Notch regulates BMP responsiveness and lateral branching in vessel networks via SMAD6

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Functional blood vessel growth depends on generation of distinct but coordinated responses from endothelial cells. Bone morphogenetic proteins (BMP), part of the TGFβ superfamily, bind receptors to induce phosphorylation and nuclear translocation of SMAD transcription factors (R-SMAD1/5/8) and regulate vessel growth. However, SMAD1/5/8 signalling results in both pro- and anti-angiogenic outputs, highlighting a poor understanding of the complexities of BMP signalling in the vasculature. Here we show that BMP6 and BMP2 ligands are pro-angiogenic in vitro and in vivo, and that lateral vessel branching requires threshold levels of R-SMAD phosphorylation. Endothelial cell responsiveness to these pro-angiogenic BMP ligands is regulated by Notch status and Notch sets responsiveness by regulating a cell-intrinsic BMP inhibitor, SMAD6, which affects BMP responses upstream of target gene expression. Thus, we reveal a paradigm for Notch-dependent regulation of angiogenesis: Notch regulates SMAD6 expression to affect BMP responsiveness of endothelial cells and new vessel branch formation.

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Blood vessel networks expand via endothelial cell (EC) sprouting, migration, anastomosis and lumination to form new conduits, a process called sprouting angiogenesis\(^1\). Heterogeneous yet coordinated responses to pro-angiogenic signals are essential for proper angiogenesis and for subsequent maintenance of a functional vasculature\(^2\). Hence, numerous signalling pathways integrate to provide this heterogeneity in ways that are not completely understood\(^3\).

Bone morphogenetic protein (BMP) signalling is essential to the proper form and function of blood vessels\(^8\)-\(^14\). BMPs belong to the transforming growth factor-β superfamily of secreted growth factors and they signal through cell-surface heterotetramers of type II and type I serine/threonine kinase receptors\(^15,16\). Phosphorylation of type I receptors by type II receptors induces phosphorylation of the receptor-associated SMADs 1, 5 and 8 (R-SMADs 1/5/8). R-SMAD phosphorylation associates with SMAD4 and this complex translocates to the nucleus, to regulate expression of target genes. SMAD6 is an inhibitory SMAD that competitively binds type I receptors or SMAD4 to inhibit SMAD1/5/8 phosphorylation or nuclear translocation, respectively, and downregulate BMP signalling\(^17,18\).

Recent studies highlight novel roles for BMPs during angiogenesis\(^8\)-\(^14\). Global genetic deletion of SMAD5 in mice led to multiple angiogenesis defects during embryogenesis\(^19\), EC-selective deletion of SMAD1 and SMAD5 severely impaired angiogenesis, resulting in defective yolk sac and cranial vasculature, and SMAD1/5 double knockout EC displayed reduced proliferation and migration, implicating BMP signalling in cellular processes essential to angiogenesis\(^8\). In zebrafish, Bmp2b is a venous-specific pro-angiogenic signal that promotes Vegfa-independent sprouting from the posterior cardinal vein\(^9\). The type I BMP receptor ALK1 is either anti-angiogenic or pro-angiogenic when inhibited by Fc-conjugation or a highly specific blocking antibody, respectively\(^10,13\). These data suggest that the angiogenic activity of BMP ligands is context dependent.

EC differentially respond to BMP signalling\(^9\), but how BMP responsiveness is set remains largely unknown. Therefore, we sought to identify a mechanism by which EC intrinsically regulate the magnitude of their responses to BMP ligands. Previous studies indicated that BMP6 and BMP2 can be pro-angiogenic\(^8,9,20\). Here we show that these ligands are pro-angiogenic, and that lateral vessel branching requires threshold levels of R-SMAD phosphorylation. EC responsiveness to pro-angiogenic BMP ligands is regulated by Notch status. Notch sets responsiveness by regulating the cell-intrinsic BMP inhibitor, SMAD6 and SMAD6 affects BMP responses upstream of target gene expression, revealing a new paradigm for Notch-dependent regulation of angiogenesis.

Results

**BMP2 and BMP6 promote lateral vessel branching.** We first determined the effects of BMP6 and BMP2 on vessel branching in a three-dimensional (3D) sprouting angiogenesis assay\(^21\) using human umbilical vein endothelial cells (HUVEC). Addition of exogenous BMP6 or BMP2 significantly increased both branching frequency and branch angle compared with controls (Fig. 1a–d and Supplementary Fig. 1a–d). Exogenous BMP2 had a similar effect on branching frequency in mouse embryonic stem (ES) cell-derived vessels (Supplementary Fig. 1e–g). Consistent with a more highly branched phenotype, both ligands increased the percentage of nuclei in the tip position of growing HUVEC sprouts (Fig. 1e–g and Supplementary Fig. 1h–j). Moreover, the BMP-induced branching phenotype was remarkably well-organized, in contrast to the effects of elevated vascular endothelial growth factor (VEGF)-A signalling, which often lead to gross dysmorphogenesis\(^22,23\). Thus, BMP6 and BMP2 are pro-angiogenic and increase vascular density by inducing new branch formation. Furthermore, the increased branch angles lead to lateralization of the branching pattern and suggest that pro-angiogenic BMP signalling tunes vessel branching between a ‘bush-like’ and a ‘bamboo-like’ vessel pattern, in a ligand-dependent manner.

We hypothesized that BMP-induced branching depended on EC sprouting in response to activation and nuclear localization of phosphorylated SMADs 1 and 5 (pSMAD1/5). We examined levels of nuclear pSMAD1/5 in EC during sprouting angiogenesis and found a striking inverse correlation between the distance of nuclei from the sprout tip and intensity of nuclear pSMAD1/5 staining (Fig. 1h,j,m). Thus, EC with the highest levels of BMP signalling were more likely to be in the tip cell position. As expected, short-term BMP6 treatment significantly increased overall nuclear pSMAD1/5 levels compared with controls (Fig. 1i–k and Supplementary Fig. 1k). However, although acute BMP6 exposure significantly increased the average nuclear pSMAD1/5 levels in stalk cell, it had only a modest and nonsignificant effect in tip cells (Fig. 1l), suggesting that EC at sprout tips have maximal BMP pathway activation. In fact, stalk EC in BMP6-treated sprouts had average nuclear pSMAD1/5 levels comparable to levels in tip EC of controls (Fig. 1i), suggesting that BMP signalling increases vessel branching by inducing additional tip cells from the pool of stalk cells. This idea is also consistent with the finding that stalk cells, even after BMP stimulation, retain extensive heterogeneity in nuclear pSMAD1/5 levels and their pSMAD1/5 levels do not as strongly correlate with position in the sprout as controls (Fig. 1l,m).

To verify the necessity of SMAD1/5 for lateral branch formation, we reduced the levels of each protein via small interference RNAs (siRNAs) and analysed effects on sprouting. Reduction of either SMAD1 or SMAD5 (Supplementary Figs 1l,m and 5b) reduced BMP6-induced lateral branching. Although SMAD1/5 signalling has also been implicated in stalk cell maintenance\(^9\), this effect is thought to be downstream of BMP9 engagement with complexes that include the type I receptor ALK1 and induce vascular quiescence\(^13,24,25\). To determine whether ALK1 signalling contributes to the effects we observed, we reduced ALK1 levels via siRNA and found no effect on BMP6-dependent increases in lateral branching (Supplementary Figs 1n and 5b), suggesting that BMP6-induced branching occurs independently of BMP9/ALK1 signalling. Thus, pSMAD1/5 is required to mediate the pro-angiogenic effects of BMP6 in EC independent of effects from ALK1 signalling. Taken together, these data suggest that BMP-dependent lateral vessel branching depends on EC that are ‘tunable’ and have an innate set point of BMP responsiveness. For each EC, this innate responsiveness sets a ligand threshold required to produce sufficient pro-angiogenic pSMAD1/5 signalling and provoke an EC response. In this scenario, short-term BMP6 treatment increases the number of EC that achieve the threshold and are capable of responding, leading to additional lateral branching.

**Notch sets EC BMP responsiveness.** If BMP responsiveness is innate to EC, then factors that set this responsiveness and the extent of R-SMAD phosphorylation are predicted to profoundly affect BMP-dependent vascular architecture. Notch signals via its intracellular domain (NICD) and this signalling represses the tip cell phenotype and supports a non-branched stalk cell phenotype\(^26–29\). Although recent work describes Notch-BMP pathway crosstalk at the level of target gene expression\(^8,13,30,31\), it is not clear whether Notch signalling alters the responsiveness of EC to BMP via effects on upstream BMP pathway components.
In the developing zebrafish, venous EC are BMP sensitive and form ectopic sprouts after heat-shock induction of Bmp2b, whereas intersegmental vessels (ISVs) are relatively unresponsive at 24–48 hpf (ref. 9). To investigate regulators of BMP responsiveness in this model, we used Tg(Tp1bglob:eGFP) (Notch reporter) zebrafish embryos and asked how Notch activity and BMP sensitivity align in the vasculature. Notch reporter activity was strong in the dorsal aorta (DA) and ISVs, consistent with other reports, and these vessels do not respond to ectopic BMP ligand; however, the reporter signal was undetectable in the BMP-responsive caudal vein plexus (CVP) (Fig. 2a,b). To determine whether Notch influences BMP responsiveness, we induced Notch signalling via heat-shock induction of NICD. As we have described, ectopic induction of BMP signalling promotes lateral branching of angiogenic sprouts. (a,b) HUVEC 3D sprouting assay with BMP6, representative of three independent experiments, stained with phalloidin (actin) and depth encoded. (c,d) Quantification of (c) branches per mm (n = 7 control and 10 BMP6 beads) and (d) branch angle (n = 43 control and 133 BMP6 angles). Error bars, mean ± 95% confidence interval (CI). **P < 0.01; ***P < 0.001 by Student’s t-test. (e,f) Sprouting HUVEC visualized with phalloidin (actin) and DRAQ7 (DNA). (g) Tip nuclei/total nuclei per field, representative of three independent experiments (n = 9 control and 7 BMP6 sprouts). Error bars, mean ± 95% CI. **P < 0.01 by Student’s t-test. (h–k) HUVEC 3D sprouting assay 6 h post-BMP6 treatment, visualized for nuclear pSMAD1/5, phalloidin (actin) and DRAQ7 (DNA). Images are three-channel compressed z-stacks (h,i) or single-channel compressed z-stack heat maps of pSMAD1/5 staining intensity (j,k). Scale bar, 50 mm. (l) Quantification of mean pSMAD1/5 fluorescence intensity per nucleus, representative of 2 independent experiments (n = 13 control tip, 50 control stalk, 15 BMP6 tip, and 58 BMP6 stalk EC). Error bars, mean ± 95% CI. NS, not significant; ***P ≤ 0.001 and ****P ≤ 0.0001 by one-way analysis of variance with Tukey’s post-hoc test. (m) Best-fit correlation (solid line) with 95% CI intervals (filled areas) of indicated parameters. *P < 0.05 by linear regression.
Bmp2b led to excessive sprouting from the CVP (Fig. 2c,e). However, concomitant induction of Bmp2b and NICD significantly reduced the frequency of CVP sprouts, suggesting that ectopic Notch signalling dampens the sensitivity of EC to BMPs (Fig. 2d,e). Conversely, to determine whether arterial EC could be sensitized to Bmp2b overexpression, we blocked Notch signalling by treatment with N-[2S-(3,5-difluorophenyl)acetyl]-L-lalanyl-2-phenyl-1,1-dimethylethyl ester-glycine (DAPT), a
γ-secretase inhibitor that prevents cleavage and release of NICD. Bmp2b induction induced a low level of ectopic vessels from arterial EC (Fig. 2f–h,) and DAPT treatment alone induced some ectopic arterial angiogenesis, consistent with previous reports (Fig. 2i,l)⁵. However, Notch inhibition combined with Bmp2b induction resulted in a significantly higher frequency of ectopic arteries compared with either manipulation alone (Fig. 2j–l). These results indicate that Notch is an intrinsic regulator of the magnitude of the BMP response in EC in vivo.

To quantitatively determine the impact of Notch manipulations on BMP pathway activation, we determined nuclear pSMAD1/5 levels on exposure of HUVEC to different amounts of ligand. A twofold serial dose–response curve to BMP6 yielded a prototypical sigmoidal semi-log curve for BMP-mediated EC activation, as measured by nuclear pSMAD1/5 levels (Supplementary Fig. 2a,b). We next tested the effect of Notch activation by plating HUVEC onto Fc-conjugated Dll4 ligand (Dll4-Fc) before short-term treatment with BMP6 and found that the EC$_{50}$ for BMP-mediated EC activation increased significantly compared with controls (Fig. 2m). We confirmed, using inducible NICD expression in HUVEC, that elevated Notch signalling increased the EC$_{50}$ (Supplementary Fig. 2c). This relationship also held at the single-cell level, as EC expressing NICD had reduced levels of pSMAD1/5 (Fig. 2n,o). Conversely, HUVEC treated with siRNA targeting Notch1 (Supplementary Figs 2d and 5c) were more sensitive to lower concentrations of BMP6 relative to controls (Supplementary Fig. 2e) and they exhibited increased branching with equivalent BMP6 stimulation (Supplementary Fig. 2f–j). The results of in vivo and in vitro Notch manipulations support our hypothesis that Notch regulates the innate BMP responsiveness of EC, and that the increased BMP responsiveness of EC with low Notch signalling promotes lateral branching.

**SMAD6 integrates Notch and pro-angiogenic BMP responsiveness.** Notch regulates VEGF signalling by modulating levels of VEGF receptor RNAs⁴,13. Therefore, we reasoned that Notch would regulate BMP responsiveness via expression of BMP receptors. Surprisingly, we detected no significant changes in expression levels of several type I and type II BMP receptors after Notch stimulation of HUVEC via Dll4–Fc plating or Notch blockade via DAPT (Supplementary Fig. 3a,b). BMP signalling is also intrinsically regulated by an intracellular inhibitory protein, SMAD6 (refs 17,18,36,37) and SMAD6 messenger RNA levels increased with Notch stimulation and decreased with Notch blockade in HUVEC (Fig. 3a). This relationship was maintained at the single-cell level, as EC expressing NICD had elevated levels of SMAD6 protein (Fig. 3b,c). In other cell types, SMAD6 inhibits BMP signalling by preventing R-SMAD phosphorylation and nuclear localization¹⁷,¹⁸, but its activity in EC and effects on angiogenesis are unknown. Therefore, we generated HUVEC expressing doxycycline-inducible SMAD6 fused to tdTomato. The tagged SMAD6 protein reacted with a SMAD6 antibody by immunofluorescence (Supplementary Fig. 3c–f) and suppressed nuclear pSMAD1/5 levels in HUVEC in a cell autonomous and dose-dependent manner (Fig. 3d,e). Conversely, reduction of SMAD6 protein levels via siRNA knockdown (Supplementary Figs 3g and 5d) increased BMP6-induced nuclear pSMAD1/5 (Fig. 3f). These findings show that an intrinsic BMP pathway inhibitor, SMAD6, modulates BMP signalling in EC. As elevated pSMAD levels were associated with increased lateral branching and SMAD6 suppressed BMP signalling, we hypothesized that loss of SMAD6 would increase BMP responsiveness of EC and promote branching. Consistent with this hypothesis, reduced SMAD6 levels via siRNA significantly increased branching of sprouting HUVEC with added exogenous BMP6 (Fig. 3g–k). These results show that SMAD6 regulates angiogenesis, probably by intrinsically modulating the magnitude of EC responses to BMP inputs.

To determine the functional relevance of SMAD6 in vivo, we first examined expression by fluorescence-activated cell sorting (FACS) in EC from zebrafish embryos that expressed the Notch signalling reporter (Fig. 2a). Zebrafish have two SMAD6 orthologues, smad6a and smad6b. We assessed expression of smad6b in EC in vivo, because its sequence is more homologous to that of human SMAD6. EC sorted for high levels of Notch reporter signalling had significantly elevated levels of RNAs that are expected to be elevated in arterial Notch-positive EC such as notch1b and ephrinb2. They also had significantly elevated levels of smad6b RNA relative to EC sorted from the same embryos with little or no Notch reporter signalling (Fig. 4a). We next manipulated embryonic SMAD6 expression. As global manipulations of smad6b are predicted to profoundly perturb dorsal–ventral axis formation in zebrafish embryos⁴⁸, we used the Tol2 system to generate F0 mosaic embryos expressing green fluorescent protein (GFP)-tagged smad6b, or GFP alone, under control of the vascular-specific fli1 promoter. We predicted that Smad6b expression would preferentially affect BMP-responsive EC with low Notch levels, such as those in the cardinal vein and the CVP. EC expressing Smad6b-eGFP were significantly reduced in the cardinal vein and CVP, and enriched in the DA and ISVs, compared with EC expressing only GFP (Fig. 4b–d), consistent with our finding that Smad6b expression is elevated in EC of embryos with high Notch signalling reporter expression, presumably including EC from the DA and ISVs. These findings suggest that forced Smad6b expression prevents EC from colonizing the vein and CVP where Notch signalling is low, but is irrelevant in arterial EC where Notch signalling is relatively high and support a role for SMAD6 in angiogenesis in vivo.

We next asked whether reduced levels of smad6b affect blood vessel branching and predicted that if SMAD6 is important for Notch-mediated effects in vivo, its reduction would more profoundly affect Notch-dependent ISV sprouting that is normally refractory to BMP⁴⁹. To overcome the effects of global smad6b manipulations on dorsal–ventral axis formation, we developed a tissue-specific mRNA knockdown system for zebrafish based on a CRISPRi strategy⁴⁹–⁵². However, CRISPRi is poorly efficient in eukaryotic cells⁵³; thus, dcsa9 was fused to the Drosophila engrailed repressor domain⁵⁴ to generate Dcsa9-Engrailed Repressor domain (EnR), a chimeric transcriptional repressor protein that is targeted by short guide (sg) RNAs. We validated the efficacy of dcsa9-EnR in global knockdown using sgRNAs targeting bmp6 or smad6b and observed the expected dorsalization (bmp6 loss-of-function) or ventralization (smad6b loss-of-function) phenotypes (Supplementary Fig. 4a–d). We also validated sgRNA effects at the mRNA level by quantitative reverse transcriptase–PCR (qRT–PCR) for the targeted genes (Supplementary Fig. 4e). Next, transgenic embryos expressing a fli1 promoter-driven dcsa9-EnR were generated using the Tol2 system. F1 embryos expressing vascular restricted Dcsa9-EnR were indistinguishable from wild-type (WT) siblings and dcsa9-EnR mRNA expression was confirmed by RT–PCR (Supplementary Figs 4f–h and 5e). To determine whether Smad6b regulates BMP responsiveness in vivo, we injected sgRNAs targeting smad6b into Tg(fli1:dCas9-EnR);Tg(hsp701: bmp2b);Tg(kdr:GFP) embryos, then heat shocked to induce ectopic Bmp2b expression at 26hpf, well beyond the early developmental window leading to axis defects. WT heat-shocked embryos without sgRNAs had normal ISV formation (Supplementary Fig. 4i,j,m). Likewise, Tg(fli1:dCas9-EnR);Tg(kdr:GFP) embryos injected with scrambled sgRNAs or smad6b sgRNAs and heat-shocked had normal ISV formation (Fig. 4e,f,i). Heat-shock induction of Bmp2b expression did not perturb axis formation,
but induced some ectopic ISV formation in controls and Tg(flic:Cas9-EnR); Tg(hsp701:bmp2b);Tg(kdrl:GFP) embryos injected with scrambled sgRNAs (Fig. 4g,i and Supplementary Fig. 4k,m). However, the frequency of ectopic ISV formation significantly increased in transgenic embryos overexpressing Bmp2b and injected with smad6b sgRNAs (Fig. 4h,i; Supplementary Fig. 4l,m). Remarkably, the phenotype of BMP-overexpressing, vascular smad6b knockdown embryos resembles that of BMP-overexpressing embryos with reduced Notch signalling. Combined, these in vivo results support a model whereby SMAD6 represses BMP responsiveness and vessel branching.

Based on the similarity of phenotypes in vivo and the Notch responsiveness of SMAD6 RNA and protein in EC, we further explored the mechanism by which Notch regulates SMAD6 gene expression. We investigated the role of SMAD6 in Notch signalling and its potential as a negative regulator of BMP signalling.

**Figure 3 | SMAD6 is Notch regulated and anti-angiogenic.** (a) qRT-PCR for HES1 or SMAD6 with Notch gain-of-function (DLL4-Fc) or loss-of-function (DAPT). Data points, independent experiments (n = 5 DLL4-Fc and 9 DAPT), log-conversion of the DDCT versus controls. Quantification (b) and panels (c) of nuclear SMAD6 expression in individual HUVEC with indicated parameters, representative of two independent experiments. Scale bar, 10 μm. Yellow arrow, EC expressing FLAG-NICD. Error bars, mean ± s.e.m., ****P < 0.0001 by one-way analysis of variance using Tukey’s post-hoc; NS, not significant. (d) Stable lentivirally transduced HUVEC infected with SMAD6-tomato conditionally expressing virus, stimulated with doxycycline (DOX) and stained as indicated. (e) Quantification of twofold dose-response (indicated on x axis) to DOX (SMAD6-tomato expression) after 90 min BMP6 treatment, representative of two independent experiments. Readout is nuclear pSMAD1/5. Data are four-parameter best-fit curves (solid lines) ± 95% confidence bands (filled areas). (f) HUVEC transfected with indicated siRNAs, then treated with 40 ng ml⁻¹ BMP6 for 90 min before fixation and staining for nuclear pSMAD1/5, representative of two independent experiments (n = 75 NT and 80 SMAD6 siRNA HUVEC). Error bars, mean ± 95% CI. ****P < 0.0001 by Student’s t-test. (g–j) HUVEC 3D sprouting assay with indicated siRNAs and treatment, visualized with phalloidin (actin) and depth-encoded in a compressed z-stack, representative of three independent experiments. (k) Quantification of branching (n = 11 NT/control, 13 NT/BMP6, 12 SMAD6 siRNA/control and 11 SMAD6 siRNA/BMP6 beads). Data points, individual beads; bars, mean ± 95% CI. **P ≤ 0.01 and ***P ≤ 0.001 by Kruskal–Wallis with Dunn’s post-hoc test.
expression. Analysis of the SMAD6 5′-promoter region revealed a consensus sequence for Recombination Signal Binding Protein for Immunoglobulin Kappa J Region (RBPI) binding to DNA, which is required for Notch-dependent transcription in cells. A chromatin immunoprecipitation (ChIP) assay using two separate antibodies to RBPI revealed that RBPI binds at the identified site in the SMAD6 promoter (Fig. 5a and Supplementary Fig. 5a), suggesting that Notch directly regulates transcription of SMAD6 RNA.

Finally, we hypothesized that SMAD6 is functionally downstream of Notch in regulating the BMP responsiveness of EC. We tested this hypothesis by activating Notch in HUVEC with reduced levels of SMAD6, and we assessed BMP pathway signalling via nuclear localization of pSMAD1/5. Short-term BMP6 treatment of HUVEC led to elevated nuclear pSMAD levels that were significantly suppressed by Notch stimulation (Fig. 5b,c,f,g,i). In contrast, HUVEC with reduced SMAD6 levels and BMP6 stimulation did not respond to Notch stimulation with suppression of SMAD1/5 activation, but had nuclear pSMAD1/5 levels equivalent to that of HUVEC without Notch stimulation (Fig. 5d,e,h–j). These data indicate that SMAD6 is functionally downstream of Notch and is required to mediate the effects of Notch on BMP responsiveness in EC.

### Discussion
Collectively, our data support a model whereby pro-angiogenic BMPs increase vascular complexity through regulation of lateral branching. The magnitude of intrinsic BMP responsiveness in EC is modulated by Notch-mediated regulation of the BMP inhibitor SMAD6 to ‘tune’ BMP responsiveness (Fig. 6), and combined effects on branch frequency and angle lead to a more arborized or ‘bush-like’ vascular network as BMP ligand levels increase. In this model, Notch signalling sets levels of SMAD6 in a given EC and SMAD6 levels regulate the amount of pSMAD1/5 that translocates to the nucleus with a given input of BMP ligand. As ligand levels increase, more EC reach a threshold that allows them to assume a tip cell phenotype and sprout to form a new branch. This includes a subset of EC in a classic ‘stalk’ position, and our finding of differential BMP responsiveness in the stalk cell compartment indicates dissimilarity among stalk cells. Notch is a major regulator of the tip cell versus stalk cell phenotype in EC of angiogenic sprouts26,44, and we now propose that Notch also differentiates stalk cells by modulating expression of SMAD6. We predict that Notch-dependent changes in SMAD6 expression among dynamically competing sprouting EC shifts BMP responsiveness relative to a threshold necessary for lateral branching. Subsequently, as arteries reach homeostasis and Notch signalling becomes more uniform, these changes may lead to more uniform SMAD6 levels and overall dampened BMP responsiveness in arteries versus veins.

![Figure 4](image-url)

**Figure 4 | SMAD6 regulates BMP-dependent angiogenesis in vivo.**

(a) Quantitative RT-PCR for indicated genes, fold-change Notch reporter (+) versus Notch reporter- (NR-) EC, relative to ef1a. Error bars, mean ± s.e.m., N = 5 replicates; one-sample Student’s T-test. **P ≤ 0.01 and ***P ≤ 0.001. (b–d) F0 mosaic transgenic zebrafish embryos (44 hpf) expressing GFP control (b) or GFP-tagged smad6b (c) from the vessel-specific fli promoter in the Tg(kdrl:mCherry) line. Arrows, GFP + EC. (d) Arterial versus venous distribution of GFP + EC quantified as percentage of total GFP + EC on a per-embryo basis. Data bars, average per cent arterial and venous GFP + EC, ± 95% CI, representative of three independent experiments (n = 18 fliiGFp and 30 flii:smad6b-GFP F0 embryos). ****P ≤ 0.0001 by χ² analysis (1 degree of freedom). (e–h) Heat-shocked F1 embryos (heat shock at 26 hpf, analysed at 44–46 hpf) from Tg(fli:dCas9-EnR) and Tg(hsp70l:bmp2b;Tg(kdrl;GFP) crosses, injected with scrambled (scram) or with smad6b sgRNAs. Arrows, ectopic ISV sprouts. (g,h) have Z-planes with ectopic venous sprouts removed. (i) Quantification of arterial vascular defects (% segments with ectopic ISVs) in heat-shocked embryos of indicated genotypes, representative of two independent experiments (vasculature (scram, n = 4; smad6, n = 6; scram/hsbmp, n = 4; smad6/hsbmp, n = 5). Error bars, mean ± s.e.m.; **P ≤ 0.05 and ****P ≤ 0.0001 by one-way analysis of variance, with Tukey’s post-hoc test.
Allthough convergence of Notch and BMP pathways at downstream target genes has been described\(^8,13,30\), our data strongly suggest that intrinsic responsiveness to BMP signals is independently preset by Notch to regulate lateral branching. Recent work suggests that Notch also maintains the stalk cell phenotype via downregulation of Neuruplin-1 (ref. 45). Low Neuruplin-1 in stalk cells relieves inhibition of ALK1 and ALK5, and promotes transforming growth factor-β-mediated pSMAD2/3 signalling to maintain the stalk cell phenotype. Our work shows that SMAD6, a Notch-regulated cell-intrinsic BMP pathway inhibitor, modulates EC responses to pro-angiogenic BMP ligands that stimulate pSMAD1/5 signalling and a tip cell phenotype. Thus, Notch-mediated SMAD6 regulation ‘tunes’ branching responses to BMP ligands among stalk cells and coordinately regulates the magnitude of BMP pathway activation and sprouting, to determine the patterning of growing vessel networks.

**Methods**

**Cell maintenance and processing.** No cell lines used in this study are found in the International cell line authentication committee (ICLAC) commonly misidentified cell line database.

**Figure 5 | SMAD6 mediates Notch-dependent suppression of BMP signalling.** (a) ChiP assay on micrococcal nuclease-digested HUVEC DNA immunoprecipitated with indicated antibodies and amplified unique primers targeting a single putative RBPI consensus sequence in the SMAD6 promoter. Left, size marker (bp). (b-i) HUVEC transfected with non-targeting (b,c,f,g) or SMAD6 siRNA (d,e,h,i), plated onto IgG (b,d,f,h) or Dll4-Fc (c,e,g,i), then treated with control (b-e) or 50 ng ml\(^{-1}\) BMP6 (f-i) for 90 min before staining for nuclear pSMAD1/5. Scale bar, 10 μm. (j) Quantification of nuclear pSMAD1/5 fluorescence intensity, representative of three independent experiments. Data points, individual nuclei (n indicated on graph); bars, mean ± 95% CI. *P ≤ 0.05, **P ≤ 0.01 and ****P ≤ 0.0001 by Kruskal–Wallis with Dunn’s post-hoc test.

**Figure 6 | Model of SMAD6-mediated BMP responsiveness in vessels.** The data support a model whereby Notch sets BMP responsiveness of sprouting EC through the intrinsic BMP inhibitor SMAD6, leading to a ‘tunable’ system that responds to increased BMP ligands with more lateral branching.
**Sprouting angiogenesis assay.** The 3D sprouting angiogenesis assay was performed as previously described. Briefly, 1–2 × 10^6 HUVEC were mixed with 100 μl of 90% suspension of cryopreserved (GE Healthcare 17-0485-01) in 5 ml HUVEC growth media (EBM-2 + EGM-2 BulletKit, Lonza CC-3156 and CC-3162), respectively, maintained in suspension for 4 h by mixing every 15 min, then placed in 60 mm dishes at 37 °C overnight. Beads were recovered from the plate by gentle rinsing and washed 3 × in HUVEC growth media, then resuspended in 10 ml of 2.2 mg/ml fibrinogen/0.15 M NaCl aprotinin. Five microlitres of a 50 U ml⁻¹ thrombin solution was added to each well of a 24-well plate, followed by gently mixing in 500 μl of HUVEC-coated beads in fibrinogen/ aprotinin per well. After 5 min at RT, the plate was transferred to 37 °C for 30 min for polymerization, then 1 ml of 20,000 cells ml⁻¹ NHLF was added to each well. Media was refreshed every 2 days until fixation. Recombinant human BMP2 and BMP6 (R&D Systems #355-BM-010 or #507-SP-020, respectively) were added at d2–d6 at 200 ng ml⁻¹. Day 7 cultures were fixed in 2% PFA (Electron Microscopy Supplies #15713) in DPBS (Fisher #MT-21-031-CV) for 30 min, then stained with Alexa Fluor 488 Phalloidin or Alexa Fluor 594 Phalloidin (Life Technologies FD0594) double digest as described above. A four-way LR reaction using p5-Stop (a gift from Nathan Lawson, Addgene plasmid 31160), pME-smad6-GFP, p3E-polyA49 and pDEST Tol2 CG2 (ref. 49) produced the final targeting vector. 

**Lentivirus production and stably infected HUVEC.** Lenti-X 293T cells (Clontech 632100) were maintained in DMEM Hi Glucose (Life Technologies 11995-065) + 10% normal bovine calf serum (NBCS) according to the manufacturer’s instructions. For lentiviral production, cells were plated onto 10 cm dishes at 50,000 cells cm⁻². The following morning, cells were co-transfected with 20 μg pLIX and 400 vectors (described above) and 6 μg pMD2.G (a gift from Didier Trono, Addgene plasmid 12253) and 10 μg pMDLg/pRRE (a gift from Didier Trono, Addgene plasmid 12251) using Lipofectamine 2000 (Life Technologies 11680-010) according to the manufacturer’s protocol. Transduction medium was aspirated 8 h later and 6 ml fresh medium added. Forty-eight hours later, medium was harvested by careful filtration through a sterile 0.22 μm filter (Millipore Scientific, 50-202-062), mixed 1:1 with HUVEC growth media (EBM-2 + EGM-2 BulletKit, Lonza CC-3156 and CC-3162, respectively) and added to 80% confluent puHVEC. The next day, medium was aspirated and fresh medium added. After 72 h, infected HUVEC were selected using 1 ng ml⁻¹ puromycin (Sigma-Aldrich P8870-20 ml) in HUVEC growth medium, changed daily, for 2 weeks. Surviving HUVEC were then trypsinated, re-plated under 0.5 ng ml⁻¹ puromycin selection and expanded.
under ultraviolet for total protein, blocked for 2 h in 5% non-fat milk (NFM) in PBS + 0.1% Tween20 (PBST), then incubated overnight at 4 °C with primary antibody at 1:5000 NF in PBST (see Supplementary and secondary antibodies and concentrations). Horseradish peroxidase-conjugated secondary antibodies (Life Technologies goat anti-rabbit, Molecular Probes) were added for 2 h at RT in 1% NFM in PBST. Clarity ECL (Bio-Rad) was used for detection. Films were digitized using an AlphaImager (Alpha Innotech), gel scanning station and band densities calculated in FIJI, following established guidelines.

Quantitative RT-PCR. Primers used in this study are listed in Supplementary Table 3. mRNA was collected from experimental samples using Trizol reagent (Life Technologies 15596026). cDNA was generated from 1 μg mRNA using iScript reverse transcription kit (Bio-Rad 170–8891) and diluted 1:3 in water. qRT–PCR was performed using iTaq Universal SYBR Green SuperMix (Bio-Rad 172–5121) on an ABI 7900HT Fast Real-Time PCR System (Life Technologies 432940). Threefold serial dilutions of pooled cDNA were used to generate standard curves for each amplicon and data were analysed via the Pfaffl method. RNA was isolated from FACS-enriched zebrafish cells using RNAeasy Micro Plus kit (Qiagen) and reverse transcribed with Invitrogen's Superscript III First-Strand Synthesis Supermix (Invitrogen) according to the manufacturer's instructions. For qRT–PCR, NCBI's Primer-BLAST was used to design exon-spanning, gene-specific SybrGreen primers (see Supplementary Table 3). All primers were validated by high-resolution melt analysis, size confirmation and no-template controls. SybrGreen real-time PCR was performed in triplicate on the Viia7 real-time PCR system (Invitrogen). For quantification, the ΔΔCT method was used where raw CT values were normalized to elongation factor alpha (ef1α1III) and pair sorting control, then calculated fold change as 2^−ΔΔCT. Statistical significance was determined by one-sample T-test comparing with a reference value of one-fold change.

Zebrafish (Danio rerio) embryos were maintained as previously described. The following transgenic lines were used: Tg(fli1ep:GFP);Tg(fli1ep:GFP::GFP)ksc6 (ref. 55), Tg(fli1ep:Smad6b-GFP);Tg(fli1ep:GFP::GFP)ksc6 (ref. 56). Two independent lines were used for each experiment and all samples were pooled from individual spawning groups. Embryos were manually sorted for Tp1:EGFP expression. For CRISPRi, stage with 450 pg dCas9-EnR mRNA alone or in conjunction with 200 pg scramble mRNA was used for later RNA purification.

Briefly, embryos were manually dechorionated, yolk removed, then dissociated into single-cell suspension using a combination of TrypLE (Gibco) and FACSMax (CytoFLEX). Cells were resuspended in ice-cold L-15 (Gibco) supplemented with 5% heat-inactivated FBS (HyClone), passed through 40 and 35 μm filters, then counterstained with Live/Dead fixable near-IR stain (Molecular Probes). A SH800 cell sorter (Sony) was used to sort live, tdTomato + cells by Tp1:EGFP expression level and harvested directly into RT Lysis buffer, then stored at −20 for later RNA purification.

ChIP assay. HUVECs were plated on 100 mm culture dishes and grown for 24 h and native protein–DNA complexes were cross-linked by treatment with 1% formaldehyde for 15 min. Simple ChiP Plus Enzymatic Chromatin IP kit (Cell Signalining 9005) was used per the manufacturer’s protocol. Briefly, equal aliquots of isolated chromatin were subjected to immunoprecipitation with anti-RBPJ antibodies (Cell Signaling 5313s, Abcam ab25949) or rabbit IgG control at 2 μg per 500 μl. PCR reactions of immunoprecipitated DNA were performed to validate RBPJ binding on the Smad6 promoter. Primers used were:

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\begin{align*}
2631 & \rightarrow 2097: & 5' & -TGCTGCATCAAGTGATTCTCGTGCCT-3' \\
2097 & \rightarrow 1575: & 5' & -TGCACTCACAGGATCTCTTGCTGCCT-3' \\
1575 & \rightarrow 1053: & 5' & -AGGGCCGATCACCTCAGGACGTCA-3' \\
1053 & \rightarrow 531: & 5' & -GCTTCGTCACTCAAGTGATTCTTGCTGCCT-3'.
\end{align*}
\]

PCR products were separated by gel electrophoresis and visualized by SYBRsafe (Invitrogen).

Imaging and quantification. All images were acquired on an Olympus Fluoview FV1200 confocal microscope, equipped with Green, Red and Far-Red lasers and detectors. Olympus OIF templates were imported into Fiji using Bio-Formats Imaging, 2009, for analysis and quantification, as described below.

Branch frequency was measured by skeletonizing ES-cell-derived vessels or HUVEC-derived 3D sprouting angiogenesis assay vessels and calculating branches per mm using Image J (NIH). For parameterization, both length and fluorescence were determined relative to the tip cell of each sprout. In zebrafish, the maximum branch angle was calculated at sprout tips, excluding branches that fused with other sprouts distal to the junction. The percentage of tip cells was quantified in all multi-nucleated sprouts. Tip cells were defined as the cell nucleus located most distal in a sprout. For branch parameterization, both length and fluorescence were determined relative to the tip cell.
8. Moya, I. M. et al. 10. van Meeteren, L. A. 7. Phng, L. K. & Gerhardt, H. Angiogenesis: a team effort coordinated by Notch. 12. Ramoshebi, L. N. & Ripamonti, U. Osteogenic protein-1, a bone morphogenetic protein-1 protein, induces angiogenesis in the chick chorioallantoic membrane and synergizes with basic fibroblast growth factor and transforming growth factor-β. 13. Larrivee, B. NATURE COMMUNICATIONS | DOI: 10.1038/ncomms13247 | www.nature.com/naturecommunications 16. Derynck, R. & Zhang, Y. E. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. 23. Chappell, J. C., Mouillesseaux, K. P. & Bautch, V. L. Flt-1 (vascular endothelial growth factor receptor-1) is essential for the vascular endothelial growth factor-Notch feedback loop during angiogenesis. Arterioscler. Thromb. Vasc. Biol. 33, 1952–1959 (2013). 24. David, L., Mallet, C., Mazetrousseau, B., Feige, J. & Baillie, S. Identification of BMP9 and BMP10 as functional activators of the orphan activin receptor-like kinase 1 (ALK1) in endothelial cells. Blood 109, 1953–1961 (2007). 25. David, L. et al. Bone morphogenetic protein-9 is a circulating vascular quiescence factor. Circ. Res. 102, 914–922 (2008). 26. Hellstrom, M. et al. Dß4 signalling through Notch1 regulates formation of tip cells during angiogenesis. Nature 445, 777–780 (2007). 27. Leslie, J. D. et al. Endothelial signalling by the Notch ligand Delta-like-4 restricts angiogenesis. Development 134, 839–844 (2007). 28. Suchting, S. et al. The Notch ligand Delta-like-4 negatively regulates endothelial tip cell formation and vessel branching. Proc. Natl Acad. Sci. USA 104, 3235–3230 (2007). 29. Jackson, L. et al. Endothelial cells dynamically compete for the tip cell position during angiogenic sprouting. Nat. Cell Biol. 12, 943–953 (2010). 30. Itoh, F. et al. Synergy and antagonism between Notch and BMP receptor signalling pathways in endothelial cells. EMBO J. 23, 541–551 (2004). 31. Maddaluno, L. et al. EndMT contributes to the onset and progression of cerebral cavernous malformations. Nature 498, 492–496 (2013). 32. Siekmann, J. et al. ETS factors regulate Vegf-dependent arterial specification. Dev. Cell 26, 45–58 (2013). 33. Quillien, A. et al. Distinct Notch signalling outputs pattern the developing arterial system. Development 141, 1544–1552 (2014).
