Vascular deficiency of Smad4 causes arteriovenous malformations: a mouse model of Hereditary Hemorrhagic Telangiectasia

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Abstract
Hereditary hemorrhagic telangiectasia (HHT) is an autosomal dominant vascular disorder that leads to abnormal connections between arteries and veins termed arteriovenous malformations (AVM). Mutations in TGFβ pathway members ALK1, ENG and SMAD4 lead to HHT. However, a Smad4 mouse model of HHT does not currently exist. We aimed to create and characterize a Smad4 endothelial cell (EC)-specific, inducible knockout mouse (Smad4f/f; Cdh5-CreERT2) that could be used to study AVM development in HHT. We found that postnatal ablation of Smad4 caused various vascular defects, including the formation of distinct AVMs in the neonate retina. Our analyses demonstrated that increased EC proliferation and size, altered mural cell coverage and distorted artery–vein gene expression are associated with Smad4 deficiency in the vasculature. Furthermore, we show that depletion of Smad4 leads to decreased Vegfr2 expression, and concurrent loss of endothelial Smad4 and Vegfr2 in vivo leads to AVM enlargement. Our work provides a new model in which to study HHT-associated phenotypes and links the TGFβ and VEGF signaling pathways in AVM pathogenesis.

Keywords Smad4 · Arteriovenous malformations (AVM) · Hereditary hemorrhagic telangiectasia (HHT) · Vegfr2 · TGFβ

Introduction
Hereditary hemorrhagic telangiectasia (HHT) is an autosomal dominant vascular disorder that affects 1 in 5000 people worldwide [1, 2]. HHT patients commonly exhibit: spontaneous, recurring nosebleeds; small lesions on mucous membranes called telangiectasias; and/or larger visceral lesions known as arteriovenous malformations (AVMs) [3, 4]. AVMs, which are direct connections between arteries and veins, are most commonly found in major organs such as the brain, liver or lungs. These lesions present a serious health risk and can lead to decreased quality of life and/or early death due to hemorrhaging, stroke and aneurysms [3, 5–8].

Approximately 85% of HHT cases are linked to heterozygous loss-of-function mutations in the transforming growth factor beta (TGFβ) cell surface receptors activin receptor-like kinase 1 (ALK1, HHT2) or endoglin (ENG, HHT1) [9, 10]. A small subset of HHT patients (~ 4%) exhibit haploinsufficiency of Mothers against decapentaplegic homolog 4 (SMAD4, JP/HHT) and commonly present with juvenile polyposis syndrome (JP) [11, 12]. SMAD4 is a transcription factor found in nearly all cell types [13, 14], where it serves as the central conduit through which canonical TGFβ signaling proceeds, including ALK1 and ENG signaling [15]. However, despite the key role of SMAD4 in the TGFβ pathway, the mechanisms by which it contributes to HHT pathogenesis remain unknown. In fact, virtually all HHT animal studies have focused on the Alk1 and Eng receptor interface of the TGFβ signaling pathway, whereby endothelial loss of Alk1, or Eng or blockade of the TGFβ pathway via Bmp9/10 ligand-blocking antibodies results in HHT-associated phenotypes [16–26]. What little we know about the in vivo role of SMAD4 in the vasculature comes from embryonic studies. These studies revealed that SMAD4 plays a critical role in blood vessel remodeling and maturation [27], integrity of the blood-brain barrier endothelium [28] and regulating coronary artery size [29]. Conversely, nothing is known about SMAD4 function in the postnatal vasculature as homozygous loss of Smad4 is embryonic lethal [27]. Therefore, due
to limited information on how SMAD4 contributes to the developing endothelium, it is unclear how SMAD4 defects lead to HHT phenotypes, such as AVM formation.

In order to better understand SMAD4’s contribution to HHT pathogenesis, we created an inducible, endothelial cell (EC)-specific Smad4 knockout mouse model (referred to as Smad4-iECKO). We find that induced deletion of Smad4 leads to various vascular defects including the formation of AVMs. In addition, we show that SMAD4 influences EC proliferation, EC size, mural cell coverage and artery–vein gene expression. Utilizing this new Smad4-iECKO model, we found that deletion of Smad4 leads to decreased levels of vascular endothelial growth factor receptor 2 (VEGFR2) expression. Furthermore, concurrent loss of endothelial Smad4 and Vegfr2 in vivo leads to an increased AVM severity. This work provides a new model for the HHT field and presents evidence that the TGFβ and VEGF pathways may be linked in AVM pathogenesis.

Results

EC-specific deletion of Smad4 causes multiple vascular defects, including AVM formation

To characterize SMAD4 function in the postnatal vasculature, Cdhl5-CreERT2 [30] and conditional Smad4-floxed (Smad4f/f) [31] mouse lines were utilized to generate homozygous Smad4-inducible, endothelial-specific knockout mice (Smad4-iECKO). Tamoxifen (Tx) injections were administered at postnatal day 1 (P1) and P4 to activate Cre-mediated deletion of Smad4 in the endothelium (Fig. 1A).

A majority of mice died around P8–P9, likely due to respiratory distress caused by defects in the lung vasculature (Fig. S1A–B’), similar to Alk1-deficient neonate mice [20]. Therefore, we utilized P7 retinas to assess blood vessel development, as the retina is a tractable system for identifying vascular defects, including AVMs, and has been used to study Alk1 and Eng mouse models of HHT.

To confirm Smad4 deletion in the endothelium lineage, quantitative PCR (qPCR) was performed using RNA from P7 Smad4f/f (control) and Smad4-iECKO isolated retinal ECs which revealed an ~ 80% reduction in Smad4 mRNA transcripts (Fig. 1B). In addition, using a Rosa26-EYFP transgenic reporter line [32] we confirmed that Cre-recombinase was specifically expressed in blood vessels, while absent in control blood vessels (Fig. S1C–D’). These data demonstrated efficient and specific Smad4 knockdown in the ECs of Smad4-iECKO retinas.

In order to assess the effects of Smad4 depletion on vascular development, Smad4f/f control and Smad4-iECKO P7 retinas were labeled with the vascular marker Isolectin-IB4. We observed numerous arteriovenous malformations (AVMs) in the retinas of Smad4 mutants (Fig. 1C–F), similar to those identified in Alk1- and Eng-deficient mice [17, 18, 20, 24, 33, 34]. Approximately 82% of our Smad4 mutants had AVMs, whereas AVMs were absent in all controls. Multiple AVMs were seen in 52% of Smad4-iECKO mice with an average of 1.732 AVMs per mutant retina (Fig. 1K). AVMs varied in morphology but were easily identifiable because the shunts appeared grossly enlarged in comparison with normal capillaries (Fig. 1C–F and Fig. S2A–H). AVMs were almost always located near the center of the retina, likely due to blood flow patterns in HHT models as previously described [35]. In Smad4-iECKO mice, AVMs form around P5 (data not shown) and either did not form or were smaller if Tx was administered after P1 (Fig. S2I–K).

Loss of Smad4 also caused a noticeable reduction in vascular outgrowth toward the retinal periphery (Fig. 1C, D, L). For this reason and because Alk1 mutant zebrafish exhibit EC migratory defects [36], we aimed to further assess Smad4 function on EC migration in vitro. We generated
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Increased cell proliferation in Smad4-deficient ECs. A–C Close-up views of Smad4<sup>fl/fl</sup> and Smad4-iECKO P7 retina stained for Kif67 (green), ERG (red) and Isolectin-IB4 (magenta; IB4). a arteries, v veins and AVM (white arrow) in A–C’ are outlined by dotted lines. Scale bars = 50 μm. D Smad4 deficiency leads to an increase in proliferating ECs (Kif67<sup>+</sup> and ERG<sup>+</sup>) in Smad4-iECKO arteries, capillaries and veins. Three fields of view were counted in each sample. E Loss of Smad4 reveals no change in the number of apoptotic cells as assessed by cleaved CASPASE-3 fluorescence in the P7 retinal vasculature. Three fields of view were counted in each sample. Sample size (n) indicates independent biological samples.

stable C166 mouse EC lines that expressed either nonsilencing-shRNAs or Smad4-shRNAs. In comparison with nonsilencing-shRNA, Smad4-shRNA C166 cells showed an approximately 60% reduction in levels of Smad4 transcripts and a diminished capacity to migrate and repopulate wounds in a scratch assay (Fig. S3A–D).

Although outgrowth was stunted, the number of tip cells was not significantly changed in Smad4 mutant retinas (data not shown). Similarly, quantification of vascular densities showed no statistical differences on average (data not shown). This is likely due to the high variability between mutants, as we observed some mutants that displayed significant increases in density in the tip region, while others were indistinguishable from controls (Fig. S4). Although there was variability among vascular density of mutants, one consistent phenotype was that of increased artery and vein diameters (Fig. 1G–J, M, N). Increases in vessel diameter and stunted outgrowth were also even seen in the ~20% of mutants that did not express AVMs, suggesting that these events may precede AVM development.

Collectively, these results demonstrate that Smad4 is required for proper vascular growth and vessel morphology in the postnatal retina. Furthermore, the presence of AVMs verifies that loss of Smad4 in mice recapitulates phenotypes associated with HHT patients, thus making this a suitable model for studying Smad4 mechanisms of HHT.

Smad4 depletion causes increases in EC proliferation and size

To determine whether vessel enlargement was caused by an increase in EC proliferation, we examined both Smad4 control and mutant retinas immunolabeled for the proliferation-associated protein, Kif67, and the EC-specific nuclear marker, ERG. Proliferating ECs, which are defined as both Kif67 and ERG positive, were quantified in the arteries, veins and capillaries (Fig. 2A–D). We found significant increases in proliferation in all vessel types, with the most drastic changes seen in the capillaries and veins. Increases in proliferation occurred in vascular regions with and without AVMs, indicating that these increases are attributable to reduction in SMAD4 levels and not an effect of AVM formation itself. Conversely, staining for the apoptotic marker cleaved CASPASE-3 revealed no changes in cells undergoing apoptosis between control and Smad4-iECKO retinal blood vessels (Fig. 2E). These data indicated that increased rates of EC proliferation, at least in part, are responsible for the increased vessel sizes in Smad4-iECKO retinas.

Relatedly, recent reports indicated that loss of either Eng or Smad4 leads to an increased EC size [25, 29], which could contribute to an increased artery and vein diameter and/or AVM formation. To determine whether Smad4-iECKO mice exhibited changes in cell size, we measured EC areas in each vessel type as marked by CD31-stained EC boundaries. Smad4 deletion caused the cell area of arterial ECs to increase by 41%, while venous ECs increased by 34% compared to their respective control vessels (Fig. 3A–F). In addition, C166 ECs with Smad4-shRNA showed a 115% increase in cell area compared to nonsilencing-shRNA C166 ECs (Fig. 3G–I). Overall, these results are consistent with a recent study showing that loss of Smad4 contributes to an increase in cell size in the developing coronary artery and in cultured ECs under high levels of shear stress [29]. Taken together, we conclude that a combination of an increased EC proliferation and an increased EC size contributes to the vessel enlargement phenotypes observed in Smad4 mutants.

Defective mural cell coverage in Smad4-iECKO mice

Alk1 and Eng models of HHT have previously noted several changes in mural cell coverage of retinal blood vessels. For instance, vascular smooth muscle cells (vSMCs) were found to accumulate ectopically around veins [17, 20, 24]. In Smad4-iECKO mice, we also observed strong, ectopic expression of alpha-smooth muscle actin (αSMA) protein around the AVMs and veins compared to control retinas which only exhibit αSMA on arteries at P7 (Fig. 4A–B’). Moreover, qPCR analysis demonstrated increased αSma transcript levels in Smad4-iECKO retinas compared to Smad4<sup>fl/fl</sup> controls (Fig. 4C).

Conversely, AVMs are associated with a reduction in pericyte coverage [20, 35]. Smad4 deficiency has been shown to affect EC–pericyte interactions resulting in loss of pericyte coverage in the developing brain vasculature [28]. To test whether this relationship exists in Smad4-iECKO retinas, we investigated pericyte coverage using an anti-neuron-glial antigen 2 (NG2) antibody. We found that compared to controls, Smad4 mutant retinas exhibit a marked reduction in NG2 protein accumulation in the retinal vasculature (Fig. 4D–E’). Furthermore, qPCR analysis on whole retina samples verified that Ng2 and Desmin (a pericyte marker) mRNA levels are significantly diminished when Smad4 is deleted (Fig. 4F, G). Since the platelet-derived growth factor (PDGF) signaling pathway plays a significant role
in recruiting pericytes to blood vessels [37], we assessed whether changes in expression of the endothelial-secreted PDGF-B ligand could account for the loss of pericyte coverage in Smad4 mutants. qPCR results showed that Pdgfb transcript levels are similar in control and Smad4-iECKO whole retinas (Fig. 4H), suggesting that other factors are responsible for the reduced pericyte presence in Smad4-deficient retinas. Overall, our results are consistent with other HHT models in that vSMC coverage inappropriately extends to AVMs and veins, while pericyte coverage is reduced in a Smad4-deficient background.

Artery–vein identity is disrupted in the absence of Smad4

The presence of vSMCs on Smad4-deficient veins suggested that these vessels may have acquired an arterial-like identity. Interestingly, alterations in artery and vein (AV) gene expression have been reported in HHT models [17, 20, 38, 39] and in non-HHT-associated AVMs triggered by disruptions in NOTCH pathway signaling components [40, 41]. Therefore, we characterized a number of AV identifiers, including NOTCH pathway members, in our Smad4-iECKO
Fig. 4 Altered mural cell coverage in Smad4-iECKO retinas. A–B' Confocal analysis of alpha-smooth muscle actin (αSMA; red) and Isolectin-IB4 (IB4; green) revealed that Smad4f/f retinas (n = 10) contain αSMA only on arteries, whereas Smad4-iECKO mice (n = 8) express αSMA on both arteries and veins. Insets show close-up views of an artery and vein, with an AVM covered by αSMA in B, B' inset. AVMs are denoted by white arrows in B. C qPCR results on RNA isolated from Smad4f/f and Smad4-iECKO P7 whole retinas confirm an increased αSma gene expression in Smad4 mutants. D–E' Immunofluorescent staining of the pericyte marker neuron-glial antigen 2 (NG2; red) and IB4 (green) revealed a striking reduction in levels of NG2 in Smad4-iECKO mice (n = 9) compared to control (n = 10). F, G qPCR analysis of Ng2 and Desmin (another pericyte marker) transcript levels in P7 Smad4f/f and Smad4-iECKO whole retinas verifies loss of pericytes in Smad4-deficient backgrounds. H qPCR analysis of secreted ligand Pdgfb transcript levels in P7 Smad4f/f and Smad4-iECKO whole retinas remains unchanged. α arteries, ν veins. The number of independent biological samples is shown as (n) on bar graphs, and all qPCR samples were normalized to the housekeeping gene Odc.
model by performing qPCR on isolated lung endothelial cells (iLECs) from Smad4 mutants and their control littermates (Fig. 5A). We found a significant reduction in the venous-associated markers CoupTfl1 and Ephb4 and arterial markers Hey1, Hey2, Notch1, and Nrpl1. Conversely, mRNA levels of the venous markers, Endomucin and Flt4, and the arterial markers Apelin (also associated with vascular tip cells) and Notch4 were upregulated in Smad4-iECKO iLECs. The following markers remained unchanged between Smad4 control and mutants: Apj, Cx40, Dil4, Ephrinb2, Jagged1, Nrpl2, and Sox17.

To further confirm changes in AV gene expression, we used immunofluorescent staining and in situ hybridization techniques on Smad4f/f and Smad4-iECKO retinas. At P7, ENDOMUCIN is largely absent from control retinal arteries (Fig. 5B); however, in Smad4 mutants, we observed distinct protein expression in the arteries (Fig. 5C), suggesting that increased Endomucin mRNA levels in iLECs (Fig. 5A) might be due to enhanced expression in arteries. Analysis of Apelin mRNA showed robust, ectopic expression in the retinal veins and capillaries of Smad4 mutants compared to controls, which completely lacked expression in these vessels (Fig. 5D, E). On the other hand, the apelin receptor, Apj, was present in the AVM but showed no noticeable changes in mRNA expression between Smad4 control and mutant retinas consistent with our qPCR results (Fig. 5F, G).

Examination of Ephb4 revealed the loss of transcripts in the capillaries and arteries, although Ephb4 mRNA remained in the veins (Fig. 5H, I). Interestingly, even though overall levels of Ephb4 mRNA were reduced, Ephb4 was still noticeably expressed within the AVM and the artery connected in the AVM. Analysis of Notch4 showed no changes in localization of transcripts between Smad4 control and mutant retinas; however, levels of Notch4 appeared markedly higher in Smad4-iECKO retinas (Fig. 5J, K). In all, the whole retina staining results are consistent with the qPCR analysis performed on iLECs. This also revealed the importance of examining both qPCR levels in the whole vasculature as well as localization changes, as some markers are gained in specific vascular beds (Apelin), while others are lost (Ephb4). More so, some markers can increase in levels without changing localization (Notch4), while others remain the same but are expressed in the AVM (Apj). Therefore, we conclude that loss of Smad4 in the endothelium alters AV gene expression and as other groups have suggested may play a contributing role in the formation of AVMs due to disruptions in vessel identity [41].

In addition, we quantified the transcript levels of Alk1 and Eng in Smad4 control and mutant iLECs, as previous reports have shown that genetic knockdown of one receptor can lead to changes in expression of the other in vivo [17, 20, 24]. Our qPCR results indicate a significant loss in expression of Eng in Smad4-depleted ECs; however, no changes in Alk1 expression were observed (Fig. 5A).

**Loss of Vegfr2 enhances Smad4 mutant phenotypes**

Recent investigations in Alk1 and Eng HHT models indicated a potential link with vascular endothelial growth factor receptor 2 (Vegfr2) [24, 34, 42], a major signaling component in the VEGF pathway. In order to determine whether Smad4 and Vegfr2 are associated in AVM pathogenesis, we first assessed whether Vegfr2 mRNA levels were altered in our Smad4-iECKO background. qPCR analysis on cultured iLECs demonstrated that, in comparison with controls, Vegfr2 mRNA levels are reduced by approximately 40% in Smad4-iECKO mice (Fig. 6A). However, no significant changes in Vegfr2 transcript levels were observed between Tx-injected Smad4f/f control and Smad4f/f;Cdh5-CreERT2 iLECs, even though Smad4 mRNA levels were reduced by approximately 50% in the heterozygous mutants (data not shown). Similarly, quantification in Smad4-shRNA C166 endothelial cells also showed a relationship whereby Vegfr2 transcripts were significantly reduced compared to nonsilencing-shRNA controls (Fig. 6B). Moreover, expression of VEGFR2 protein was dramatically diminished in Smad4-deficient iLECs (Fig. 6C, D) and appeared reduced throughout all retinal blood vessels in Smad4 mutants as compared to controls (Fig. 6E–H). In all, these data demonstrated that impaired SMAD4 function leads to significantly reduced expression of Vegfr2 mRNA and protein.

Since our data indicated that Vegfr2 expression decreases upon loss of Smad4, we hypothesized that the incremental losses of VEGFR2 in the Smad4-iECKO background would lead to enhanced HHT-like phenotypes during retinal vascular development. To test this possibility, we crossed our Smad4-iECKO mouse line to Vegfr2-foxed mice (Vegfr2f/f, referred to as Vegfr2f/f-, iECKO or Vegfr2f/f-;iECKO in the presence of Cdh5-CreERT2) [43]. The different genetic combinations of mice were given Tx at P1, and retinas were collected at P7. Similar to control Smad4f/f mice lacking Cdh5-CreERT2, a single allelic deletion of Smad4 (Smad4f/-;iECKO) had no noticeable effects on retinal vascular development, while Vegfr2 (Vegfr2f/-;iECKO) retinas only showed a reduction in vascular outgrowth (Fig. 7A, B, D). On the other hand, the combined loss of a single allele of Smad4 and Vegfr2 (Smad4f/f;Vegfr2f/-;iECKO) resulted in increases in vascular density at the growing front (although inconsistent), which were never observed in Smad4f/f;iECKO or Vegfr2f/f-;iECKO retinas, but were similar to Smad4f/f;iECKO retinas (compare Fig. 7 B–E and Fig. S4). More compellingly, loss of a single copy of the Vegfr2 allele in the Smad4-iECKO background (Smad4f/f;Vegfr2f/-;iECKO) revealed dramatic vascular
phenotypes beyond those observed in Smad4-iECKO mice alone (compare Fig. 7C and F). Vascular outgrowth was significantly inhibited, and the vascular front showed reliable and marked increases in density (Fig. 7J). Even more noticeable was the increased number and striking enlargement of AVMs. We quantified severity of AVMs by measuring the diameter of the AVM in Smad4-iECKO and Smad4nullVegfr2f/f-iECKO mice. On average, Smad4nullVegfr2f/f-iECKO AVMs were ~75 µm wider than Smad4-iECKO AVMs, suggesting that more blood is able to be shunted from artery to vein in these AVMs leading to a more severe phenotype (Fig. 7K–M). Together, these experiments implied that overall levels of VEGFR2 have an effect on severity of Smad4-mediated HHT phenotypes.

Interestingly, reciprocal experiments in Vegfr2 null backgrounds showed slightly different results. As previously observed, complete loss of Vegfr2 (Vegfr2f/f-iECKO) in the retina led to severe vascular defects, including an overall reduction in the vasculature with fewer vessels and a lack of definitive capillaries (compare Fig. 7A, G) [44]. Loss of a single allele of Smad4 in the Vegfr2 null background (Smad4f/fVegfr2f/f-iECKO) resulted in similar phenotypes, suggesting that heterozygous loss of Smad4 had little effect on the overall vascular phenotype (Fig. 7H). In addition, combinatorial deletion of Smad4 and Vegfr2 (Smad4f/fVegfr2f/f-iECKO) led to AVM formation, but these retinas exhibited very little vascular coverage (Fig. 7I). This suggests that there is a threshold for which loss of Vegfr2 becomes dominant to Smad4 deletion, as was previously reported in Alk1 mutants [24]. In conclusion, our results indicate that reduction in VEGF signaling may contribute to heightened HHT phenotypes during developmental angiogenesis.

**Discussion**

Our studies are the first to report a Smad4 animal model of HHT (Smad4-iECKO). We showed that endothelial loss of Smad4 recapitulates vascular phenotypes seen in other HHT mouse models, particularly AVM formation. To better understand Smad4’s role in HHT pathogenesis, we performed a comprehensive characterization of Smad4-iECKO mice. Our results demonstrated that increased EC proliferation and size, alterations in mural cell coverage and disruption in AV gene expression are associated with Smad4-deficient blood vessels. We also provided evidence that loss of SMAD4 causes decreased VEGFR2 expression, and that loss of a single allele of Vegfr2 in the Smad4 null background leads to an increased severity of AVMs.

Considering Smad4’s centralized role in TGFβ signaling, we aimed to test the universality of our Smad4-iECKO model in relationship to HHT phenotypes. Consistent with previous reports, loss of TGFβ signaling through Smad4 leads to vascular defects similar to those found in Eng and Alk1 mouse retinal models (Fig. 1; summarized comparisons of HHT models in Table 1). For example, blood vessel enlargement in Alk1 and Eng mutant mice has been linked to increases in EC proliferation [17, 20, 34, 45], which we also observed in our Smad4 mutants (Fig. 2). Moreover, loss of Smad4 led to an increase in EC cell size both in vivo and in vitro, which was unreported in Alk1 and Eng models (Fig. 3). These findings are supported by a recent study showing that loss of Smad4 caused an increase in the size and rates of proliferation of ECs in the coronary artery and under in vitro flow conditions [29]. Furthermore, work in zebrafish has shown that in response to increases in flow, Eng-deficient blood vessels enlarge [25]. Interestingly, our in vitro data suggest that, in Smad4 mutants, cell size changes may also occur in the absence of flow. Taken together, it appears that blood vessel enlargement in HHT models is affected not only by increases in EC proliferation but also by an increase in EC size itself. How these alterations may lead to AVM formation is unclear, although it is notable that defects in the NOTCH pathway (both gain-of-function and loss-of-function) cause AVM formation [40, 41, 46, 47] via an initial increase in size of ECs [48]. Whether AVMs in HHT patients form in a similar manner remains an open question, as evidence in zebrafish suggests that HHT-associated AVMs are not directly caused by alterations in NOTCH signaling [49].

Nonetheless, our work demonstrated that expression of NOTCH signaling components, which are associated with arterial identity, as well as genes connected to venous and tip cell identity, are disrupted in the absence of Smad4 (Fig. 4). We also revealed that these changes can occur in arteries, veins and/or capillaries; however, it is important to note that the AVMs themselves expressed all genes examined regardless of whether the marker was up- or downregulated in other vessel types. When comparing these results to those obtained in Alk1 and Eng mouse models, we noted variations in AV gene expression between all three mutant backgrounds [17, 20, 24, 34, 38]. These differences could be due to tissue-specific effects related to the source tissues examined and/or the vascular expression patterns of Alk1, Eng and Smad4. For instance, some studies examined gene expression in isolated lung ECs [24], while others utilized brain and/or retinal ECs [20, 34]. Additionally, it is possible that expression levels in various vessel types play a role, as Alk1 is highly expressed in arterial ECs [50], while Eng is only moderately expressed in arteries [51]. Eng also is expressed highly in capillaries and weakly in veins [52]. In comparison, Smad4 is present in virtually all tissues [13, 14]. However, despite these differences, it is clear that overall disruptions in AV gene expression are consistent between all three mouse models of HHT. Further examination is
needed to address whether alterations in AV identity are a primary cause or secondary effect of AVM formation. To this point, our work does not address whether the observed phenotypes and molecular changes are a cause or an effect of AVM formation, as experiments were performed after AVMs developed. This cause/effect relationship has not been explored in \textit{Alk1} and \textit{Eng} models of HHT either. Therefore, future studies addressing this issue will be important for identifying the underlying molecular defects that drive AVM formation.

**Fig. 5** \textit{Smad4}-iECKO mice display alterations in artery and vein gene expression profiles. A qPCR analysis of 12 arterial (red), 5 venous (blue) and 2 HHT (purple)-associated genes on isolated, cultured lung ECs (iLECs) from P7 \textit{Smad4}^{f/f} \textit{(n} = 3) and \textit{Smad4}-iECKO \textit{(n} = 3) mice revealed altered AV- and HHT-associated gene expression. All transcripts were normalized to \textit{Cd31/Pecam} mRNA levels (*$p < 0.05$; **$p < 0.005$; ***$p \leq 0.0001$). B–C Qualitative evaluation of \textit{Smad4} control (\textit{n} = 6) and mutant (\textit{n} = 6) P7 retinas revealed increased levels of ENDOMUCIN protein (red) in arteries of \textit{Smad4}-iECKO mice (white arrows; IB4, green). D–K In situ hybridization analysis of various artery–vein mRNAs substantiates qPCR results from iLECs. D, E \textit{Apelin} mRNA is localized to the vascular tip cells (black arrows) and arteries of \textit{Smad4}^{f/f} mice (\textit{n} = 3); however, in \textit{Smad4}-iECKO mice \textit{Apelin} is also robustly and ectopically expressed in the capillaries and veins (\textit{n} = 3). F–G \textit{Apj} expression remains unchanged in \textit{Smad4}^{f/f} (\textit{n} = 3) and \textit{Smad4}-iECKO (\textit{n} = 3) littermate retinas. Arrow in G points to an AVM expressing \textit{Apj}. H–I \textit{Ephb4} mRNA is expressed strongly in the veins and moderately in the arteries and capillaries (yellow arrow) of \textit{Smad4}^{f/f} retinas (\textit{n} = 3). In \textit{Smad4}-iECKO retinas, \textit{Ephb4} is largely absent in the capillary beds (yellow arrow) and in most arteries, excluding the artery connected to the AVM (black arrow) (\textit{n} = 3). Note that \textit{Ephb4} mRNA is expressed in the AVM. J–K \textit{Notch4} mRNA expression patterns remain the same in \textit{Smad4}^{f/f} (\textit{n} = 3) and \textit{Smad4}-iECKO (\textit{n} = 3) littermate retinas, but overall levels are increased in all vessel types of \textit{Smad4} mutants. a arteries, v veins, \textit{n} the number of independent biological samples.
Fig. 6 Postnatal ablation of Smad4 leads to reduced levels of Vegfr2. A, B qPCR analysis of Smad4 and Vegfr2 mRNA levels in isolated lung endothelial cells (A, normalized to Cd31) and C166 cells (B, normalized to Odc) reveals a significant reduction of Vegfr2 transcripts when Smad4 is knocked down. C Representative western blot shows reductions in SMAD4 and VEGFR2 proteins in Smad4-iECKO isolated lung ECs compared to Smad4f/f controls. D Western blot quantifications verify decreased VEGFR2 protein levels in Smad4-depleted iLECs. E–F Immunofluorescent staining of IB4 (red) and VEGFR2 (green) on P7 Smad4f/f (n = 3) and Smad4-iECKO (n = 4) retinas indicates reduced expression of VEGFR2 protein in Smad4-depleted blood vessels. Scale bar represents 200 µm. Sample size (n) represents the number of independent biological samples.
pathogenesis versus those that are secondary effects of AVM formation.

It is also important to note that tamoxifen-inducible murine models of HHT have several limitations. HHT phenotypes arise in patients due to mutations (most commonly missense mutations) that lead to haploinsufficiency [10]. In contrast, mouse models of HHT often utilize null genetic backgrounds because loss of one allele of \textit{Alk1}, \textit{Eng} or \textit{Smad4} does not result in consistent presence of AVMs in predictable locations [21, 26, 53–55]. Furthermore, HHT
patients harbor germline mutations, which manifest from gestation and remain throughout adulthood. However, in mice, complete loss of \textit{Alk1}, \textit{Eng} or \textit{Smad4} during gestation results in embryonic lethality making it impossible to study their postnatal impact on HHT [16, 27, 33, 56, 57]. For this reason, the mouse retina has become an effective model to study AVM formation; the retinal vasculature forms directly after birth allowing researchers to assess developmental angiogenesis, similar to vessel growth that would be seen in a developing human. Although these models do not perfectly mimic the genetic background of HHT patients, retinal AVMs form at consistent rates and locations providing a reliable model to investigate the mechanisms of AVM formation.

In our \textit{Smad4-}iECKO retinas we noted delayed angiogenic outgrowth similar to \textit{Eng} mutants [17, 34], while \textit{Alk1} mutant retinas did not exhibit reduction in vascular outgrowth [20]. Interestingly, our \textit{Smad4-}iECKO mice exhibit a significant reduction in \textit{Eng} transcript levels but show no changes in \textit{Alk1} mRNA levels (Fig. 5A). This could account for the observed similarities in reduced vascular outgrowth between \textit{Smad4} and \textit{Eng}, but not \textit{Alk1} mice. However, this result also illustrates the complex association

### Table 1 Comparison of HHT mouse models

| Mutation          | \textit{Alk1} | \textit{Eng} | \textit{Smad4} |
|-------------------|---------------|--------------|---------------|
| Associated with   | HHT2          | HHT1         | HHT/JP        |
| Percentage of mutants with AVMs | 60% [20] | 70% [17] | 82% |
| Angiogenic delay  | No reduction [20] | Reduced [1, 34] | Reduced |
| Vessel size       |               |              |               |
| Artery size       | Enlarged [34] | Enlarged     | Enlarged      |
| Vein size         | Enlarged [17] | Enlarged     | Enlarged      |
| Proliferation     |               |              |               |
| Artery            | Increased [17, 34] | Increased     | Increased     |
| Vein              | Increased [20] | Increased [17], not changed [34] | Increased |
| Capillary         | Increased [17], not changed [34] | Increased     | Increased     |
| Cell size         |               |              |               |
| Smooth muscle coverage | Increased [20] | Increased [17] | Increased |
| Pericytes         | Decreased (only in capillaries) [20] | Increased | Decreased |
| Artery identity   |               |              |               |
| \textit{Dll4}     | No change [34]; not expressed in AVM | No change     | No change     |
| \textit{Ephrinb2} | Downregulated [77] | No change [17], downregulated [34] | No change     |
| \textit{Hey1}     | Downregulated [20, 24] | Downregulated [34] | Downregulated |
| \textit{Jagged1}  | Downregulated [20, 24] | No change [17]; increased [34] | No change     |
| \textit{Notch1}   | Downregulated [20, 24] | Downregulated | Downregulated |
| Venous identity   |               |              |               |
| \textit{Apj}      | No change [17]; upregulated [24] | No change (Expressed in AVM) [34] | No change; expressed in AVM |
| \textit{Ephb4}    | No change [24] | No change (Expressed in AVM) [34] | No change |
| VEGFR2 levels     | No change [24] | Altered VEGFA-induced kinetics [34] | Reduced expressed in AVM |
| Respiratory distress | 24–48 h post-Tx Inj [20] | 168–192 h post-Tx Inj |               |

This table combines data from references using the inducible, endothelial-specific Cre-driver line Cdh5-Cre\textsuperscript{ERT2}: Mahmoud et al. [17], Tual-Chalot et al. [20], Ola et al. [24], Jin et al. [34] and Poduri et al. [29]

\textsuperscript{a}Note that Poduri et al. [29] used a ubiquitous \textit{Rosa-Cre}\textsuperscript{ERT2} driver line. No in vitro data were included in this table.
between the TGFβ pathway and HHT, as Eng expression levels are reduced in the Alk1 mouse models of HHT [20, 24], yet show no changes in outgrowth. To our knowledge, it is unknown what happens to levels of Alk1 expression in the Eng HHT model, or whether Smad4 levels are affected in either Alk1 or Eng mouse models. Moving forward, it will be important to understand the association between Alk1, Eng and Smad4 in HHT because even though it is expected that all three cooperate in a linear manner in the TGFβ pathway, differences in phenotypes (Table 1) suggest this might not be the case.

The overall objective of our work was to develop a Smad4 model of HHT that could be used to identify the TGFβ targets that drive AVM formation, as almost nothing is known about these downstream effectors. To this end, we explored a possible link with the vascular endothelial growth factor (VEGF) signaling pathway that has been previously suggested in other HHT models [21, 24, 26, 34, 42, 53]. For instance, homozygous-induced deletion of Alk1 or Eng in adult mice requires the presence of exogenous VEGF before AVMs will form in the brain, suggesting that activation of the VEGF pathway is needed for AVM formation [21, 26]. To this end, VEGF neutralizing antibodies have been shown to prevent wound-induced skin AVMs from developing in Alk1-deficient mice [53]. Furthermore, in the absence of Alk1 and Eng, several studies have reported increased Vegfr2 expression and altered VEGFR2 kinetics in vitro [24, 34, 42]. In contrast, our data showed that loss of Smad4 led to a reliable and significant decrease in Vegfr2 expression both in vitro and in vivo (Fig. 6). This is consistent with a previous study on human patients with cerebral brain AVMs where there was a marked decrease in Vegfr2 expression [58]. Contrary to other HHT studies, the reduction of Vegfr2 in Smad4-iECKO mice could potentially be attributed to the downregulation in Nrp1, a VEGFR2 co-receptor. Studies have shown that decreased Nrp1 levels correlate with reduced Vegfr2 expression [59, 60]. Although other HHT studies did not find reduced Vegfr2 levels, homozygous deletion of both Smad4 and Vegfr2 produced similar results to those obtained in double Alk1- and Vegfr2-deficient retinas [24]. In each study, deletion of both alleles of Vegfr2 in the Alk1 or Smad4 null backgrounds resulted in inhibition of retinal vascular development, suggesting that appreciable loss of Vegfr2 in the absence of either Alk1 or Smad4 overrides HHT-like phenotypes because the vasculature is severely underdeveloped (Fig. 7). We did note that AVMs still formed in both experiments at fewer and similar rates in Alk1 and Smad4 mutants, respectively. However, in further studies we demonstrated that loss of a single Vegfr2 allele in the Smad4 mutant background led to an enhancement of vascular phenotypes associated with Smad4-iECKO retinas; the vascular front exhibited a consistent increase in density and AVMs showed a substantial enlargement. Alternatively, increased AVM size could be attributed to altered blood flow rates, hemodynamics forces and/or rates of oxygen diffusion caused by the overall stunted growth of the mutant blood vessels, rather than due to the loss of VEGFR2 directly. Future studies will be needed to understand how these processes are altered in TGFβ mutant backgrounds and how those contributions may affect severity of AVMs.

This SMAD4-VEGFR2 association is somewhat contrary to the clinical use of bevacizumab (also known as Avastin), which is a humanized anti-VEGF monoclonal antibody that sequesters VEGF to prevent it from binding both VEGFR1 and VEGFR2 subsequently hindering angiogenesis [61, 62]. Bevacizumab is currently used as a palliative therapy for HHT where it alleviates symptoms such as chronic nosebleeds but is not considered a long-term therapy [63]. Studies on the use of bevacizumab have been performed in mature vascular networks, namely that of adult humans and mice [64, 65]. Little information is known about the effects of bevacizumab in children or developing/remodeling vascular networks. Our work suggests that the connection between SMAD4 and VEGFR2 is different during developmental angiogenesis, when AVMs are thought to form, as compared to mature, established vascular networks. Therefore, further research on the effects of bevacizumab in developing vascular networks is needed, as our results indicate that bevacizumab may enhance developmental HHT phenotypes.

**Materials and methods**

**Mice**

All animal experiments were performed in accordance with Tulane University’s Institutional Animal Care and Use Committee policy. To create our Smad4-iECKO mouse model, we crossed an endothelial-specific, tamoxifen-inducible Cre-driver line (Tg(Cdh5-CreERT2)1Rha, further referred to as Cdh5-CreERT2) [30] with a conditional Smad4 mouse (Smad4fl) [31]. To confirm that Smad4 was being knocked out only in ECs, we mated Smad4-iECKO mice with a Rosa26-EYFP reporter mouse (Gt(Rosa)26Sor<sup>tm1(EYFP)Cos</sup>) [32]. Induction of tamoxifen was done using 0.075 mg tamoxifen (Sigma T5648) per gram of body weight on postnatal days 1 and 4. Note: For Vegfr2-iECKO mice only one injection of Tx was given on P1. For experiments, Smad4<sup>iECKO</sup>:Cdh5-Cre<sup>ERT2</sup> (otherwise referred to as Smad4-iECKO) mice were the experimental group, while Smad4<sup>iECKO</sup> littermates were used as controls. Genotyping primers and conditions can be found in supplemental methods.
Hematoxylin and eosin staining of murine lungs

Neonatal lungs were dissected from postnatal day 8 pups and fixed for 4 h in 4% PFA at 4 °C. The lungs were then embedded in paraffin and sectioned at 10 µm. Sections were washed in xylene twice then put through a rehydration series. Slides were placed into hematoxylin solution for ~ 1 min and then rinsed with water for several minutes. This process was repeated for eosin staining. Slides were then mounted with Permount (Thermo).

Retinal whole mount stains

Retinas were dissected and stained as previously described [66]. The following antibodies were used at a 1:100 concentration: aSMA (Sigma C6198), cleaved CASPASE-3 (Cell signaling 9661), COLLAGEN IV (Millipore AB756P), ENDOMUCIN (Santa Cruz 6415), ERG (Abcam 92513), GFP (Aves GFP-1020), KI67 (Cell Signaling 9449), NG2 (Millipore 5320), PECAM/CD31 (BD 553370), VEGFR2 (BD 555307). Additionally, the following immunofluorescent stains were performed according to the manufacturer’s instructions: Dapi (Life Technologies R37606), Isolectin-488 (Invitrogen 21411), Isolectin-594 (Invitrogen 21413), Isolectin-647 (Invitrogen 32450). Confocal images were taken at the same exposure settings for both mutant and control retinas, so fluorescent intensity could be compared.

In situ hybridizations

In situ hybridizations were performed as previously described [66]. In situ hybridizations were performed in batches where mutants and controls were subjected to the colorimetric reaction for the same period of time so that results could be compared. The following probes were synthesized from plasmids containing: Apelin (Dharmacon), Apj (Dharmacon), Ephb4, Notch4. Images were taken using a Leica M205 FA stereomicroscope.

Isolation of endothelial cells

Retinal and lung endothelial cell isolation were performed as previously described [67]. Briefly, tissue (either lung or retina) was digested in a collagenase/dispase solution and minced into fine pieces. After obtaining a single cell suspension, sheep anti-rat IgG dynabeads (Invitrogen 11035) coated with PECAM/CD31 antibody (BD 553370) were used to isolate endothelial cells. Cells were either used for RNA collection immediately or allowed to grow in EGM-2 medium for one week before being used for protein or RNA collection.

qPCR and analysis

All quantitative real-time PCR (qPCR) experiments were performed using RNA isolated with a GeneJET RNA Purification Kit (Thermo K0732) and quantified using a Nanodrop (Thermo). For each sample, 500–1000 ng of RNA was used for cDNA synthesis using a iScript cDNA Synthesis Kit (Bio-Rad). qPCRs were run using SYBR green mastermix (Thermo K0221) on a Bio-Rad CFX96 Touch Real-Time PCR Detection machine. Analysis was performed using the double delta Ct method, and statistics were generated using GraphPad Prism. For all qPCR experiments, three independent biological replicates were used and three technical replicates were performed per sample. Primers were verified for specificity and efficiency and can be found in supplemental materials.

Quantification of retinal images

All retina images were analyzed using Nikon NIS-Elements AR Analysis 64-bit software, and ImageJ software was used to measure vascular outgrowth, cell area and cell density.

Statistical analysis

GraphPad Prism software was used for all statistical analysis. For all statistics, sample size (n) indicates the number of independent biological samples. A minimum of three technical replicates was included per sample. For statistical analysis, we ran unpaired two-tailed Student’s t test where a p value of < 0.05 was considered significant.

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Compliance with ethical standards

Conflict of interest

We have no competing financial interests.

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