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Alk2/ACVR1 and Alk3/BMPR1A Provide Essential Function for Bone Morphogenetic Protein Induced Retinal Angiogenesis

Heon-Woo Lee^{1,6}, Diana C. Chong^{2,6}, Roxana Ola^{1,6}, William P. Dunworth¹, Stryder Meadows³, Jun Ka⁴, Vesa M. Kaartinen⁵, Yibing Qyang¹, Ondine Cleaver³, Victoria L. Bautch^{2,7}, Anne Eichmann^{1,7}, and Suk-Won Jin^{1,4,7}

¹Yale Cardiovascular Research Center, Section of Cardiovascular Medicine, Department of Internal Medicine, Yale University School of Medicine, New Haven, CT, USA

²Department of Biology and McAllister Heart Institute, University of North Carolina, NC, USA

³Department of Molecular Biology, UT Southwestern Medical Center, 5323 Harry Hines Blvd, Dallas, TX 75390, USA

⁴School of Life Sciences and Cell Logistics Research Center, Gwangju Institute of Science and Technology, Gwangju, Korea

⁵Department of Biologic & Materials Sciences, School of Dentistry, University of Michigan, Ann Arbor, MI, USA

Abstract

Objective—Increasing evidence suggests that Bone Morphogenetic Protein (BMP) signaling regulates angiogenesis. Here, we aimed to define the function of BMP receptors in regulating early post-natal angiogenesis by analysis of inducible, endothelial specific deletion of the BMP receptor components *Bmpr2*, *Alk1*, *Alk2* and *Alk3* in mouse retinal vessels.

Approach and Results—Expression analysis of several BMP ligands showed that pro-angiogenic BMP ligands are highly expressed in postnatal retinas. Consistently, BMP receptors are also strongly expressed in retina with a distinct pattern. To assess the function of BMP signaling in retinal angiogenesis, we first generated mice carrying an endothelial-specific inducible deletion of BMP Type 2 receptor (*Bmpr2*). Postnatal deletion of *Bmpr2* in endothelial cells substantially decreased the number of angiogenic sprouts at the vascular front and branchpoints behind the front, leading to attenuated radial expansion. To identify critical BMPR1s associated with BMPR2 in retinal angiogenesis, we generated endothelial-specific inducible deletion of three BMPR1s abundantly expressed in endothelial cells and analyzed the respective phenotypes. Among these, endothelial specific deletion of either *Alk2/acvr1* or *Alk3/Bmpr1a* caused a delay in radial expansion, reminiscent of vascular defects associated with postnatal endothelial specific deletion

⁷To whom correspondence should be addressed: bautch@unc.edu, anne.eichmann@yale.edu, suk-won.jin@yale.edu, or sukwonjin@gist.ac.kr.

⁶These authors contribute equally

DISCLOSURE

NONE

of BMPR2, suggesting that ALK2/ACVR1 and ALK3/BMPR1A are likely to be the critical BMPR1s necessary for pro-angiogenic BMP signaling in retinal vessels.

Conclusions—Our data identify BMP signaling mediated by coordination of ALK2/ACVR1, ALK3/BMPR1A, and BMPR2 as an essential pro-angiogenic cue for retinal vessels.

Keywords

Angiogenesis; BMP signaling; retina; vertebrate development

INTRODUCTION

Bone Morphogenetic Protein (BMP) signaling has been implicated as a key regulator for angiogenesis¹. Depending on the nature of the ligands, BMP signaling can either promote or inhibit angiogenesis²; Pro-angiogenic BMP2/4 augments vessel sprouting in a matrigel plug assay³, and stimulation with BMP2 promotes angiogenic responses such as filopodia extension and migration in human umbilical vein endothelial cells (HUVEC) by inducing the expression of target genes, such as *MYOX*⁴. In addition, its zebrafish orthologue, *Bmp2b*, functions as the predominant angiogenic cue for veins^{5, 6}. Similar pro-angiogenic effects have been demonstrated for BMP6^{4, 7}. In contrast, BMP9 and BMP10 induce quiescence of endothelial cells, and therefore function as anti-angiogenic cues^{8–10}. Consistent with the idea that BMP9 and BMP10 modulate the homeostasis of mature vessels, it has been shown that BMP9 can promote differentiation of endothelial progenitors during ischemic neovascularization¹¹

On the cell membrane of the signal receiving cells, BMP ligands interact with tetra-heteromeric receptor complexes composed of two BMP Type 1 Receptors (BMPR1s) and two BMP Type 2 Receptors (BMPR2s). The signaling specificity of each BMP ligand is determined by the interaction between BMP ligand and its cognate BMPR1, since BMPR2 can only serve as a low affinity receptor^{12, 13}. Therefore, BMPR1s are essential for the outcomes of BMP signaling. In the mammalian genome, four BMPR1s, *Alk1/Acvr11*, *Alk2/Acvr1*, *Alk3/Bmpr1a*, and *Alk6/Bmpr1b*, have been annotated^{14, 15}. Previous work showed that endothelial-specific deletion of *Alk1* generates exuberant angiogenesis, indicating that ALK1 is likely to mediate anti-angiogenic BMP9/10 signaling in endothelial cells^{9, 16, 17}. Considering that ALK2, ALK3, and ALK6 bind to BMP2, BMP4, and BMP6 in other circumstances¹⁸, these receptors are likely to mediate pro-angiogenic BMP signaling. However, since global deletion of these receptors leads to early embryonic lethality^{19–22}, surprisingly little is known about the individual function of these BMPR1s in endothelial cells. In addition, previous attempts to elucidate the role of each BMPR1 in endothelial cells failed to provide comprehensive analyses due to the lack of suitable endothelial-specific Cre driver lines^{23, 24}. Most importantly, manipulations of BMPR1 in endothelial cells were not performed in the same way in the same vascular bed in previous studies, making it difficult to determine the role of each BMPR1s in endothelial cells^{23, 24}.

In this study, we investigated the function of each BMPRI in angiogenesis using postnatal mouse retinal vessels to better understand the molecular and cellular underpinning of pro-angiogenic BMP signaling. To assess the contribution of BMPs in retinal angiogenesis, we

showed BMP signaling reporter activity in the early postnatal retina, and found that BMP6 and BMP7 are the most abundant BMP ligands in that organ. We found that several BMPR1s were expressed in distinct regions of the retinal vasculature. Conditional deletion of *Bmpr2* and three highly expressed BMPR1s, *Alk1/Acvr11*, *Alk2/Acvr1*, and *Alk3/Bmpr1a*, in endothelial cells of postnatal mice showed that mice deficient for *Alk2/Acvr1* or *Alk3/Bmpr1a* partially phenocopied the vascular defects of mice with an endothelial-specific deletion of *Bmpr2*. Taken together, our data indicate that the pro-angiogenic BMP signaling mediated by ALK2 and ALK3 receptors, likely in conjunction with BMPR2, is essential for proper retinal angiogenesis.

MATERIALS AND METHODS

Materials and Methods are available in the online-only Data Supplement.

RESULTS

Diverse BMP ligands and receptors are expressed during retinal angiogenesis

To determine the role of pro-angiogenic BMP signaling, we first examined the expression of GFP in retinal vessels of BRE-GFP mice, where the expression of GFP is regulated by BMP Responsive Elements (BRE) isolated from the *Id1* promoter²⁵. As previously reported²⁶, BRE-GFP expression was broadly detected in P5 retinal vessels (Figure 1A). Within the vascular front, BRE-GFP was highly expressed in both tip and stalk cells (Figure 1B). While the expression of BRE-GFP in stalk cells can be attributed to the ALK1-mediated anti-angiogenic BMP signaling activity^{9, 26}, it is not clear how BRE-GFP expression in tip cells is induced.

To identify which BMP ligands and receptors induce BRE-GFP expression in the retinal vessels, we performed *in situ* hybridization analyses on mouse retinas and quantitative RT-PCR using cells isolated from postnatal retinas at different developmental stages (Figure 1C and Online Figure I). A number of pro-angiogenic BMP ligands were highly expressed during the stages coincident with extensive retinal angiogenesis (Figure 1C and Online Figure II). In particular, the expression of BMP6 and BMP7, was increased over two folds. In contrast, expression of BMP9 and 10, which are ligands for ALK1 and are produced by liver and heart respectively, were not detected in the retina (Online Figure I). However, given that they are delivered by circulation, it is likely that the mature BMP9 and BMP10 protein are present in the retina. In endothelial cells, only three BMPR1s, ALK1, ALK2, and ALK3, but not ALK6, were highly expressed. Therefore, ALK6 was excluded from further analyses (Online Figure III).

Next, we analyzed the expression of BMPR2 and the three BMPR1s that are abundant in endothelial cells, ALK1/ACVRL, ALK2/ACVR1A, and ALK3/BMPR1A by immunostaining in retinas (Figure 1D–G and data not shown). BMPR2 staining is vascular and appears in all vessels, with heightened expression at the vascular front (Figures 1D and H). Interestingly, each BMPR1 has distinct expression pattern; ALK1 and ALK3 proteins are found in both plexus and vascular front, with ALK1 being enriched in the arteries of the plexus region. ALK2 staining is highly enriched in veins in the plexus region and not

associated with the vascular front or arteries (Figures 1E–G and I–K). These findings show that pro-angiogenic BMP ligands and receptors are highly expressed in blood vessels during retinal development, and the receptors have distinct expression patterns. Therefore, it is likely that BRE-GFP reporter activity in tip cells at the vascular front results from the activity of pro-angiogenic BMP ligands.

BMPR2 activity promotes retinal angiogenesis

To examine the requirement for pro-angiogenic BMP signaling during retinal angiogenesis, we generated mice carrying an inducible, endothelial-specific *Bmpr2* deletion by crossing *Bmpr2^{fl/fl}* and *Cdh5(Pac)Cre^{ERT2}* mice²⁷. Given that BMPR2 is the predominant binding partner for BMPR1s mediating pro-angiogenic BMP activity, we postulated that deletion of *Bmpr2* is likely to attenuate signaling outcomes downstream of most pro-angiogenic BMP ligand engagement during retinal angiogenesis. Consistent with our hypothesis, endothelial cell deletion of *Bmpr2* significantly decreased the radial expansion of the retinal vessels (Figures 2A and 2C), indicating that BMP signaling is essential for retinal angiogenesis. *Bmpr2^{fl/fl};Cdh5(Pac)Cre^{ERT2}* mice also displayed a significant reduction in vascular density (% vascularized area) compared to wild-type littermates (Figures 2B and 2C). This data shows that pro-angiogenic BMP signaling mediated by BMPR2 is a critical positive regulator of retinal angiogenesis.

Endothelial specific deletion of *Alk2/ACVR1A* or *Alk3/BMPR1A* but not *Alk1/ACVRL* recapitulates vascular defects of endothelial specific deletion of *Bmpr2*

Next, we investigated critical BMPR1 interactions mediating pro-angiogenic BMP signaling during retinal angiogenesis. Endothelial deletion of *Alk1*, *Alk2*, or *Alk3* during postnatal retinal angiogenesis was induced by crossing homozygous mice carrying a floxed allele of each *BMPR1* and *Cdh5(Pac)Cre^{ERT2}* mice (Online Figure IV). As previously reported^{9, 27}, tamoxifen-mediated deletion of *Alk1* in endothelial cells at P1 did not exhibit severe defects in radial extension, but caused exuberant angiogenesis in developing retinas compared to phenotypic wild-type littermates and therefore substantially increased vascular density (% vascularized area) (Figure 3A, D). In *Alk1^{fl/fl};Cdh5(Pac)Cre^{ERT2}* mice, the vascular phenotype was more pronounced in the vascular front than in the plexus region (Figure 3A). Considering the distinct phenotypes between *Bmpr2^{fl/fl};Cdh5(Pac)Cre^{ERT2}* and *Alk1^{fl/fl};Cdh5(Pac)Cre^{ERT2}* mice, it is unlikely that ALK1 is involved in pro-angiogenic BMP signaling mediated by BMPR2.

In contrast, tamoxifen-mediated deletion of *Alk2* or *Alk3* in endothelial cells at P1 led to a substantial reduction in the radial expansion of retinal vessels (Figures 3B–C and Online Figure IV), reminiscent of the defects in *Bmpr2^{fl/fl};Cdh5(Pac)Cre^{ERT2}* mice (Figure 2A). In addition, vascular density was substantially reduced in tamoxifen-injected *Alk2^{fl/fl};Cdh5(Pac)Cre^{ERT2}* or *Alk3^{fl/fl};Cdh5(Pac)Cre^{ERT2}* retinas compared to wild-type littermates (Figure 3D). These findings show that both ALK2 and ALK3 are important for proper retinal vessel morphogenesis, likely in conjunction with BMPR2.

Endothelial specific deletion of *Alk3/BMPR1A* and *Bmpr2* but not *Alk2/ACVR1A* affect angiogenic sprouts at the vascular front

Considering that both *Alk2* and *Alk3* deletion in endothelial cells significantly delayed radial expansion but only *Alk3* is expressed at the vascular front, we hypothesized that ALK2 and ALK3 receptors would differentially affect angiogenic sprouts. To examine this notion, we first examined the phenotype of endothelial deletion of *Bmpr2* and found that lack of *Bmpr2* significantly decreased the number of angiogenic sprouts (Figure 4A). Endothelial deletion of *Alk3/BMPR1A* similarly decreased the number of angiogenic sprouts present at the vascular front compared to phenotypic wild-type littermates (Figures 4B to 4C), while deletion of *Alk2* did not significantly alter the number of angiogenic sprouts at the vascular front. Considering that the vascular coverage (% vascularized area) was significantly decreased in retinas of mice with deletion of either receptor (Figure 3B–D), it is likely that different BMPR1 receptors mediate pro-angiogenic BMP signaling at the vascular front and behind the front.

DISCUSSION

Our results identify BMPR2/ALK2 and BMPR2/ALK3 as key receptors that mediate pro-angiogenic BMP signaling in the early post-natal retina, and reveal regional differences among BMPR1s by analysis of parallel genetic experiments in a defined vascular bed. Deletion of the common BMPR2 receptor reduced vascular sprouting and density. Deletion of ALK3, which is ubiquitously expressed in retinal endothelial cells, also dramatically reduced vascular sprouting and density, while loss of ALK2, which is enriched behind the vascular front, did not significantly affect sprouting but reduced overall vessel density. Therefore, we propose that spatially regulated BMPR1 expression fine-tunes endothelial cell responses to pro-angiogenic BMP ligands in development. Since expression of BMP ligands selective for ALK2 and ALK3 is elevated during retinal angiogenesis, it is tempting to speculate that BMP6/7-ALK2/3-BMPR2 signaling axis may provide essential input for the developing retina.

Since the phenotype of endothelial-specific deletion of *Bmpr2* is quite distinct from the vascular phenotype caused by endothelial-specific deletion of *Alk1*, it is likely that BMPR2-mediated pro-angiogenic signaling is dominant over interactions with ALK1 in the early postnatal retina. In contrast, ALK2 and ALK3 appear to be essential for BMPR2-mediated pro-angiogenic signaling. These receptors function similarly but non-redundantly behind the vascular front in mediating branching, as deletion of either Type 1 receptor partially phenocopies the phenotype of *Bmpr2* deletion and results in reduced branching. This finding suggests that both ALK2/BMPR2 and ALK3/BMPR2 complexes contribute to branching morphogenesis, which is consistent with our previous finding that pro-angiogenic BMP signaling leads to increased branching *in vitro* and *in vivo*^{5, 6}. Angiogenic sprouting at the vascular front is likely to selectively utilize ALK3/BMPR2 complexes, since genetic deletion of *Alk2* did not significantly affect angiogenic sprouting. This is consistent with the expression patterns of the receptors in the early postnatal retina, as ALK3 but not ALK2 reactivity was found at the vascular front. ALK2 reactivity is enriched in veins, while ALK3 expression is not enriched in larger vessels, suggesting potential differences in ALK2 and

ALK3 mediated pro-angiogenic BMP signaling. ALK1, which does not appear to be essential for BMPR2-mediated pro-angiogenic BMP signaling, may provide an additional regulation for vessel remodeling and homeostasis at later stages.

Previously, it has been shown that BMPR1s can promiscuously form heterodimeric complex^{16, 28–30}. For instance, it has been shown that ALK1 and ALK2 can form heterodimers under certain circumstances^{29, 30}. Similarly, ALK2 and ALK3 are proposed to form heterodimers to mediate BMP2 signaling²⁸. Therefore, it is possible that they may influence the signaling property of each other. While we cannot rule out this possibility due to the lack of appropriate reagents, given the distinct expression pattern of BMPR1s, we believe that the vascular phenotype caused by endothelial specific deletion of single BMPR1 is likely to represent the unique signaling property of each receptor.

Our analyses indicate that ALK3/BMPR2 mediated BMP signaling regulates angiogenic sprouting at the vascular front, while both ALK3/BMPR2 and ALK2/BMPR2 regulate branching behind the vascular front. This is consistent with our finding that BMP signaling, as read out by BRE-EGFP, is active throughout the retinal vasculature, and suggests that pro-angiogenic BMP signaling regulates both sprouting and branching. These results are consistent with a previous report describing ubiquitous expression of BRE-EGFP in retinal vessels²⁶.

Our work presents compelling evidence that pro-angiogenic BMP signaling is important for angiogenesis. Here we show that expression of Type 1 receptors, specifically ALK2 and ALK3, may explain the different effects of BMP signaling on developing vessels, and they each likely complex with BMPR2 to mediate pro-angiogenic effects in blood vessels in a non-redundant way. While the molecular underpinning of the phenotypic differences between *Alk2^{fl/fl}; Cdh5(Pac)Cre^{ERT2}* and *Alk3^{fl/fl}; Cdh5(Pac)Cre^{ERT2}* mice needs further analyses, careful phenotypic comparisons between these mice will help elucidate how angiogenesis is regulated in development and disease, and may provide useful information in developing therapeutic strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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LIST OF NON-STANDARD ABBREVIATIONS AND ACRONYMS

ALK Activin receptor-like kinase

| | |
|--------------|---------------------------------------|
| BMP | Bone Morphogenetic Protein |
| BMPR1 | BMP Type 1 Receptor |
| BMPR2 | BMP Type 2 Receptor |
| BRE | BMP Response Element |
| HUVEC | Human Umbilical Vein Endothelial Cell |

References

1. Cai J, Pardali E, Sanchez-Duffhues G, ten Dijke P. Bmp signaling in vascular diseases. *FEBS letters*. 2012; 586:1993–2002. [PubMed: 22710160]
2. David L, Feige JJ, Bailly S. Emerging role of bone morphogenetic proteins in angiogenesis. *Cytokine & growth factor reviews*. 2009; 20:203–212. [PubMed: 19502096]
3. Langenfeld EM, Langenfeld J. Bone morphogenetic protein-2 stimulates angiogenesis in developing tumors. *Molecular cancer research : MCR*. 2004; 2:141–149. [PubMed: 15037653]
4. Pi X, Ren R, Kelley R, Zhang C, Moser M, Bohil AB, Divito M, Cheney RE, Patterson C. Sequential roles for myosin-x in bmp6-dependent filopodial extension, migration, and activation of bmp receptors. *The Journal of cell biology*. 2007; 179:1569–1582. [PubMed: 18158328]
5. Wiley DM, Kim JD, Hao J, Hong CC, Bautch VL, Jin SW. Distinct signalling pathways regulate sprouting angiogenesis from the dorsal aorta and the axial vein. *Nature cell biology*. 2011; 13:686–692. [PubMed: 21572418]
6. Wakayama Y, Fukuhara S, Ando K, Matsuda M, Mochizuki N. Cdc42 mediates bmp-induced sprouting angiogenesis through fmnl3-driven assembly of endothelial filopodia in zebrafish. *Developmental cell*. 2015; 32:109–122. [PubMed: 25584797]
7. Ren R, Charles PC, Zhang C, Wu Y, Wang H, Patterson C. Gene expression profiles identify a role for cyclooxygenase 2-dependent prostanoid generation in bmp6-induced angiogenic responses. *Blood*. 2007; 109:2847–2853. [PubMed: 17119124]
8. David L, Mallet C, Mazerbourg S, Feige JJ, Bailly S. Identification of bmp9 and bmp10 as functional activators of the orphan activin receptor-like kinase 1 (alk1) in endothelial cells. *Blood*. 2007; 109:1953–1961. [PubMed: 17068149]
9. Larrivee B, Prahst C, Gordon E, del Toro R, Mathivet T, Duarte A, Simons M, Eichmann A. Alk1 signaling inhibits angiogenesis by cooperating with the notch pathway. *Developmental cell*. 2012; 22:489–500. [PubMed: 22421041]
10. Ricard N, Ciaia D, Levet S, Subileau M, Mallet C, Zimmers TA, Lee SJ, Bidart M, Feige JJ, Bailly S. Bmp9 and bmp10 are critical for postnatal retinal vascular remodeling. *Blood*. 2012; 119:6162–6171. [PubMed: 22566602]
11. Kim J, Kim M, Jeong Y, Lee WB, Park H, Kwon JY, Kim YM, Hwang D, Kwon YG. Bmp9 induces cord blood-derived endothelial progenitor cell differentiation and ischemic neovascularization via alk1. *Arteriosclerosis, thrombosis, and vascular biology*. 2015; 35:2020–2031.
12. Pouliot F, Blais A, Labrie C. Overexpression of a dominant negative type ii bone morphogenetic protein receptor inhibits the growth of human breast cancer cells. *Cancer research*. 2003; 63:277–281. [PubMed: 12543773]
13. Townson SA, Martinez-Hackert E, Greppi C, Lowden P, Sako D, Liu J, Ucran JA, Liharska K, Underwood KW, Sehra J, Kumar R, Grinberg AV. Specificity and structure of a high affinity activin receptor-like kinase 1 (alk1) signaling complex. *The Journal of biological chemistry*. 2012; 287:27313–27325. [PubMed: 22718755]
14. Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. The protein kinase complement of the human genome. *Science*. 2002; 298:1912–1934. [PubMed: 12471243]
15. Mazerbourg S, Sangkuhl K, Luo CW, Sudo S, Klein C, Hsueh AJ. Identification of receptors and signaling pathways for orphan bone morphogenetic protein/growth differentiation factor ligands

- based on genomic analyses. *The Journal of biological chemistry*. 2005; 280:32122–32132. [PubMed: 16049014]
16. Aspalter IM, Gordon E, Dubrac A, Ragab A, Narloch J, Vizan P, Geudens I, Collins RT, Franco CA, Abrahams CL, Thurston G, Fruttiger M, Rosewell I, Eichmann A, Gerhardt H. Alk1 and alk5 inhibition by nrp1 controls vascular sprouting downstream of notch. *Nature communications*. 2015; 6:7264.
 17. Zhu Q, Kim YH, Wang D, Oh SP, Luo K. Snr facilitates alk1-smad1/5 signaling during embryonic angiogenesis. *The Journal of cell biology*. 2013; 202:937–950. [PubMed: 24019535]
 18. ten Dijke P, Yamashita H, Sampath TK, Reddi AH, Estevez M, Riddle DL, Ichijo H, Heldin CH, Miyazono K. Identification of type i receptors for osteogenic protein-1 and bone morphogenetic protein-4. *The Journal of biological chemistry*. 1994; 269:16985–16988. [PubMed: 8006002]
 19. Komatsu Y, Scott G, Nagy A, Kaartinen V, Mishina Y. Bmp type i receptor alk2 is essential for proper patterning at late gastrulation during mouse embryogenesis. *Developmental dynamics : an official publication of the American Association of Anatomists*. 2007; 236:512–517. [PubMed: 17117439]
 20. Gu Z, Reynolds EM, Song J, Lei H, Feijen A, Yu L, He W, MacLaughlin DT, van den Eijnden-van Raaij J, Donahoe PK, Li E. The type i serine/threonine kinase receptor actria (alk2) is required for gastrulation of the mouse embryo. *Development*. 1999; 126:2551–2561. [PubMed: 10226013]
 21. Mishina Y, Crombie R, Bradley A, Behringer RR. Multiple roles for activin-like kinase-2 signaling during mouse embryogenesis. *Developmental biology*. 1999; 213:314–326. [PubMed: 10479450]
 22. Mishina Y, Suzuki A, Ueno N, Behringer RR. Bmpr encodes a type i bone morphogenetic protein receptor that is essential for gastrulation during mouse embryogenesis. *Genes & development*. 1995; 9:3027–3037. [PubMed: 8543149]
 23. Song L, Fassler R, Mishina Y, Jiao K, Baldwin HS. Essential functions of alk3 during av cushion morphogenesis in mouse embryonic hearts. *Developmental biology*. 2007; 301:276–286. [PubMed: 16959237]
 24. Wang J, Sridurongrit S, Dudas M, Thomas P, Nagy A, Schneider MD, Epstein JA, Kaartinen V. Atrioventricular cushion transformation is mediated by alk2 in the developing mouse heart. *Developmental biology*. 2005; 286:299–310. [PubMed: 16140292]
 25. Monteiro RM, de Sousa Lopes SM, Bialecka M, de Boer S, Zwijsen A, Mummery CL. Real time monitoring of bmp smads transcriptional activity during mouse development. *Genesis*. 2008; 46:335–346. [PubMed: 18615729]
 26. Moya IM, Umans L, Maas E, Pereira PN, Beets K, Francis A, Sents W, Robertson EJ, Mummery CL, Huylebroeck D, Zwijsen A. Stalk cell phenotype depends on integration of notch and smad1/5 signaling cascades. *Developmental cell*. 2012; 22:501–514. [PubMed: 22364862]
 27. Benedito R, Roca C, Sorensen I, Adams S, Gossler A, Fruttiger M, Adams RH. The notch ligands *dll4* and *jagged1* have opposing effects on angiogenesis. *Cell*. 2009; 137:1124–1135. [PubMed: 19524514]
 28. Little SC, Mullins MC. Bone morphogenetic protein heterodimers assemble heteromeric type i receptor complexes to pattern the dorsoventral axis. *Nature cell biology*. 2009; 11:637–643. [PubMed: 19377468]
 29. Luo J, Tang M, Huang J, He BC, Gao JL, Chen L, Zuo GW, Zhang W, Luo Q, Shi Q, Zhang BQ, Bi Y, Luo X, Jiang W, Su Y, Shen J, Kim SH, Huang E, Gao Y, Zhou JZ, Yang K, Luu HH, Pan X, Haydon RC, Deng ZL, He TC. Tgfbeta/bmp type i receptors alk1 and alk2 are essential for bmp9-induced osteogenic signaling in mesenchymal stem cells. *The Journal of biological chemistry*. 2010; 285:29588–29598. [PubMed: 20628059]
 30. Scharpfenecker M, van Dinther M, Liu Z, van Bezooijen RL, Zhao Q, Pukac L, Lowik CW, ten Dijke P. Bmp-9 signals via alk1 and inhibits bfgf-induced endothelial cell proliferation and vegf-stimulated angiogenesis. *Journal of cell science*. 2007; 120:964–972. [PubMed: 17311849]

HIGHLIGHTS

- Pro-angiogenic BMP signaling is essential for retinal angiogenesis.
- Alk2/Acvr1 and Alk3/Bmpr1a relays distinct aspects of pro-angiogenic effects of BMP.

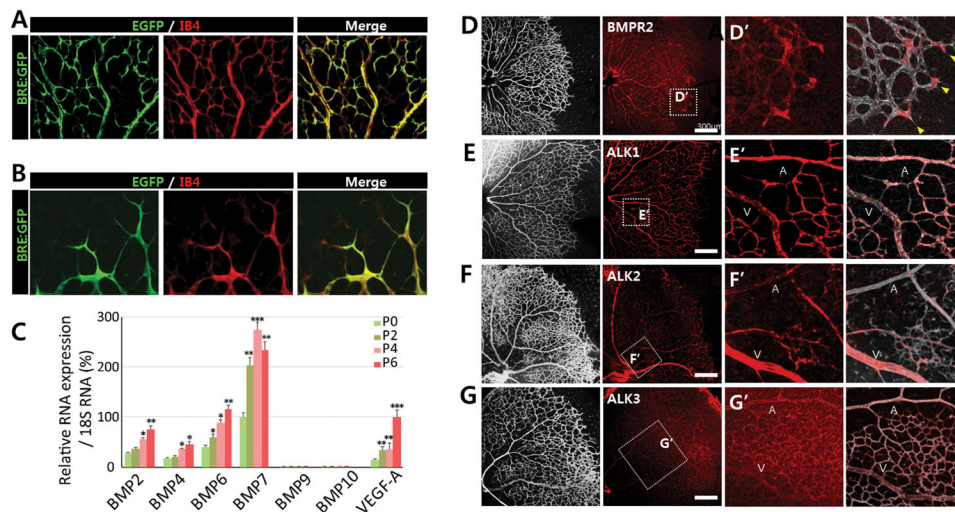


FIGURE 1. Diverse BMP ligands and receptors are expressed during retinal development (A–B) Representative overview of retinal vessels at (A) the retinal center and (B) vascular front shown in higher magnification in postnatal day P5 *BRE-gfp* transgenic mice. Endothelial cells are shown in red. BRE-GFP is highly expressed in both tip cells and stalk cells. (C) qRT-PCR for *Bmp* ligands and *Vegfa* in P0, P2, P4, and P6 retina. *Bmp6* and *Bmp7* are the most abundant BMP ligands between P0 and P6 (n=5). (D–G) Immunohistochemistry of P5 retina showing BMPR2 (D), ALK1/ACVRL (E), ALK2/ACVR1 (F), and ALK3/BMPRI1A (G). While BMPR2, ALK1 and ALK3 are expressed ubiquitously, ALK2 expression is enriched in the veins within the plexus region; scale bar, 300 μ m. (H–K) Areas within the white rectangles in panels D to G are shown in higher magnification.

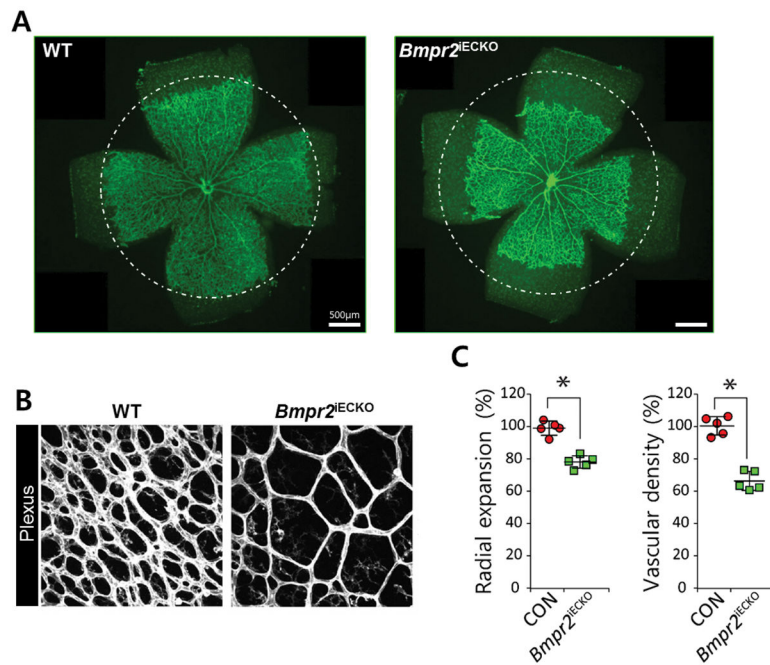


FIGURE 2. Bmpr2 is essential for retinal angiogenesis

(A) Representative overview of P5 retinal vessels taken from inducible endothelial specific knock out of *Bmpr2^{fl/fl}; Cdh5(Pac)Cre^{ERT2}* (right) and their phenotypic wild-type littermates (left); scale bar, 500 μm. Mean radial expansion of the retinal vessels in P5 *Bmpr2^{fl/fl}* retinas was decreased to 78.9±9.8% of wildtype littermates. (B) Representative overview of plexus region of P5 retina in *Bmpr2^{fl/fl}; Cdh5(Pac)Cre^{ERT2}* (right) and their phenotypic wild-type littermates (left). Mean vascular density in the plexus region in *Bmpr2^{fl/fl}* retinas was reduced to 65.7±8.3% of wildtype littermates; scale bar, 500 μm. Endothelial cells are visualized by anti- Isolectin B4 (IB4) staining. (C) Quantification of radial expansion and vascular density (% vascularized area) in *Bmpr2^{fl/+}; Cdh5(Pac)Cre^{ERT2}* (gray) and *Bmpr2^{fl/fl}; Cdh5(Pac)Cre^{ERT2}* mice injected with 100 μg tamoxifen at P1, retinas were assayed P5 (n=5). $P < 0.05$. Statistical significance was assessed using a Student's unpaired *t*-test.

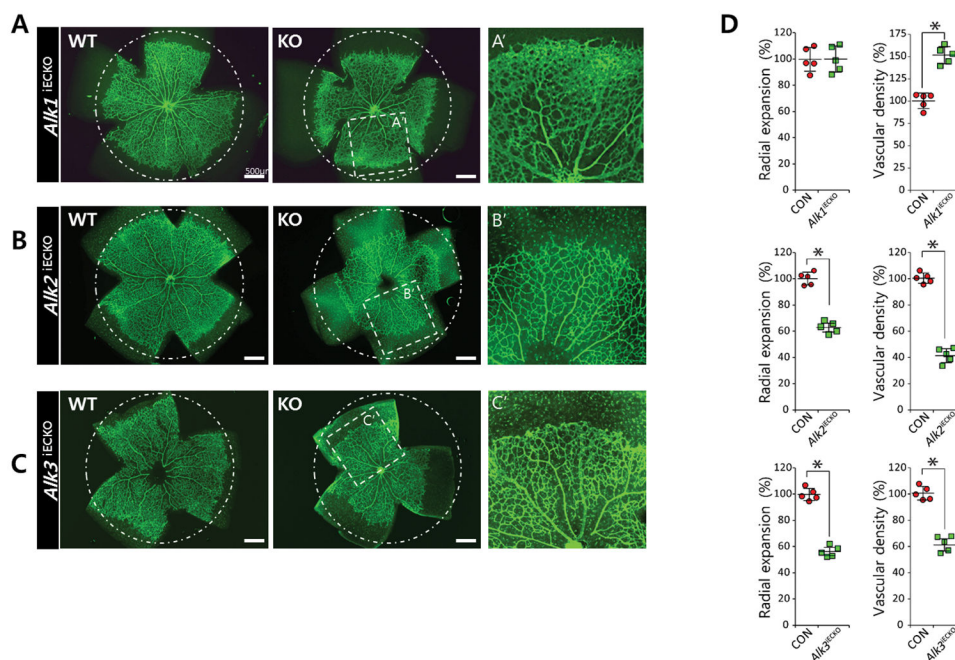


FIGURE 3. ALK2 and ALK3 promotes retinal angiogenesis

(A–C) Representative overview of retinal vessels taken from inducible endothelial specific knock out of *Alk1*^{fl/fl}; *Cdh5(Pac)Cre*^{ERT2} (A), *Alk2*^{fl/f}; *Cdh5(Pac)Cre*^{ERT2} (B), or *Alk3*^{fl/fl}; *Cdh5(Pac)Cre*^{ERT2} (C) and their phenotypic wild-type littermates. Mice were injected with 50µg tamoxifen at P1, retinas were assayed P5; scale bar, 500µm. Both radial expansion and vascular density in the plexus region were significantly increased in P5 *Alk1*^{fl/fl}; *Cdh5(Pac)Cre*^{ERT2} retinas (101±6.7% for radial expansion and 155±6.2% for vascular density compared to wildtype littermates). By contrast, deletion of either *Alk2* or *Alk3* significantly decreased both radial expansion (63.2±17.1% for *Alk2*^{fl/f}; *Cdh5(Pac)Cre*^{ERT2} retinas and 56.4±14.2% for *Alk3*^{fl/fl}; *Cdh5(Pac)Cre*^{ERT2} retinas compared to wildtype littermates) and vascular density in the plexus region (42.3±15.9% for *Alk2*^{fl/f}; *Cdh5(Pac)Cre*^{ERT2} retinas, and 61.9±12.8% for *Alk3*^{fl/fl}; *Cdh5(Pac)Cre*^{ERT2} retinas compared to wildtype littermates). Areas within the white rectangle in middle column are shown in higher magnification (right column). While radial expansion was similarly affected by deletion of either *Alk2* or *Alk3*, vascular density in the plexus region is more severely affected by the deletion of *Alk2* than *Alk3*. Endothelial cells are visualized by anti- Isolectin B4 (IB4) staining. (D) Quantification of radial expansion and vascular density (% vascularized area) in inducible endothelial specific knockout of each *BMPRI* (pink) and their littermates (gray) (n=5). *P*<0.05. Statistical significance was assessed using a Student's unpaired *t*-test.

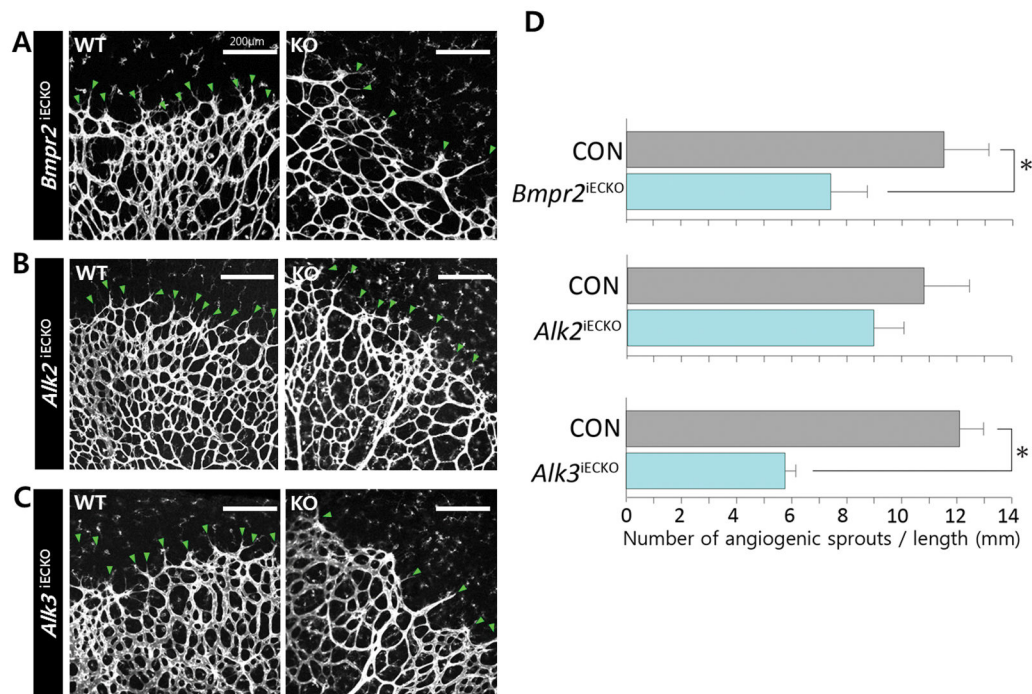


FIGURE 4. ALK3/BMPR2 signaling is required for the formation of angiogenic sprouts in the vascular front

(A) Representative image of vascular front in postnatal day P5 *Bmpr2^{fl/fl}; Cdh5(Pac)Cre^{ERT2}* (A), *Alk2^{fl/fl}; Cdh5(Pac)Cre^{ERT2}* (B), or *Alk3^{fl/fl}; Cdh5(Pac)Cre^{ERT2}* Endothelial cells are visualized by anti- Isolectin B4 (IB4) staining. (C) and their phenotypic wild-type littermates. Green arrows point the angiogenic sprouts in the vascular front (n=5); scale bar, 200 μm. (D) Quantification of angiogenic sprouts. The number of angiogenic sprouts in the vascular front was measured by dividing the number of angiogenic sprouts by the total length of vascular front (See Supplemental methods for detail). While the number of angiogenic sprouts in the vascular front was mildly decreased by 20% to in P5 *Alk2^{fl/fl}* retinas (8.8±0.2 compared to 10.8±1.9 in wildtype littermates), it was decreased by 45% in *Bmpr2^{fl/fl}* (6.5±1.3 in *Bmpr2^{fl/fl}* retinas compared to 11.8±1.6 in wildtype littermates), and by 53% in *Alk3^{fl/fl}* retinas (5.3±0.4 in *Alk3^{fl/fl}* retinas compared to 11.2±0.9 in wildtype littermates). *P*<0.05. Statistical significance was assessed using a Student's unpaired *t*-test.