Circulating Bmp10 acts through endothelial Alk1 to mediate flow-dependent arterial quiescence

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SUMMARY
Blood flow plays crucial roles in vascular development, remodeling and homeostasis, but the molecular pathways required for transducing flow signals are not well understood. In zebrafish embryos, arterial expression of activin receptor-like kinase 1 (alk1), which encodes a TGFβ family type I receptor, is dependent on blood flow, and loss of alk1 mimics lack of blood flow in terms of dysregulation of a subset of flow-responsive arterial genes and increased arterial endothelial cell number. These data suggest that blood flow activates Alk1 signaling to promote a flow-responsive gene expression program that limits nascent arterial caliber. Here, we demonstrate that restoration of endothelial alk1 expression to flow-deprived arteries fails to rescue Alk1 activity or normalize arterial endothelial cell gene expression or number, implying that blood flow may play an additional role in Alk1 signaling independent of alk1 induction. To this end, we define cardiac-derived Bmp10 as the crucial ligand for endothelial Alk1 in embryonic vascular development, and provide evidence that circulating Bmp10 acts through endothelial Alk1 to limit endothelial cell number in and thereby stabilize the caliber of nascent arteries. Thus, blood flow promotes Alk1 activity by concomitantly inducing alk1 expression and distributing Bmp10, thereby reinforcing this signaling pathway, which functions to limit arterial caliber at the onset of flow. Because mutations in ALK1 cause arteriovenous malformations (AVMs), our findings suggest that an impaired flow response initiates AVM development.

KEY WORDS: Bmp10, Alk1/ACVRL1, Hereditary hemorrhagic telangiectasia, Arteriovenous malformation, Zebrafish, Flow response

INTRODUCTION
Blood flow imparts mechanical forces and distributes endocrine factors that influence vascular development and remodeling and allow maintenance of arterial-venous identity, but the molecular pathways that govern these flow-dependent processes are not fully understood (for a review, see Roman and Pekkan, 2012). Among the physical forces imparted by blood flow, shear stress, the frictional force that acts in the direction of blood flow, is the most extensively studied. Laminar shear stress induces expression of the transcription factor Klf2 which coordinates expression of numerous flow-responsive genes that promote cell cycle arrest and vasodilation, thereby favoring vascular quiescence and conferring atheroprotection (Dekker et al., 2002; Dekker et al., 2005; Dekker et al., 2006; Parmar et al., 2006). By contrast, disturbed (low or oscillatory) shear stress activates the transcription factors NF-κB and AP-1, which promote an atherogenic response characterized by inflammation and vasoconstriction (Lan et al., 1994; Khachigian et al., 1995; Bhullar et al., 1998; Hay et al., 2003). However, the repertoire of flow responses that exist in vivo clearly extends well beyond these pathways.

Mounting evidence implicates the TGFβ family type I receptor, Alk1, as a key player in a flow-responsive signaling pathway that functions to promote quiescence in nascent arteries. In zebrafish embryos, alk1 (acvrl1 – Zebrafish Information Network) is expressed predominantly in arteries proximal to the heart, which experience relatively high magnitudes of mechanical forces; preventing heartbeat eliminates alk1 mRNA expression (Corti et al., 2011). Furthermore, either loss of blood flow or loss of alk1 results in increased expression of cxcr4a, which encodes a pro-angiogenic chemokine receptor, and decreased expression of endothelin 1 (edn1), which encodes a vasoconstrictive peptide (Corti et al., 2011). Both of these genes are flow responsive in cultured endothelial cells (Bussmann et al., 2011), and zebrafish alk1 mutants, which exhibit abnormally high levels of arterial cxcr4a, develop enlarged arteries containing supernumerary endothelial cells, suggestive of failed flow-induced suppression of endothelial cell migration or proliferation (Roman et al., 2002; Corti et al., 2011). Evidence from mice further supports the idea that Alk1 functions in a flow-responsive pathway to promote quiescence of nascent arteries. In mice, Alk1 is expressed predominantly in embryonic arterial endothelial cells, with weak expression in adults (Seki et al., 2003; Seki et al., 2004). However, Alk1 expression can be induced in adult mice during periods of active angiogenesis in arterial endothelial cells exposed to high shear stress (Seki et al., 2003). Furthermore, recent mouse studies have implicated bone morphogenetic protein (BMP) signaling in general or Alk1 signaling in particular in the maintenance of a quiescent endothelial stalk cell fate (Larrivée et al., 2012; Moya et al., 2012). Together, these data from both mouse and zebrafish support the hypothesis that Alk1 signaling mediates flow-dependent arterial endothelial cell quiescence. Notably, alk1 acts independently of klf2a in zebrafish (Corti et al., 2011), suggesting that multiple flow-dependent pathways coordinate in vivo to control the activation state of the endothelium.

Alk1 signaling is crucial for normal vascular development and homeostasis in mice and zebrafish, with loss of function resulting in embryonic lethality associated with development of direct
connections between arteries and veins, or arteriovenous malformations (AVMs) (Oh et al., 2000; Urness et al., 2000; Roman et al., 2002). In humans, ALK1 heterozygosity results in hereditary hemorrhagic telangiectasia type 2 (HHT2), a vascular disorder characterized by predisposition to development of telangiectases and AVMs (Guttmacher et al., 1995; Johnson et al., 1996). However, despite the clear link between ALK1 signaling and AVM prevention, the ALK1 signaling pathway remains poorly defined in vivo. In TGFB family signaling, ligands bind to a heterotrimeric complex of two type II receptors and two type I receptors, both of which are serine/threonine kinases. The type II receptors phosphorylate and thus activate the type I receptors, and the type I receptors then phosphorylate receptor-specific Smad proteins. Phosphorylated Smads complex with the common partner Smad, Smad4, enter the nucleus and, together with a variety of transcription factors, regulate transcription of target genes (for a review, see Xu et al., 2012). With respect to ALK1, the ligands TGFB1, TGFB3, BMP9 and BMP10 can induce ALK1-dependent phosphorylation of Smad1, Smad5 and/or Smad9 (hereafter referred to as Smad1/5/9) and stimulate activity of a phospho-Smad1/5/9 (pSmad1/5/9)-responsive reporter in cultured endothelial cells (ten Dijke et al., 1994; Lux et al., 1999; Oh et al., 2000; Goumans et al., 2002; Goumans et al., 2003; Brown et al., 2005; David et al., 2007; Scharpenecker et al., 2007; David et al., 2008; Mitchell et al., 2010). However, although TGFB-mediated activation of ALK1 requires ALK5 (canonical TGFB1 type I receptor) activity in cultured endothelial cells (Goumans et al., 2003), endothelial cell-specific deletion of ALK5 in mice or ALK5 inhibition in zebrafish embryos does not affect Alk1 activity (Park et al., 2008), suggesting that TGFB subfamily ligands are not relevant to ALK1 signaling in embryonic development. In fact, BMP9 and BMP10 are the only TGFB superfAMILY ligands that bind to ALK1 with high affinity in vitro, and they circulate at physiologically relevant concentrations (Brown et al., 2005; David et al., 2008; Mitchell et al., 2010; Ricard et al., 2012). However, neither Bmp9- (Ricard et al., 2012) nor Bmp10- (Chen et al., 2004) null mice phenocopy Alk1-null mice (Oh et al., 2000; Urness et al., 2000), which present with AVMs. Although the lack of AVMs could reflect ligand redundancy, interference with both ligands via blocking antibodies and/or ligand traps impairs mouse retinal angiogenesis but does not produce retinal AVMs (Larrivée et al., 2012; Ricard et al., 2012). As such, the identity of the activating ALK1 ligand in vivo has remained elusive.

In this work, we use zebrafish embryos to demonstrate that Alk1 kinase activity, in addition to alk1 mRNA expression, requires blood flow, and we provide evidence that this newly defined role for blood flow stems not from mechanical force but from distribution of the cardiac-derived circulating ligand, Bnp10. Taken together, our data define a novel endocrine pathway in which circulating Bnp10 binds to endothelial cell Alk1 to induce phosphorylation of Smad1/5/9, which promotes a program of gene expression that limits endothelial cell number within nascent arteries in response to blood flow. Abrogation of this flow response results in enlarged arteries and ultimately AVMs.

**MATERIALS AND METHODS**

**Zebrafish lines and maintenance**

Adult zebrafish (*Danio rerio*) were maintained according to standard protocols (Westerfield, 1995). When appropriate, embryo medium was supplemented with 0.003% phenylthioioura (PTU) (Sigma, St Louis, MO, USA) at 24 hours post-fertilization (hpf) to prevent melanin synthesis. Mutant line alk1<sup>pt5</sup> and the alk1<sup>pt5</sup> genotyping assay have been described previously (Roman et al., 2002). Transgenic lines Tg(fli1a:egfp)<sup>pt5</sup>, Tg(kdrl:egfp)<sup>pt16</sup>, Tg(gata1a:DsRed)<sup>pt5</sup> and Tg(fli1a.ep:mrfp-CAAX)<sup>pt5</sup> have been described previously (Roman et al., 2002; Traver et al., 2003; Choi et al., 2007; Corti et al., 2011). To drive wild-type alk1 in endothelial cells, we generated ptol-fli1a.ep:alk1-myc by Gateway cloning (Invitrogen, Carlsbad, CA, USA), recombining pDESTtol2pA2 (Kwan et al., 2007) with pSE fli1a.ep (Villefranc et al., 2007), pME-alk1 and pSE-MTPa (Kwan et al., 2007). To drive ligand- and type II receptor-independent, constitutively active alk1 (Roman et al., 2002) in endothelial cells, we generated ptol-fli1a.ep:alk1<sup>pt5</sup>-mCherry, hereafter referred to as Tg(fli1a:alk1-myc), and a series of mosaic founders for Tg(fli1a.ep:alk1<sup>pt5</sup>-mCherry), hereafter referred to as Tg(fli1a:alk1<sup>pt5</sup>-mCh). The constitutively active alk1 transgene causes severe vascular defects and is embryonic lethal in F1 embryos.

**Morphonols**

Translation blocking (TB) and splice blocking (SB) morpholino-modified antisense oligonucleotides (GeneTools, Philomath, OR, USA) were as follows: bmp9<sup>pt5</sup>, 5'-GGAGCAAAATGCTCAGGCCACAT-3'; bmp9<sup>pt5</sup>, 5'-CTCTTTATGTGACTCCCTCGAG-3'; bmp10<sup>pt5</sup>, 5'-AAAAATGGTTACGTTGACCCAGGAC-3'; bmp10<sup>pt5</sup>, 5'-AGGGAAATATCCGATTTACCTTCAT-3'; and bmp10-like<sup>pt5</sup>, 5'-CGACGAGAGAAATCAGCATGACTGC-3'. For TB morpholinos, efficacy and specificity were evaluated by injecting into one-cell embryos CMV-driven EGFP DNA containing morphonolo-binding sites upstream of the initiator methionine, with or without cognate or non-cognate morpholino, and assessing EGFP expression at ~6 hpf. We could not rescue bmp10 or bmp10-like morphant phenotypes by injecting morpholino-resistant mRNA because embryos ventralized (data not shown). The alk1, tnn2a and control morpholinos have been described previously (Schnett et al., 2002; Corti et al., 2011).

**In situ hybridization and immunostaining**

Digoxigenin-labeled riboprobe (Roche, Indianapolis, IN, USA) for cdh5, cxxcr4a, edn1 and myl7 (myosin light chain 7, previously known as cardiac myosin light chain) have been described previously (Yelon et al., 1999; Corti et al., 2011). Zebrafish bmp10 was amplified from cDNA using primers 5'-ACCACAGCTGAACCTCCGAC-3' and 5'-TCCACATGTGGCCAGC-CTAC-3'; and bmp10-like was amplified using primers 5'-CGGATGAAGACGACGAG-3' and 5'-CCGTACGTCTCTCTCACT-3'. These fragments were cloned into pCRII-TOPO (Invitrogen). Whole-mount in situ hybridization was performed as described previously (Roman et al., 2002). Immunohistochemistry was performed using primary antibodies MF20 (sarcomeric myosin, Developmental Studies Hybridimda Bank, Iowa City, IA, USA) or 9E10 at 1:200 (myc, Covance, Princeton, NJ, USA), biotinylated horse anti-mouse Ig at 1:200, ABC reagent (Vector Laboratories, Burlingame, CA), 3,3'-diaminobenzidine (Sigma, St Louis, MO, USA). Embryos were fixed using an MVX-10 MacroView microscope and DP71 camera (Olympus America, Center Valley, PA, USA). For sections, embryos were embedded in JB4 (Polysciences, Warrington, PA, USA), sectioned at 8 μm with a VT1000S vibratome, stained with anti-smad1/5/9 (also known as anti-phospho-Smad1/5,8, Cell Signaling Technology, Beverly, MA, USA), and sectioned at 8 μm with a VT1000S vibratome, stained with anti-smad1/5/9 (also known as anti-phospho-Smad1/5,8, Cell Signaling Technology, Beverly, MA, USA), and sectioned at 8 μm. Whole-mount in situ hybridization was performed as described previously (Roman et al., 2002). Immunofluorescence was performed in 4% paraformaldehyde in PBS overnight at 4°C, embedded in 4% NuSieve GTG agarose (Lonza, Rockland, ME, USA), and sectioned at 50 μm with a VT1000S vibratome (Leica Microsystems, Buffalo Grove, IL, USA). Rabbit anti-phospho-Smad1/5/9 (also known as anti-phospho-Smad1/5/8, 9511, Cell Signaling Technology, Beverly, MA, USA) was used at 1:100 and goat-anti-rabbit Alexa Fluor 647 at 1:200. Sections were mounted with Vectashield HardSet Mounting Medium (Vector) and imaged with an Olympus Fluoview 1000 confocal microscope outfitted with a UPFLN 40× oil immersion objective, with scan speed 244 Hz. Two-dimensional projections were generated from Adobe Photoshop CS2 version 9.0.2 (Adobe Systems, San Jose, CA, USA).
presentation, images were pseudocolored and colocalization highlighted using the ‘boost colocalization’ function. For quantitation of nuclear phospho-Smad1/5/9, threshold images were created for summed projections of nuclear EGFP panels, alk1-positive arteries were traced with the ‘trace region’ tool, and all nuclei were selected within the traced region using the ‘create regions around objects’ tool. Traced nuclei were transferred to corresponding pSmad1/5/9 summed projections, and average nuclear pixel intensity was measured and normalized to the average pixel intensity for an adjacent non-vascular pSmad1/5/9-positive domain. Results are expressed as percentage of corresponding controls.

Microinjection of rhBMP10 and rhBMP9
Embryos were anesthetized in 160 μg/ml tricaine and inserted into 500 μm troughs in 2% SeaKem LE agarose (Lonza)/30% Danieau. Z-series (0.5-2 μm steps) were collected using a TCS SP5 multiphoton/confocal microscope (Leica Microsystems, Wetzlar, Germany) outfitted with an APO L 20×/1.00 water immersion objective, non-descanned detectors and spectral detectors. EGFP was excited with a Mai Tai DeepSee Ti:Sapphire laser (Newport/Spectra Physics, Santa Clara, CA, USA) at 900 nm, whereas dsRed and mCherry were excited with a 561 nm multiphoton/confocal microscope (Leica Microsystems, Wetzlar, Germany) at 900 nm, whereas dsRed and mCherry were excited with a 561 nm multiphoton/confocal microscope (Leica Microsystems, Wetzlar, Germany). To determine more directly whether restoration of endothelial alk1 expression is sufficient to restore Alk1 signaling in the absence of flow, we assessed a proximal read-out of Alk1 activity: nuclear pSmad1/5/9. pSmad1/5/9 is present in endothelial cell nuclei within the alk1-positive CdIs and BCA in 36 hpf wild-type embryos, is decreased in these arteries in alk1−/− and is restored in alk1+/−, Tg(fli1a:alk1-myc) transgenics. This transgene restores normal expression of cxcr4a and edn1 in alk1 mutants (Fig. 1B), rescues alk1 mutants to adulthood [n=23 alk1−/− of 130 adults from alk1+/−, Tg(fli1a:alk1-myc) crosses; 71% rate of rescue], and has no untoward effects on growth and development. However, flow-responsive expression of endothelial cell alk1 fails to normalize expression of cxcr4a or edn1 in 36 hpf tntt2a morphants (Fig. 1B). There are two plausible explanations for this observation: Alk1 signaling may not be sufficient downstream of blood flow to control expression of these mechanoresponsive genes, or flow may be required for some aspect of Alk1 signaling in addition to alk1 expression.

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**RESULTS**

**Blood flow is required not only for alk1 expression but also for Alk1 activity**

In 36 hpf zebrafish embryos, alk1 is expressed predominantly in endothelial cells within arteries proximal to the heart: the first aortic arch; the cranialward internal carotid artery, caudal division of the internal carotid artery (CaDI) and basal communicating artery (BCA); and the caudalward lateral dorsal aorta (Fig. 1A). We have previously demonstrated that alk1 expression requires blood flow, and that loss of alk1 mimics loss of blood flow in terms of changes in expression of cxcr4a and edn1: both alk1 mutants, which have high flow through assayed vessels, and cardiac troponin-Ia (tntt2a) morphants, which lack heartbeat and blood flow, exhibit increased cxcr4a expression and decreased edn1 expression in alk1-expressing cranial arteries at 36 hpf compared with corresponding controls (Fig. 1B) (Corti et al., 2011). Given that mammalian orthologs CXCR4 and EDN1 are flow responsive in cultured human endothelial cells (Wang et al., 1993; Melchionna et al., 2005), these data suggest that Alk1 might act downstream of blood flow to control expression of these mechanoresponsive genes. Accordingly, if Alk1 signaling is sufficient downstream of blood flow, then restoration of alk1 in the absence of flow might be expected to rescue expression of cxcr4a and edn1. To test this hypothesis, we generated a stable transgenic line, Tg(fli1a:alk1-myc), that expresses Alk1-myc in all endothelial cells regardless of the presence of blood flow (supplementary material Fig. S1). This transgene restores normal expression of cxcr4a and edn1 in alk1 mutants (Fig. 1B), rescues alk1 mutants to adulthood [n=23 alk1−/− of 130 adults from alk1+/−, Tg(fli1a:alk1-myc) crosses; 71% rate of rescue], and has no untoward effects on growth and development. However, flow-independent expression of endothelial cell alk1 fails to normalize expression of cxcr4a or edn1 in 36 hpf tntt2a morphants (Fig. 1B). There are two plausible explanations for this observation: Alk1 signaling may not be sufficient downstream of blood flow to control expression of these genes, or flow may be required for some aspect of Alk1 signaling in addition to alk1 expression.

Endothelial cell numbers and pSmad1/5/9 intensities were compared by two-tailed, unpaired Student’s t-test with significance set at P<0.05.

**Statistics**

Endothelial cell numbers and pSmad1/5/9 intensities were compared by two-tailed, unpaired Student’s t-test with significance set at P<0.05.
To investigate the effect of blood flow on Alk1 ligand availability, we first needed to determine the physiologically relevant ligand during zebrafish embryonic vascular development. Therefore, we assessed vascular gene expression and architecture between 36 and 48 hpf in \textit{bmp9} (ENSDARG00000059173) and \textit{bmp10} (ENSDARG00000061769) morphants, and compared results with \textit{alk1} mutants/morphants. Translation blocking (TB) morpholinos were validated \textit{in vivo} as described in the Materials and methods (supplementary material Fig. S3A). Injection of \textit{bmp9}\textsuperscript{TB} morpholino had no effect on nuclear pSmad1/5/9 in the 36 hpf CaDI/BCA (supplementary material Fig. S3B) nor any effect on cranial vascular architecture at 48 hpf (supplementary material Fig. S3C), but reproducibly generated a venous remodeling defect in the tail that was phenocopied by injection of \textit{bmp9} splice blocking (SB) morpholino (data to be presented in a separate manuscript). By contrast, at 36 hpf, \textit{bmp10}\textsuperscript{TB} morphant CaDI/BCAs were enlarged and contained supernumerary endothelial cells, a phenotype indistinguishable from \textit{alk1} morphants (Fig. 3A,B). Furthermore, in the 36 hpf CaDI/BCA, \textit{bmp10}\textsuperscript{TB} morphants exhibited decreased nuclear pSmad1/5/9 (Fig. 3C; supplementary material Table S1), increased expression of \textit{cxcr4a} (50/53, 94% Fig. 3D), and decreased expression of \textit{edn1} (15/15, 100%; Fig. 3D). These effects were similar to effects observed in \textit{alk1} mutants (supplementary material Fig. S2; Fig. 3D). In addition, by 48 hpf, \textit{bmp10}\textsuperscript{TB} morphants developed A VMs connecting the arterial system underlying the midbrain and hindbrain to adjacent veins, strongly resembling \textit{alk1} mutants/morphants (Fig. 3E). However, the \textit{bmp10} morpholino-induced AVM phenotype was observed with relatively weak penetrance (21/43, 49%; Fig. 3F) and low expressivity at a maximum tolerated dose (20 ng), suggesting that an additional ligand might be compensating for \textit{bmp10} at later time points.

\textbf{bmp10 knockdown phenocopies \textit{alk1} mutants}

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Examination of the zebrafish genome uncovered \textit{bmp10-like} (ENSDARG00000045632), which is most closely related to \textit{bmp10} (64% identity in the active peptide) and likely represents a \textit{bmp10} paralog that arose during a teleost whole-genome duplication (Woods et al., 2005). Therefore, we knocked down both \textit{bmp10-like} and \textit{bmp10}, and assessed the zebrafish vasculature at 48 hpf. Injection of \textit{bmp10-like} morpholino (3 ng) or \textit{bmp10}\textsuperscript{TB} morpholino (10-15 ng) alone had almost no effect on cranial vascular
Fig. 2. Alk1 activity is dependent on blood flow. (A-E) pSmad1/5/9 (middle column) in endothelial cell nuclei (marked by fli1a:.negfp transgene, left column); in merge (right column), EGFP-expressing endothelial cell nuclei are green and pSmad1/5/9 immunofluorescence is magenta. (A) 24 hpf wild type, prior to blood flow; (B) 36 hpf tntnt2a morphant (no flow); (C) 36 hpf wild type; (D) 36 hpf tntnt2a morphant harboring a fli1a:alk1-myc transgene; (E) 36 hpf tntnt2a morphant harboring a fli1a:alk1-CA-mCh transgene. Tg(fli1a:alk1-CA-mCh) embryos do not have lumenized vessels. In merge (right column), EGFP-expressing endothelial cell nuclei are green, pSmad1/5/9 immunofluorescence is magenta. Yellow and blue arrows indicate endothelial cells in the caudal division of the internal carotid artery (CiDi) and basal communicating artery (BCA), respectively. 2D confocal projections of 50 μm frontal sections, dorsal upwards. Scale bar: 50 μm. See supplementary material Table S1 for fluorescence quantitation.

architecture (n=61 and 47, respectively), whereas co-injection of these two morpholinos at these same doses robustly phenocopied alk1 morphants in terms of AVM development (122/146, 84%; Fig. 3E,F), suggesting a strong genetic interaction. Similar results were observed with a bmp10TB morpholino, with no AVMs at 15 ng (n=48) but AVMs upon co-injection with 3 ng bmp10-like morpholino (46/57, 81%). By contrast, co-injection of bmp9TB morpholino (7 ng) with bmp10TB morpholino (15 ng) failed to elicit AVMs (0/29), and co-injection of bmp9TB morpholino (7 ng), bmp10TB morpholino (15 ng) and bmp10-like morpholino (3 ng) did not increase the percentage phenotype (20/31, 65%) beyond combined injection of bmp10TB morpholino and bmp10-like morpholino. These results demonstrate that bmp10-like acts redundantly with bmp10 and that Bmp10 but not Bmp9 is the crucial in vivo Alk1 ligand required for arterial quiescence and AVM prevention during zebrafish embryonic vascular development.

bmp10 paralogs are expressed exclusively in the heart

We have demonstrated that concomitant knockdown of bmp10 and bmp10-like phenocopies alk1 mutants. bmp10 is undetectable at 22 hpf but expressed in the developing heart as early as 24 hpf, with predominant expression in the ventricle by 36 hpf (Fig. 4A; supplementary material Fig. S4A). By 48 hpf, bmp10 is expressed in both heart chambers but appears to be absent from the atrioventricular canal (Fig. 4A). Double staining for bmp10 mRNA and sarcomeric myosin protein revealed that bmp10 is expressed strongly in endocardium but is largely absent from myocardium at 36 hpf (Fig. 4B). bmp10-like is undetectable in the heart at 28 hpf but is expressed at 36 and 48 hpf in distal ventricular myocardium (Fig. 4A,B). Both bmp10 and bmp10-like are expressed independently of heartbeat (supplementary material Fig. S4B), and no discrete extracardiac expression domains of either bmp10 or bmp10-like are observed (data not shown). The temporal difference in ligand expression suggests that Bmp10-like might compensate for Bmp10 after 36 hpf, providing a plausible explanation for the observation that knockdown of bmp10 alone robustly phenocopies alk1 mutants at 36 hpf, but double knockdown of bmp10 and bmp10-like is required for robust phenocopy at 48 hpf (Fig. 3). Furthermore, these data demonstrate that the heart is the most likely source of both Bmp10 and Bmp10-like, and suggest that cardiac-derived Bmp10 ligands might be carried by blood flow to arterial endothelial Alk1.

Circulating Bmp10 acts via Alk1 to limit endothelial cell number in nascent arteries

Given that bmp10 paralogs are detectable only in the heart in zebrafish (Fig. 4) and mouse (Neuhaus et al., 1999; Chen et al., 2004), and that Bmp10 circulates in embryonic mouse plasma (Ricard et al., 2012), it stands to reason that blood flow may be required to transport Bmp10 to arterial endothelial cell Alk1, explaining why restoration of alk1 gene expression alone is insufficient to rescue defects in arterial endothelial cell cxcr4a, edn1 and pSmad1/5/9 in the absence of flow (Figs 1, 2). To test this hypothesis, we injected nntnt2a morpholino into Tg(fli1a:alk1-myc) embryos, once again generating embryos that lack heartbeat but nonetheless express endothelial alk1. Non-transgenic siblings injected with nntnt2a morpholino served as blood flow deficient and therefore alk1-deficient controls. At 28 hpf, we injected tracer (phosph red or quantum dots) alone or together with recombinant human (rh) BMP10 directly into the base of one CaDi. rhBMP10 can act through zebrafish Alk1 to activate BMP responsive element (BRE)-driven luciferase activity in transected C2C12 cells with an EC50 similar to that required for activation of human ALK1 (Fig. 5A). Restoration of either alk1 alone [nntnt2a morphant, Tg(fli1a:alk1-myc)] or rhBMP10 alone (nntnt2a morphant + rhBMP10) failed to rescue nuclear pSmad1/5/9 in the CaDi, whereas restoration of both alk1 and rhBMP10 increased nuclear pSmad1/5/9 ~3-fold near the site of rhBMP10 injection (Fig. 5B; supplementary material Table S1). Injection of rhBMP10 into alk1 mutants failed to rescue pSmad1/5/9, supporting the idea that rhBMP10 is not acting through other endothelial receptors besides
Alk1 (supplementary material Fig. S5; Table S1). Furthermore, injection of rhBMP10 into *tnnt2a* morphant;*Tg(fli1a:alk1-myc)* embryos decreased expression of *cxcr4a* (13/23, 57%) and increased expression of *edn1* (14/22, 64%) on the injected side, driving expression of these genes toward levels observed in the presence of normal blood flow (Fig. 5C). In addition, similarly restoring rhBMP10/Alk1 signaling to one CaDI in flow-deprived *tnnt2a* morphant embryos significantly decreased endothelial cell number within the injected CaDI and BCA but not the uninjected CaDI, normalizing endothelial cell number in the injected...
Early embryonic blood vessel development is controlled largely by paracrine signaling interactions that orchestrate endothelial cell migration and proliferation, and coalescence into vascular cords, as well as subsequent angiogenic sprouting that serves to elaborate the primitive vascular network. However, once blood vessels form a lumen, biomechanical forces and endocrine factors also come into play. In this work, we present evidence that blood flow is crucial for limiting endothelial cell number within nascent arteries, and that Alk1 acts downstream of blood flow in mediating this effect. Furthermore, we demonstrate that blood flow is required not only for alk1 expression but also for Alk1 activity, and that the latter requirement is met by provision of circulating ligand, Bmp10. Thus, flow-dependent induction of alk1 and distribution of ligand synergize to enhance Alk1 activity.

It is well established that both BMP9 and BMP10 bind ALK1 with high affinity and can induce Smad1/5/9 phosphorylation and pSmad1/5/9-dependent transcriptional responses in cultured endothelial cells (David et al., 2007; David et al., 2008; Mitchell et al., 2010). However, our work is the first to define Alk1 ligands unequivocally in vivo. We have demonstrated in zebrafish that bmp10 paralogs are required for Alk1 function during embryonic development, whereas bmp9 is dispensable. By contrast, neither Bmp9 nor Bmp10 mouse nulls have been reported to exhibit AVMs. Bmp9-null mice live to adulthood without apparent vascular abnormalities (Ricard et al., 2012), whereas ventricular hypoplasia and impaired trabeculation but not AVMs have been reported in Bmp10 nulls (Chen et al., 2004). There are several possible explanations for this discrepancy between zebrafish and mice. First, Bmp9 and Bmp10 may act redundantly in mouse embryonic vascular development, reflecting a difference in the spatial and/or temporal ligand requirement in mouse versus zebrafish vasculature. Indeed, treatment of Bmp9-null mice with Bmp10 blocking antibodies results in early postnatal retinal vascular defects, hinting at functional redundancy, although AVMs were not noted in these vessels (Ricard et al., 2012). Furthermore, like rhBMP10, rhBMP9 was effective in restoring nuclear pSmad1/5/9 in the CaDI/BCA in vivo if available to Alk1 contemporaneously. In zebrafish, bmp9 is first detected in liver around 48 hpf (C.J.M. and B. Shravage, unpublished) and the liver is first vascularized around 55 hpf (Korzh et al., 2008), supporting the idea that Bmp9 is not redundant with Bmp10 between 24 and 48 hpf, the time period during which AVMs develop in alk1 mutants (Roman et al., 2002; Corti et al., 2011). A second possible reason for the discordance between mice and zebrafish in terms of definition of Alk1 ligand stems from the fact that slowed heartbeat and impaired circulation preceede death of Bmp10 mouse nulls at embryonic day (E) 9.5-10.5 (Chen et al., 2004). Thus, it is reasonable to hypothesize that the absence of AVMs in Bmp10 nulls is due to the earlier death of Bmp10 nulls versus Alk1 nulls (E10.5-11.5) and/or insufficient circulation to precipitate AVM formation, which we have demonstrated to be a flow-dependent process (Corti et al., 2011). Because trabeculation in zebrafish does not occur until 60-72 hpf in zebrafish (Liu et al., 2010; Peshkovsky et al., 2011), it is not surprising that we noted no defects in heart development or function at these ages.

In cranial arterial endothelial cells, both blood flow and Bmp10/Alk1 function are required for phosphorylation of Smad1/5/9, repression of ccr4a and induction of edn1. Concomitantly restoration of endothelial alk1 expression and injection...
rhBMP10 normalizes these molecular endpoints and restores arterial endothelial cell number in the absence of flow. These data strongly suggest that Alk1 acts downstream of blood flow to limit endothelial cell migration, proliferation and/or vascular tone, and thus limit arterial endothelial cell number and vessel caliber. However, changes in expression of cxcr4a and edn1 cannot fully

**Fig. 5.** Bmp10/Alk1 lies downstream of blood flow in regulation of pSmad1/5/9, cxcr4a and edn1. (A) BREluciferase activity in C2C12 cells transfected with human (h) ALK1, zebrafish (z) alk1, hALK7 kinase dead mutant (R411Q), hALK3 (which does not bind BMP10) or lipofectamine (lipo), and treated with rhBMP10. Data are normalized to pTK-Renilla luciferase activity. Values are mean±s.e.m., n=3 biological replicates, each representing 3 technical replicates. (B) pSmad1/5/9 (middle column) in endothelial cells (nuclei marked by fli1a:negfp transgene, left column) of the CaDI (caudal division of the internal carotid artery) of 36 hpf tnt2a morphants in which endothelial cell alk1 expression [Tg(fli1a:alk1-myc)], BMP10 availability (rhBMP10; 2 nl of 10 μM rhBMP10 injected into base of CaDI at 28 hpf), or both, are restored. In merge (right column), EGFP-expressing endothelial cell nuclei are green, pSmad1/5/9 immunofluorescence is magenta. Yellow arrows indicate endothelial cells in the injected CaDI. 2D confocal projections of 50 μm frontal sections, dorsal upwards. Scale bar: 10 μm. See supplementary material Table S1 for fluorescence quantitation. (C) Whole-mount in situ hybridization for cxcr4a, edn1 and cdh5 (pan-endothelial control) in 36 hpf tnt2a morphant; Tg(fli1a:alk1-myc) embryos injected at 28 hpf into the base of the left CaDI with 2 nl tracer with or without 10 μM rhBMP10. Yellow arrow indicates injected CaDI, red arrow indicates uninjected CaDI. Asterisk indicates injection site. Frontal views, dorsal upwards; lateral views, right (uninjected) side or left (injected) side. Scale bar: 50 μm. (D) Quantitation of CaDI endothelial cell number in 36 hpf Tg(fli1a:alk1-myc) embryos left uninjected (white bars) or injected at the one-cell stage with tnt2a morpholino (gray, black bars). At 28 hpf, tnt2a morphants were injected in the right CaDI with 2 nl tracer (gray bars) or 2 nl 10 μM rhBMP10 (black bars). tnt2a morphants injected intravascularly with tracer exhibited increased endothelial cell number throughout the CaDI/BCA, whereas intravascular injection of rhBMP10 normalized endothelial cell number in the injected CaDI and BCA (basal communicating artery). n=10–12 per group in two independent experiments. Values are mean±s.e.m. Student’s t-test: *P<0.05; **P<0.001; ns, not significant.
explain defects in vessel architecture resulting from loss of \textit{alk1}. Although ALK1 signaling can repress \textit{CXCR4} or induce \textit{EEN1} in cultured endothelial cells (Star et al., 2010; Park et al., 2012; Young et al., 2012), supporting our in \textit{vivo} data, previous work has demonstrated that increased \textit{cxc4a} is not necessary nor is loss of \textit{edn1} sufficient for AVM development (Corti et al., 2011), and additional work has demonstrated that concomitant increase in \textit{cxc4a} and loss of \textit{edn1} is insufficient to generate AVMs (E. Rochon and B.L.R., unpublished). Thus, further work is required to define the molecular mechanisms and cellular behaviors that lead to arterial enlargement in the absence of \textit{alk1}.

The multifaceted regulation of Alk1 signaling by blood flow is remarkable, with flow required for both \textit{alk1} expression and \textit{alk} activity. Given that mammalian \textit{CXCR4} and \textit{EDN1} respond to shear stress and/or cyclic stress in cultured endothelial cells (Wang et al., 1993; Melchionna et al., 2005), it seems likely that Alk1 is important in transducing mechanical force into a biochemical signal in \textit{vivo}. However, the mechanism by which blood flow upregulates \textit{alk1} expression is currently unknown, and it remains formally possible that the flow dependence of \textit{alk1} expression stems at least in part from a circulating factor. Circulation of ligand clearly contributes to the dependence of Alk1 activation on blood flow: blood flow distributes cardiac-derived circulating Bmp10 to arterial endothelial cell Alk1, thereby explaining the blood flow dependence of Smad1/5/9 phosphorylation in these cells. However, a recent study reported that mechanical force induces Smad1/5/9 phosphorylation in intact mouse endothelium and cultured endothelial cells in a ligand-independent manner (Zhou et al., 2012), contradicting our conclusions. This discrepancy could possibly be explained by the fact that ligand independence in that study was examined via treatment with Noggin, which sequesters most BMP ligands, but not BMP9 or BMP10 (Seemann et al., 2009). Alternatively, oscillatory shear stress applied in that study may have different effects on BMP signaling than pulsatile laminar shear stress, which acts within zebrafish cranial arteries (Chen et al., 2011), or the type I receptor responsible for oscillatory shear-induced \textit{p5E}\textsubscript{Smad1/5/9} may not be Alk1. Further work is required to better define the roles of and probe interactions between endocrine factors and mechanical force in the regulation of Alk1 signaling.

In summary, our data demonstrate that blood flow induces \textit{alk1} expression and provides Bmp10 to arterial endothelial cell Alk1, thereby activating Smad1/5/9 phosphorylation, decreasing \textit{cxc4a} expression and inducing \textit{edn1} expression. These changes in gene expression, along with changes in expression of yet to be identified genes, serve to dampen angiogenic behavior and to stabilize arterial endothelial cell number and caliber at the onset of blood flow. Taken together with our previous work (Corti et al., 2011), our data suggest that loss of Alk1 function abrogates this important flow response and results in increased nascent arterial caliber, which in turn leads to increased hemodynamic forces within downstream arteries. In an attempt to normalize these hemodynamic forces, downstream vessels mount an Alk1-independent flow response that causes normally transient conduits between this overloaded arterial system and neighboring veins to be retained and enlarged, thereby forming high flow AVMs. Thus, our model suggests that in individuals with HHT, abrogation of one flow response – due to impaired Alk1 signaling – leads to activation of an independent flow response that acts to normalize hemodynamic forces, ultimately leading to AVMs.

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Competing interests statement
The authors declare no competing financial interests.

Author contributions
D.W.L. performed the majority of the zebrafish experiments, generated all zebrafish data figures and wrote the paper. S.Y. performed bmp9 morpholino studies, generated the Tg{\textit{fltl1a.eds};\textit{alk1}}\textsuperscript{myc\textsubscript{ires}} line and assisted in most zebrafish experiments; P.D. analyzed \textit{bmp10} and \textit{bmp10}\textsuperscript{like} morphant shunting phenotype; C.J.M. performed preliminary characterization of \textit{bmp9} and \textit{bmp10} expression patterns and morphant phenotypes; P.D.U. performed luciferase assays and generated the accompanying figure; B.L.R. conceived and directed the study, generated \textit{ptel-fltl1a.eds-alk1}{\textsubscript{CF}}\textsuperscript{icer}\textsuperscript{mCherry} and wrote the paper.

Supplementary material
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