors (VEGFs) and their receptors play key roles in angiogenesis and lymphangiogenesis 2, 3. We observe that human vascular EC (HUVEC) express both VEGFR-1 and VEGFR-2, two VEGF receptors involved in angiogenesis. In contrast, human lymphatic EC (HLEC) express VEGFR-3 and VEGFR-2, two VEGF receptors critical for lymphangiogenesis. Consistent with previous results 31, 32, VEGF-A activates VEGFR-2 whereas VEGF-C activates VEGFR-2 and VEGFR-3. By taking an siRNA knockdown approach in HLEC, we demonstrate that Bmx is necessary for maximal responses of both VEGF-A and VEGF-C-induced signaling cascades including phosphorylation of VEGFR-2, PLC-γ and Akt. Other signaling mediators have also been found to be important downstream of VEGFR-2/3 such as small GTPases 40. Interestingly, RhoA, which is selectively activated by Bmx amon the Small GTPases 41, is important for LEC tube formation 42. Indeed, we detected an increase in an active form of Rho A after stimulation with VEGF-A and C (Supplemental Fig. V a,b). More importantly, we observed a decreased amount of active RhoA after Bmx silencing and inhibition, relative to control (Supplemental Fig. Va,b). Interestingly, we could detect a basal association of Bmx with VEGFR-2 in resting lymphatic EC. This is similar to our previous finding that Bmx associates with TNFR2 in resting blood EC 24. The ability of Bmx to bind membrane through its PH domain allows rapid involvement in VEGFR-2/3 related signaling. Further

studies are needed to determine if Bmx is involved in VEGFR-2/3 cellular trafficking and endocytosis, a critical step in regulating VEGFR-2 activation 43, 44.

Notably, VEGF-C was more active in inducing lymphangiogenesis in vivo (cornea assay) and in vitro (the HLEC tube formation assay) compared to VEGF-A. Relative to VEGF-A, VEGF-C induces a weaker phosphorylation of the VEGFR-2-PLC-γ axis, but shows a stronger activation of VEGFR3-Akt. These results suggest that a robust response of Akt by VEGF-C is likely through VEGFR-3. Importantly, our data support that Bmx, in addition to regulating VEGFR-2, also plays a direct role in VEGFC/VEGFR-3-induced Akt activation. Since Bmx knockdown has no effect on VEGFR-3 phosphorylation, it appears that Bmx mediates VEGFR-3-dependent Akt activation at a point downstream of the receptor activation. It is known that upon VEGFR-3 ligand (e.g., VEGF-C) binding, autophosphorylation of VEGFR-3 at several tyrosine residues in the intracellular domain results in recruitment of signaling adaptor proteins such as Src homology containing protein (Shc) and growth factor receptor binding protein 2 (Grb-2), inducing the activation of downstream cascades including PI3K/Akt signaling 34, 35. The exact mechanism by which Bmx mediates Akt activation is not known. Our previous studies demonstrated that Bmx could directly associate with PI3K p85 subunit, transducing TNFR2-dependent Akt activation 24. It is conceivable that Bmx play a similar role in VEGFR-3-mediated Akt activation. It has been recently shown that both VEGFR-2 and VEGFR-3 stimulation activates eNOS in LEC 45. It remains to be determined whether Bmx is important for VEGFR-2/3-dependent eNOS activation through activating the Akt pathway.

Previously, we have shown that Bmx is among many genes that play important roles in pathological but not physiological angiogenesis 23. One reason is that Bmx is normally expressed in aortas/arteries, but ischemia strongly upregulates Bmx expression in angiogenic vessels 23. Similarly, Bmx-deficient mice have normal lymphatic vasculature under physiological settings as compared to WT mice (in lymph nodes and cornea as we observed). Bmx is not or only weakly expressed in normal lymphatic vessels, but is highly induced in response to VEGF. Upregulation of Bmx suggests that Bmx may be directly responsible for lymphangiogenesis by enhancing lymphangiogenic signaling in inflammatory settings. In vitro, VEGF-A or VEGF-C did not significantly enhance Bmx expression (supplementary figure II). It remains to be determined how Bmx expression is regulated by ischemia and VEGF. Furthermore, the incomplete loss of lymphangiogenesis suggests there are other redundant mediators of lymphangiogenesis, but Bmx is necessary for a full response in our models. In summary, we conclude that Bmx is important for maximal adult lymphangiogenesis in using the models described here. Our data provide a new potential therapeutic target for the treatment of human lymphangiogenesis-associated diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

a. Acknowledgements: We thank C. Smith for helpful discussion.

b. Source of funding: This work was supported by NIH grants R01 HL077357 and P01HL070295 and a National Nature Science Foundation of China (30828032) to WM; NIH P20 RR018758-06 to HC; a National Research Service Award (National Cancer Institute, Ruth Kirschstein Predoctoral Fellowship F31 CA 136316) to D. Jones.

c. Disclosure: none.

REFERENCES

1. Oliver G. Lymphatic vasculature development. Nat Rev Immunol. 2004; 4:35–45. [PubMed: 14704766]
2. Alitalo K, Tammela T, Petrova TV. Lymphangiogenesis in development and human disease. Nature. 2005; 438:946–953. [PubMed: 16355212]
3. Cueni LN, Detmar M. The lymphatic system in health and disease. Lymphat Res Biol. 2008; 6:109–122. [PubMed: 19093783]
4. Kunstfeld R, Hirakawa S, Hong YK, Schacht V, Lange-Asschenfeldt B, Velasco P, Lin C, Fiebiger E, Wei X, Wu Y, Hicklin D, Bohlen P, Detmar M. Induction of cutaneous delayed-type hypersensitivity reactions in VEGF-A transgenic mice results in chronic skin inflammation associated with persistent lymphatic hyperplasia. Blood. 2004; 104:1048–1057. [PubMed: 15100155]
5. Stacker SA, Caesar C, Baldwin ME, Thornton GE, Williams RA, Prevo R, Jackson DG, Nishikawa S, Kubo H, Achen MG. VEGF-D promotes the metastatic spread of tumor cells via the lymphatics. Nat Med. 2001; 7:186–191. [PubMed: 11175849]
6. Mandriota SJ, Jussila L, Jeltsch M, Compagni A, Baetens D, Prevo R, Banerji S, Huarte J, Montesano R, Jackson DG, Orci L, Alitalo K, Christofori G, Pepper MS. Vascular endothelial growth factor-C-mediated lymphangiogenesis promotes tumour metastasis. Embo J. 2001; 20:672–682. [PubMed: 11179212]
7. Dadras SS, Lange-Asschenfeldt B, Velasco P, Nguyen L, Vora A, Muzikansky A, Jahnke K, Hauschild A, Hirakawa S, Mihm MC, Detmar M. Tumor lymphangiogenesis predicts melanoma metastasis to sentinel lymph nodes. Mod Pathol. 2005; 18:1232–1242. [PubMed: 15803182]
8. Wigle JT, Oliver G. Prox1 function is required for the development of the murine lymphatic system. Cell. 1999; 98:769–778. [PubMed: 10499794]
9. Breiteneder-Geleff S, Soleiman A, Horvat R, Amann G, Kowalski H, Kerjaschki D. [Podoplanin--a specific marker for lymphatic endothelium expressed in angiosarcoma]. Verh Dtsch Ges Pathol. 1999; 83:270–275. [PubMed: 10714221]
10. Banerji S, Ni J, Wang SX, Clasper S, Su J, Tammi R, Jones M, Jackson DG. LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. J Cell Biol. 1999; 144:789–801. [PubMed: 10037799]
11. Tammela T, Zarkada G, Wallgard E, Murtomaki A, Suchting S, Wirzenius M, Waltari M, Hellstrom M, Schomber T, Peltonen R, Freitas C, Duarte A, Isoniemi H, Laakkonen P, Christofori G, Yla-Herttuala S, Shibuya M, Pytowski B, Eichmann A, Betsholtz C, Alitalo K. Blocking VEGFR-3 suppresses angiogenic sprouting and vascular network formation. Nature. 2008; 454:656–660. [PubMed: 18594512]
12. Karpanen T, Alitalo K. Molecular biology and pathology of lymphangiogenesis. Annu Rev Pathol. 2008; 3:367–397. [PubMed: 18039141]
13. Kaipainen A, Korhonen J, Mustonen T, van Hinsbergh VW, Fang GH, Dumont D, Breitman M, Alitalo K. Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. Proc Natl Acad Sci U S A. 1995; 92:3566–3570. [PubMed: 7724599]
14. Kukk E, Lymboussaki A, Taira S, Kaipainen A, Jeltsch M, Joukov V, Alitalo K. VEGF-C receptor binding and pattern of expression with VEGFR-3 suggests a role in lymphatic vascular development. Development. 1996; 122:3829–3837. [PubMed: 9012504]
15. Achen MG, Jeltsch M, Kukk E, Makinen T, Vitali A, Wilks AF, Alitalo K, Stacker SA. Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). Proc Natl Acad Sci U S A. 1998; 95:548–553. [PubMed: 9435229]
16. Halin C, Tobler NE, Vigl B, Brown LF, Detmar M. VEGF-A produced by chronically inflamed tissue induces lymphangiogenesis in draining lymph nodes. Blood. 2007; 110:3158–3167. [PubMed: 17625067]
17. Bjorndahl MA, Cao R, Burton JB, Brakenhielm E, Religa P, Galter D, Wu L, Cao Y. Vascular endothelial growth factor-a promotes peritumoral lymphangiogenesis and lymphatic metastasis. Cancer Res. 2005; 65:9261–9268. [PubMed: 16230387]

18. Nagy JA, Vasile E, Feng D, Sundberg C, Brown LF, Detmar MJ, Lawitts JA, Benjamin L, Tan X, Manseau EJ, Dvorak AM, Dvorak HF. Vascular permeability factor/vascular endothelial growth factor induces lymphangiogenesis as well as angiogenesis. J Exp Med. 2002; 196:1497–1506. [PubMed: 12461084]
19. Hirakawa S, Brown LF, Kodama S, Paavonen K, Alitalo K, Detmar M. VEGF-C-induced lymphangiogenesis in sentinel lymph nodes promotes tumor metastasis to distant sites. Blood. 2007; 109:1010–1017. [PubMed: 17032920]
20. Hong YK, Lange-Asschenfeldt B, Velasco P, Hirakawa S, Kunstfeld R, Brown LF, Bohlen P, Senger DR, Detmar M. VEGF-A promotes tissue repair-associated lymphatic vessel formation via VEGFR-2 and the alpha1beta1 and alpha2beta1 integrins. Faseb J. 2004; 18:1111–1113. [PubMed: 15132990]
21. Schmidt U, Boucheron N, Unger B, Ellmeier W. The role of Tec family kinases in myeloid cells. Int Arch Allergy Immunol. 2004; 134:65–78. [PubMed: 15133303]
22. Rajantie I, Ekman N, Iljin K, Arighi E, Gunji Y, Kaukonen J, Palotie A, Dewerchin M, Carmeliet P, Alitalo K. Bmx tyrosine kinase has a redundant function downstream of angiopoietin and vascular endothelial growth factor receptors in arterial endothelium. Mol Cell Biol. 2001; 21:4647–4655. [PubMed: 11416142]
23. He Y, Luo Y, Tang S, Rajantie I, Salven P, Heil M, Zhang R, Luo D, Li X, Chi H, Yu J, Carmeliet P, Schaper W, Sinusas AJ, Sessa WC, Alitalo K, Min W. Critical function of Bmx/Etk in ischemia-mediated arteriogenesis and angiogenesis. J Clin Invest. 2006; 116:2344–2355. [PubMed: 16932810]
24. Zhang R, Xu Y, Ekman N, Wu Z, Wu J, Alitalo K, Min W. Etk/Bmx transactivates vascular endothelial growth factor 2 and recruits phosphatidylinositol 3-kinase to mediate the tumor necrosis factor-induced angiogenic pathway. J Biol Chem. 2003; 278:51267–51276. [PubMed: 14532277]
25. Zhang H, He Y, Dai S, Xu Z, Luo Y, Wan T, Luo D, Jones D, Tang S, Chen H, Sessa WC, Min W. AIP1 functions as an endogenous inhibitor of VEGFR2-mediated signaling and inflammatory angiogenesis in mice. J Clin Invest. 2008; 118:3904–3916. [PubMed: 19033661]
26. Prevo R, Banerji S, Ferguson DJ, Clasper S, Jackson DG. Mouse LYVE-1 is an endocytic receptor for hyaluronan in lymphatic endothelium. J Biol Chem. 2001; 276:19420–19430. [PubMed: 11278811]
27. Cursiefen C, Chen L, Borges LP, Jackson D, Cao J, Radziejewski C, D'Amore PA, Dana MR, Wiegand SJ, Streilein JW. VEGF-A stimulates lymphangiogenesis and hemangiogenesis in inflammatory neovascularization via macrophage recruitment. J Clin Invest. 2004; 113:1040–1050. [PubMed: 15057311]
28. Nakao S, Maruyama K, Zandi S, Melhorn MI, Taher M, Noda K, Nusayr E, Doetschman T, Hafezi-Moghadam A. Lymphangiogenesis and angiogenesis: concurrence and/or dependence? Studies in inbred mouse strains. Faseb J. 24:504–513. [PubMed: 19858096]
29. Murakami M, Zheng Y, Hirashima M, Suda T, Morita Y, Ooehara J, Ema H, Fong GH, Shibuya M. VEGFR1 tyrosine kinase signaling promotes lymphangiogenesis as well as angiogenesis indirectly via macrophage recruitment. Arterioscler Thromb Vasc Biol. 2008; 28:658–664. [PubMed: 18174461]
30. Tu T, Thotala D, Geng L, Hallahan DE, Willey CD. Bone marrow X kinase-mediated signal transduction in irradiated vascular endothelium. Cancer Res. 2008; 68:2861–2869. [PubMed: 18413754]
31. Adams RH, Alitalo K. Molecular regulation of angiogenesis and lymphangiogenesis. Nat Rev Mol Cell Biol. 2007; 8:464–478. [PubMed: 17522591]
33. Goldman J, Rutkowski JM, Shields JD, Pasquier MC, Cui Y, Schmokel HG, Willey S, Hicklin DJ, Pytowski B, Swartz MA. Cooperative and redundant roles of VEGFR-2 and VEGFR-3 signaling in adult lymphangiogenesis. Faseb J. 2007; 21:1003–1012. [PubMed: 17210781]
33. Fournier E, Blaikie P, Rosnet O, Margolis B, Birnbaum D, Borg JP. Role of tyrosine residues and protein interaction domains of SHC adaptor in VEGF receptor 3 signaling. Oncogene. 1999; 18:507–514. [PubMed: 9927207]

34. Salameh A, Galvagni F, Bardelli M, Bussolino F, Oliviero S. Direct recruitment of CRK and GRB2 to VEGFR-3 induces proliferation, migration, and survival of endothelial cells through the activation of ERK, AKT, and JNK pathways. Blood. 2005; 106:3423–3431. [PubMed: 16076871]
35. Wissmann C, Detmar M. Pathways targeting tumor lymphangiogenesis. Clin Cancer Res. 2006; 12:6865–6868. [PubMed: 17145802]
36. Dixelius J, Makinen T, Wirzenius M, Karkkainen MJ, Wernstedt C, Alitalo K, Claesson-Welsh L. Ligand-induced vascular endothelial growth factor receptor-3 (VEGFR-3) heterodimerization with VEGFR-2 in primary lymphatic endothelial cells regulates tyrosine phosphorylation sites. J Biol Chem. 2003; 278:40973–40979. [PubMed: 12881528]
37. Pan S, An P, Zhang R, He X, Yin G, Min W. Etk/Bmx as a tumor necrosis factor receptor type 2-specific kinase: role in endothelial cell migration and angiogenesis. Mol Cell Biol. 2002; 22:7512–7523. [PubMed: 12370298]
38. Dai B, Chen H, Guo S, Yang X, Linn DE, Sun F, Li W, Guo Z, Xu K, Kim O, Kong X, Melamed J, Qiu S, Chen H, Qiu Y. Compensatory upregulation of tyrosine kinase Etk/BMX in response to androgen deprivation promotes castration-resistant growth of prostate cancer cells. Cancer Res. 2010; 70:5587–5596. [PubMed: 20570899]
39. Cohen I, Maoz M, Turm H, Grisaru-Granovsky S, Maly B, Uziely B, Weiss E, Abramovitch R, Gross E, Barzilay O, Qiu Y, Bar-Shavit R. Etk/Bmx regulates proteinase-activated-receptor1 (PAR1) in breast cancer invasion: signaling partners, hierarchy and physiological significance. PLoS One. 5:e11135. [PubMed: 20559570]
40. Larrieu-Lahargue F, Welm AL, Thomas KR, Li DY. Netrin-4 induces lymphangiogenesis in vivo. Blood. 2010; 115:5418–5426. [PubMed: 20407033]
41. Kim O, Yang J, Qiu Y. Selective activation of small GTPase RhoA by tyrosine kinase Etk through its pleckstrin homology domain. J Biol Chem. 2002; 277:30066–30071. [PubMed: 12023958]
42. Navarro A, Perez RE, Rezaiekhaligh M, Mabry SM, Ekekezie II. T1alpha/podoplanin is essential for capillary morphogenesis in lymphatic endothelial cells. Am J Physiol Lung Cell Mol Physiol. 2008; 295:L543–L551. [PubMed: 18658274]
43. He Y, Zhang H, Yu L, Gunel M, Boggon TJ, Chen H, Min W. Stabilization of VEGFR2 signaling by cerebral cavernous malformation 3 is critical for vascular development. Sci Signal. 2010; 3:ra26. [PubMed: 20371769]
44. Lanahan AA, Hermans K, Claes F, Kerley-Hamilton JS, Zhuang ZW, Giordano FJ, Carmeliet P, Simons M. VEGF receptor 2 endocytic trafficking regulates arterial morphogenesis. Dev Cell. 2010; 18:713–724. [PubMed: 20434959]
45. Lahdenranta J, Hagendoorn J, Padera TP, Hoshida T, Nelson G, Kashiwagi S, Jain RK, Fukumura D. Endothelial nitric oxide synthase mediates lymphangiogenesis and lymphatic metastasis. Cancer Res. 2009; 69:2801–2808. [PubMed: 19318557]

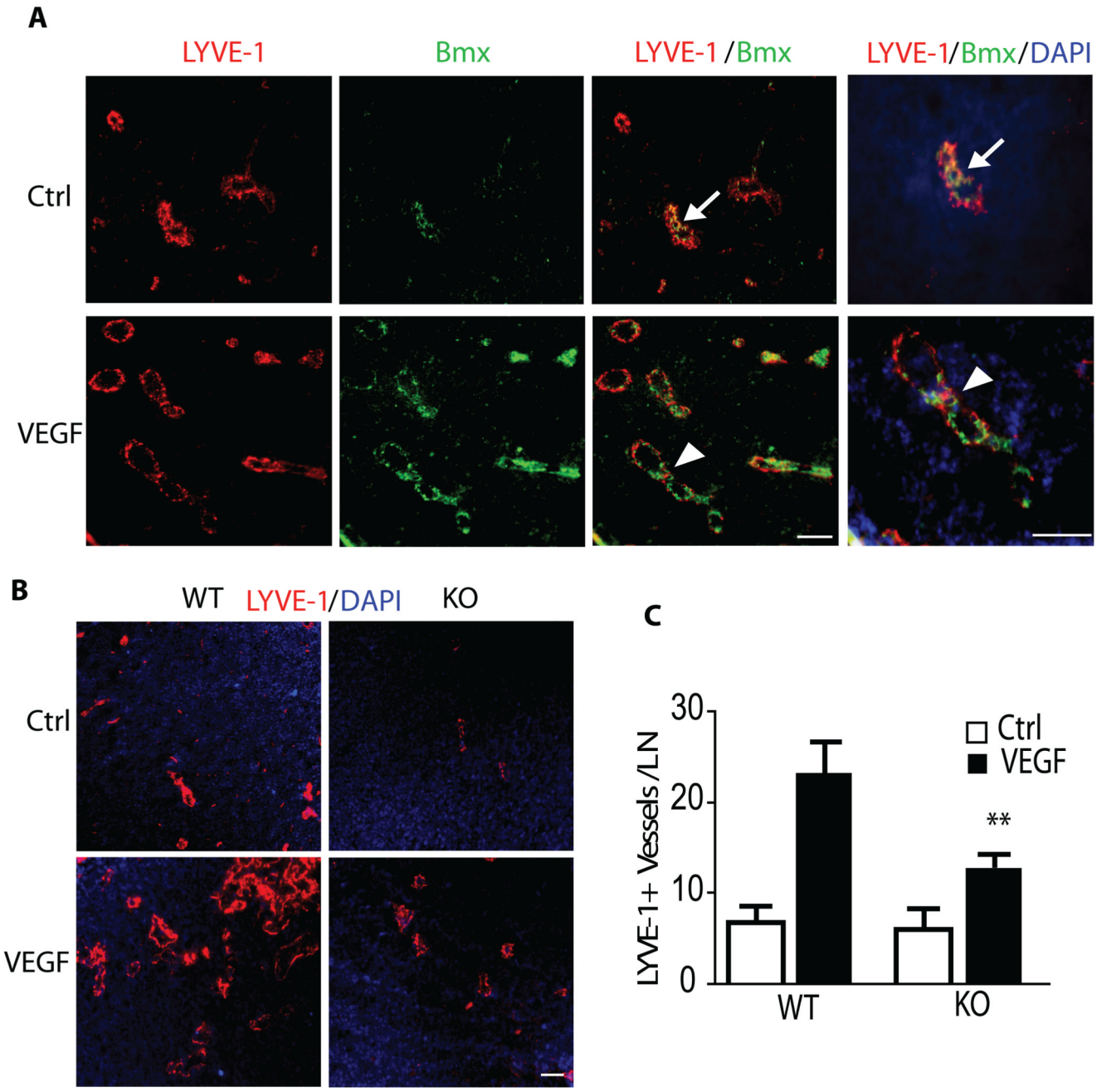

Fig. 1. Bmx is upregulated in lymphatic vessels by VEGF and contributes to VEGF-induced lymphangiogenesis

A. Induction of Bmx in lymphatic EC by VEGF-A. 1×10^8 pfu of adenoviruses encoding lacZ (Ctrl) or VEGF-A were injected intravenously into WT mice. Inguinal lymph nodes were harvested on day 5 post-injection and Bmx expression in lymphatic vessel was detected by immunostaining of frozen sections with anti-Bmx and anti-LYVE-1 followed by Alexa Fluor 488 (donkey anti-rabbit) and 594 (donkey anti-rat conjugated secondary antibodies), respectively. Sections were counterstained with DAPI and images were merged. Basal and VEGF-induced Bmx expression in LYVE-1 positive lymphatic vessels are indicated by arrows and arrowheads, respectively. Higher magnification images are shown

on the right panels. **B–C**. Reduction of VEGF-induced lymphangiogenesis in Bmx-KO mice. Lymphatic vessels were visualized by immunohistochemistry using anti-LYVE-1 (**C**). LYVE-1-positive vessels were quantified in **D**. Data are the mean ± SEM, n=6 for each strain. **, $p<0.01$. Scale bar, 50 μm.

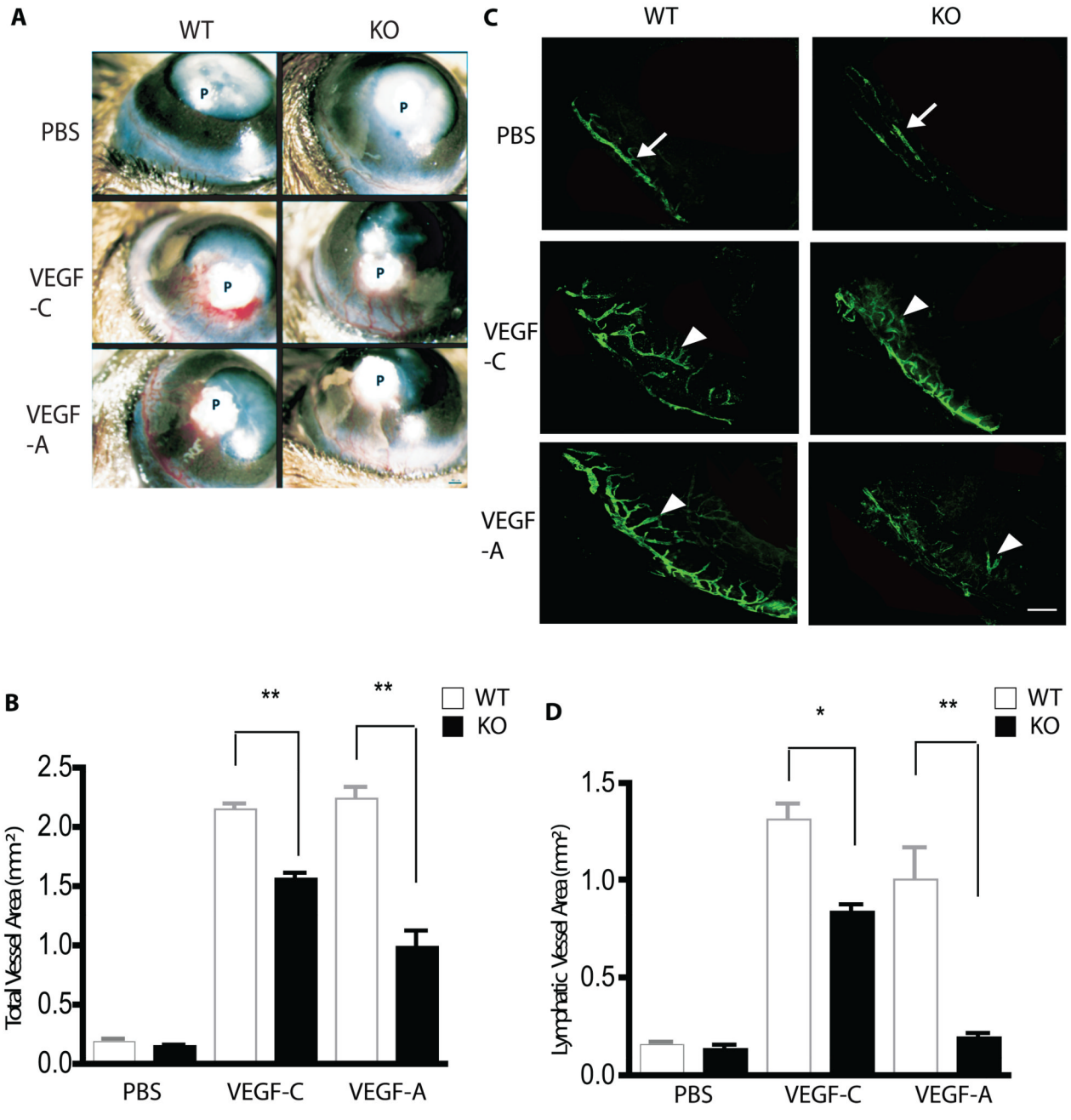

Fig. 2. VEGF-induced cornea lymphangiogenesis is attenuated in Bmx-KO mice

Hydron pellet containing PBS, VEGF-A or VEGF-C was implanted into the cornea of WT and Bmx-KO mice. Neovacularization was assessed using stereomicroscopy on day 7 following implantation (**A**) and vascular density was quantified in **B**. Corneas were harvested on day 14, and lymphatic vessels were stained with LYVE-1 (**C**) and quantified in (**D**). Basal and VEGF-induced LYVE-1 positive lymphatic vessels are indicated by arrows and arrowheads, respectively. Data are mean ± SEM, n=6 for each strain, **, $p<0.01$. Scale bar, 100 μm.

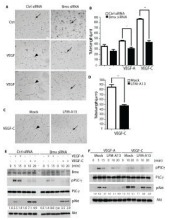

Fig. 3. Bmx mediates VEGF-A and VEGF-C-induced lymphangiogenic signaling

A–B. Effects of Bmx knockdown on VEGF-induced lymphatic tube formation. HLEC were transfected with a human Bmx siRNA or control siRNA (20 nM) for 24 h, subsequently serum-starved overnight. Cells were seeded to Matrigel and cultured with 3% serum alone or 3% serum + VEGF-A (50 ng/ml) or VEGF-C (100 ng/ml) for 6 h. Representative images are shown in **A** with quantification in **B** for tube length per field (20x). Individual EC and tube-like structures are indicated by arrows and arrowheads, respectively. Data are mean ± SEM from three independent experiments, *, $p<0.05$. Scale bar, 100 μm. **C–D**. Effects of the Bmx inhibitor on lymphatic tube formation. Parental HLEC were subjected to VEGF-C-induced tube formation in the presence of DMSO (mock) or a Bmx inhibitor LFM-A13 (30 μmol/L). Representative images are shown in **C** with quantification in **D** for tube length per field (20x). Individual EC and tube-like structures are indicated by arrows and arrowheads, respectively. Data are mean ± SEM from three independent experiments, *, $p<0.05$. Scale bar, 100 μm. **E**. Effects of Bmx siRNA on VEGF signaling. siRNA-transfected HLEC were serum-starved overnight and then treated with VEGF (50 ng/ml) or VEGF-C (100 ng/ml) for indicated times. Knockdown of Bmx was determined by Western blot with anti-Bmx. Phosphorylations of PLC-γ and Akt were determined by Western blot with phospho-specific antibodies. Total levels of PLC-γ and Akt were determined by Western blot with respective antibodies. Similar results were obtained from additional two experiments. **F**. Effects of the Bmx inhibitor on VEGF signaling. Parental HLEC were serum-starved overnight. Cells were pretreated with DMSO (mock) or a Bmx inhibitor LFM-A13 (30 μmol/L) for 1 h followed by treatment with VEGF (50 ng/ml) or VEGF-C (100 ng/ml) for indicated times. Phosphorylations of PLC-γ and Akt were determined. Similar results were obtained from additional two experiments.

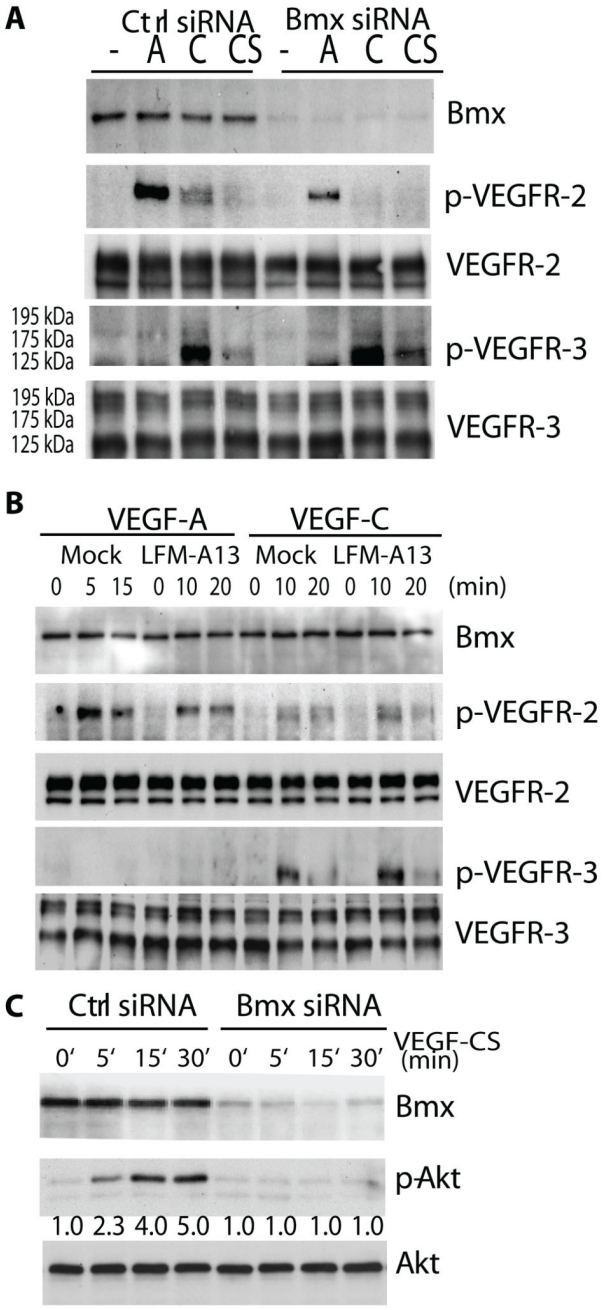

Fig. 4. Bmx differentially regulates VEGFR-2 and VEGFR-3-dependent signaling in human lymphatic endothelial cells

A. Bmx siRNA reduces phosphorylation of VEGFR-2 but not VEGFR-3. HLEC were transfected with a human Bmx siRNA or control siRNA (20 nM) for 24 h, subsequently serum-starved overnight and then treated with VEGF-A (50 ng/ml), VEGF-C (100 ng/ml) or VEGF-CS(*Cys156Ser*) (500 ng/ml) for 5, 10 and 10 min, respectively. Phospho- and total VEGFR-2 and VEGFR-3 were determined by Western blot with phospho-specific antibodies. Similar results were obtained from additional two experiments. **B**. The Bmx inhibitor reduces phosphorylation of VEGFR-2 but not VEGFR-3. Parental HLEC were serum starved overnight. Cells were pretreated with DMSO (mock) or a Bmx inhibitor

LFM-A13 (30 μmol/L) for 1 h followed by treatment with VEGF (50 ng/ml) or VEGF-C (100 ng/ml) for indicated times. Phospho- and total VEGFR-2 and VEGFR-3 were determined. Similar results were obtained from additional two experiments. **C**. Bmx mediates VEGR-3 downstream signaling. HLEC were transfected with a human Bmx siRNA or control siRNA (20 nM) for 24 h, subsequently serum-starved overnight and then treated with VEGFR-3-specific ligand VEGF-CS(*Cys156Ser*) (500 ng/ml) for indicated times. Phospho- and total Akt were determined. Similar results were obtained from additional two experiments.

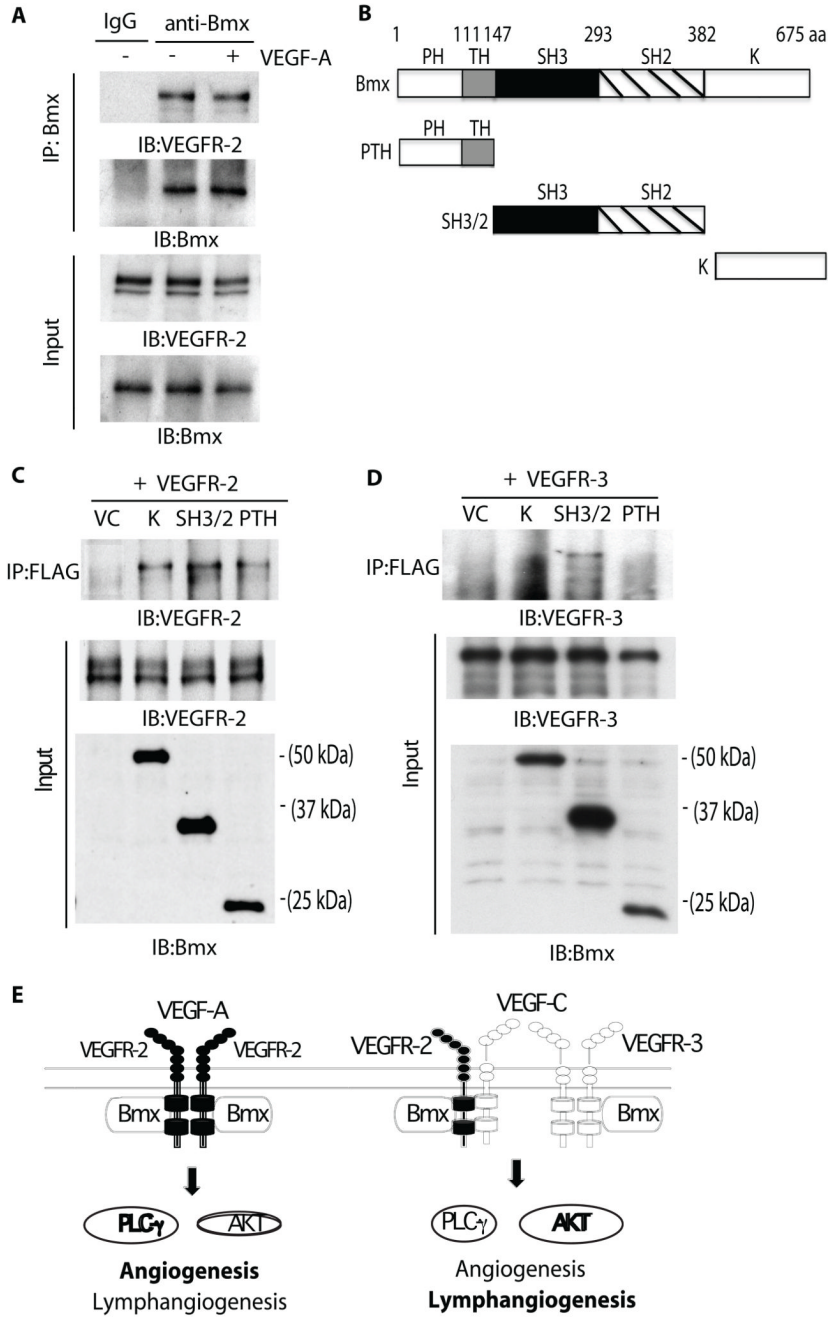

Fig. 5. Bmx via multiple domains associates with VEGFR-2 in HLEC

A. Association of endogenous Bmx with VEGFR-2. HLEC were serum-starved and treated with VEGF-A (50 ng/ml for 5 min). Association of Bmx with VEGFR-2 was determined by a co-immunoprecipitation assay with anti-Bmx followed by Western blot with anti-VEGFR2 or anti-Bmx. An isotope IgG was used as a control. Proteins in the input were determined by Western blot with respectively antibodies. Experiments were repeated twice. **B**. Schematic diagram for Bmx structural domains and expression constructs. PH: pleakstrin homolog domain; TH: the Tec homology domain; SH3: the Src homolog 3 domain; SH2: the Src homology 2 domain; K: the kinase domain. **C**. Bmx via multiple domains binds to VEGFR-2. VEGFR-2 expression plasmid was co-transfected with FLAG-tagged Bmx

mutants into 293T cells, and interaction of Bmx proteins with VEGFR-2 was performed by co-immunoprecipitation with anti-FLAG followed by Western blot with anti-VEGFR-2. VC: vector control. **D**. Bmx via the SH3/2 domains weakly associates with VEGFR-3. VEGFR-3 and FLAG-tagged Bmx mutants were co-expressed in 293T cells, and interaction of Bmx proteins with VEGFR-3 was performed by a co-immunoprecipitation assay with anti-FLAG followed by Western blot with anti-VEGFR-3. **E**. A model for the role of Bmx in VEGF-A and VEGF-C signaling. Bmx differentially regulates VEGFR-2 and VEGFR-3-dependent lymphangiogenic pathways. See text for details.