Pathogenesis of Arteriovenous Malformations in the Absence of Endoglin

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Rationale: Arteriovenous malformations (AVMs) result in anomalous direct blood flow between arteries and veins, bypassing the normal capillary bed. Depending on size and location, AVMs may lead to severe clinical effects including systemic cyanosis (pulmonary AVMs), hemorrhagic stroke (cerebral AVMs) and high output cardiac failure (hepatic AVMs). The factors leading to AVM formation are poorly understood, but patients with the familial disease hereditary hemorrhagic telangiectasia (HHT) develop AVMs at high frequency. As most HHT patients have mutations in ENG (endoglin) or ACVRL1 (activin receptor-like kinase 1), a better understanding of the role of these genes in vascular development is likely to reveal the etiology of AVM formation.

Objective: Using a mouse with a conditional mutation in the Eng gene, we investigated the sequence of abnormal cellular events occurring during development of an AVM.

Methods and Results: In the absence of endoglin, subcutaneous Matrigel implants in adult mice were populated by reduced numbers of new blood vessels compared with controls, and resulted in local venous enlargement (venomegaly). To investigate abnormal vascular responses in more detail, we turned to the more readily accessible vasculature of the neonatal retina. Endoglin-deficient retinas exhibited delayed remodeling of the capillary plexus, increased proliferation of endothelial cells and localized AVMs. Muscularization of the resulting arteriovenous shunts appeared to be a secondary response to increased blood flow.

Conclusions: AVMs develop when an angiogenic stimulus is combined with endoglin depletion. Moreover, AVM formation appears to result from the combination of delayed vascular remodeling and an inappropriate endothelial cell proliferation response in the absence of endoglin. (Circ Res. 2010;106:1425-1433.)

Key Words: mouse models of cardiovascular disease ■ angiogenesis ■ endothelial cells

Arteriovenous malformations (AVMs) are the most frequent cause of hemorrhagic stroke in young adults.1,2 The factors leading to AVM formation are unknown, but a number of inherited diseases leading to vascular malformations have now been identified and the causal mutations mapped.3 Of these familial disorders, hereditary hemorrhagic telangiectasia (HHT) patients have a strikingly high frequency of AVMs. Because HHT is most frequently associated with mutations in the endoglin or ACVRL1 (activin receptor-like kinase 1) gene,4,5 it is likely that these genes have important roles in preventing AVMs during normal development. HHT is an autosomal dominant disorder affecting approximately 1 in 10 000 people and is characterized by bleeding from small superficial AVMs (known as telangiectases) in the nose and gastrointestinal tract, as well as larger AVMs that may occur in major organs including lung, brain and liver.6 At present, there is limited understanding of how deficiencies in endoglin or ACVRL1 lead to disease pathology, although their role in transforming growth factor (TGF)β family signaling has been the subject of considerable investigation.7,8

Endoglin is an auxiliary receptor for members of the TGFβ family of ligands and is expressed primarily on vascular endothelial cells (ECs). It has no signaling kinase domain itself, but can promote TGFβ signaling through the ACVRL1 receptor to promote cell proliferation and migration.9,10 ACVRL1 signals by phosphorylating Smad1/5/8 transcription factors which then translocate to the nucleus to regulate expression of downstream genes.7 Recently, endoglin and ACVRL1 have been shown to respond to bone morphogenetic protein (BMP)9 and BMP10 ligands of the TGFβ family to promote endothelial cytostasis, even in the presence of angiogenic growth factors.11–13 How these different in vitro responses link to the normal role of these genes in averting AVM formation in development is not yet clear.
To address this issue, we, and others, have previously derived mouse models with endoglin mutations and found that Endoglin null embryos die halfway through gestation from developmental defects in the cardiovascular system. Mice that are heterozygous for endoglin-null mutations survive and model some features of HHT, but AVMs occur at an extremely low frequency. With the aim of developing a more reproducible model of AVM formation, and bypassing embryonic lethality of the endoglin null mouse, we have taken a conditional knockout approach combining our recently generated endoglin-floxed mouse model with an endothelial specific Cdh5(PAC)-CreERT2 transgenic line. As CreERT2 is inactive until exposed to tamoxifen, this combination of alleles allows endoglin depletion in ECs at different stages of development and adult life, permitting a high degree of manipulability with which to investigate the role of endoglin in vivo.

Methods

Mice

All animal experiments were performed under United Kingdom home office license, with approval from Newcastle University ethical review committee. The alleles and transgenes used have been previously described [Eng2fl/2fl; Cdh5(PAC)-CreERT2; Rosa26R, and the tamoxifen inducible EndoEC line, Cdh5(PAC)-CreERT2]. Endothelial-specific endoglin knockout (Eng-iKOe) mice were generated endoglin-floxed mouse model [18] with an endothelial specific Cdh5(PAC)-CreERT2 transgenic line. [19] As CreERT2 is inactive until exposed to tamoxifen, this combination of alleles allows endoglin depletion in ECs at different stages of development and adult life, permitting a high degree of manipulability with which to investigate the role of endoglin in vivo.

Results

Endoglin Depletion Leads to Reduced Angiogenesis and Venomegaly in Adult Mice

To determine the effect of endoglin loss on angiogenesis in vivo, we first used the subdermal Matrigel angiogenesis assay in adult mice. Tamoxifen treatment efficiently activated Cdh5(PAC)-CreERT2 in vascular ECs leading to lacZ expression in the presence of the Rosa26R Cre reporter allele (Online Figure I, B). Tamoxifen treatment also leads to loss of endoglin expression in Eng-iKO mice (Figure 1A through 1D). To investigate the effect of endoglin loss on angiogenesis in adult mice, a Matrigel plug, supplemented with angiogenic growth factors (VEGF and basic fibroblast growth factor), was injected subdermally into control and Eng-iKO mice and examined for neovascularization after 15 days. Reduced neoangiogenesis of the Matrigel plug was observed.
in Eng-iKO mice (Figure 1A through 1E), that was similar to a phenotype previously reported for Eng$^{+/}$ mice.\textsuperscript{23} However, an additional phenotype of venous enlargement adjacent to the Matrigel occurred to a far greater extent in Eng-iKO mice than in controls (Figure 1F through 1H). Vessels in the contralateral flank of Eng-iKO mice appeared normal, suggesting the excessive venous enlargement was a local response to growth factor-supplemented Matrigel, in the absence of endoglin. Venous enlargement was also accompanied by a significant increase in the number of venous ECs in Eng-iKO mice (Figure II) indicating this was not primarily a vasoregulatory response, rather this was a venomegaly. Reconstructed serial sections further illustrate this response (Online Figure II).

**Loss of Endoglin Leads to Retinal Vascular Abnormalities and Delayed Vascular Remodeling**

To investigate the abnormal vascular responses in the Eng-iKO mice during angiogenesis in a more readily visualized physiological setting, we examined vascular development in the neonatal retina. Development of retinal blood vessels in the mouse occurs in the first week of postnatal life to form a highly organized vascular plexus within a 2D plane. Blood vessels begin to form at the center of the retina and the vascular plexus grows outwards toward the periphery to generate a regular alternating pattern of arteries and veins with an intervening capillary network.\textsuperscript{24} Endoglin is expressed in all retinal blood vessels at P7 but expression is markedly higher in the veins and in the reorganizing capillaries ahead of the vein, but behind the tip cells (Figure 2A). In contrast, Acvrl1 was expressed fairly uniformly in ECs of retinal arteries, veins, and capillaries (not shown).

Figure 1. Angiogenesis-dependent venous enlargement in skin of adult Eng-iKO mice. A through D, Immunohistochemical staining for CD31 and endoglin on serial adult skin sections confirm that blood vessels in Eng-iKO show efficient loss of endoglin protein. The Matrigel-skin boundary is indicated by dashed lines (A through D) and the extent of migration of new blood vessels into the Matrigel is indicated by arrows (A and C). E, Neovascularization of Matrigel implants is significantly reduced in Eng-iKO mice ($n=6$) compared with control mice ($n=6$). $^{**}P=0.016$. F, Skin sections stained with hematoxylin/eosin from a control mouse show normal appearance of postcapillary venule (boxed area, and in digital zoom) adjacent to a small artery (a) running next to the Panniculus carnosus (pc). G, A similar section from an Eng-iKO mouse shows major venous enlargement (boxed area, and in digital zoom) adjacent to Matrigel plug. H, $t$ test of differences between average cross sectional area of venules in the presence and absence of Matrigel in control ($n=4$) and Eng-iKO ($n=6$) mice show that the combination of local angiogenic stimulation (Matrigel +VEGF/FGF) and endoglin loss leads to gross venous enlargement ($^{*}P=0.023$). I, $t$ test of differences between average endothelial cell number in venules, in the presence and absence of local angiogenic stimulation, show that the combination of local angiogenic stimulation and endoglin loss leads to an increase in venous EC number ($^{*}P=0.049$).

Tamoxifen treatment leads to Cdh5(PAC)-Cre\textsuperscript{ERT2} activation in retinal ECs at high efficiency to activate the Rosa26R reporter and to deplete endoglin protein levels (Figure 2B and 2C). The earliest defect observed in the Eng-iKO retinal vasculature was seen at P4 in the form of a dense capillary

Figure 2. Delayed progression of the capillary plexus in Eng-iKO retinas. A, Endoglin expression in normal retinas at P7 is higher in veins (v) than in arteries (a) and is also strongly expressed in the remodeling capillaries (*) behind the tip cells (arrows). B, Endoglin is efficiently knocked down in Eng-iKO retinal ECs following tamoxifen treatment. C, X-gal staining shows efficient Cdh5(PAC)-Cre\textsuperscript{ERT2} activation of the Rosa26R reporter in retinal blood vessels following tamoxifen treatment. D through G, Defects in capillary plexus of Eng-iKO retinas. Isoclinin staining shows that progression of the vascular plexus to retinal periphery is delayed in Eng-iKO at P4 (E) and at P9 (G) compared with age-matched controls (D and F). Dashed arrows indicate distance between edge of retinal plexus and edge of retina at P4 (D and E). By P9, the vascular plexus has reached the edge of the retina in controls (F) but is delayed in the Eng-iKO (G). Scale bars, 200 $\mu$m (A, B, D, and E) and 100 $\mu$m (F and G).
plexus with significantly reduced progression to the retinal periphery suggesting delayed remodeling and reduced migration of the newly formed capillaries (Figure 2D and 2E). This delayed progression continued as the retina developed such that at P9 the retinal plexus had reached the retinal periphery in controls, but not in Eng-iKO^e mice (Figure 2F and 2G). This difference was statistically significant (P<0.001) and was not attributable to altered retinal size in Eng-iKO^e neonates (Online Figure III).

There was a striking increase in vascular density in the peripheral region of the retinal plexus of Eng-iKO^e neonates (Figure 3A and 3B), which may have resulted from delayed remodeling of the plexus and which precluded accurate branch counts. However, the increased vascular coverage in the Eng-iKO^e mutants is clearly seen from quantification of isolecitin staining (Figure 3C). Furthermore the thickness of the capillary plexus at the growing front was significantly increased in mutants compared with controls (Figure 3D). To investigate whether the increased vascular density was attributable to an abnormally high branching phenotype, similar to Dil4 mutants, we examined filopodia in tip and stalk cells, but observed no difference between mutants and controls (Online Figure IV). Furthermore, the branching frequency and vascular density appeared similar in controls and mutants after the initial remodeling phase was complete, as seen in the central region of the plexus (Figure 3E through 3H). Overall this phenotype would be consistent with normal branching combined with delayed remodeling of neocapillaries in the plexus.

Abnormal Cell Changes During AVM Formation in Eng-iKO^e Retinas

Major arteriovenous malformations (AVMs) occurred in 70% of Eng-iKO^e retinas (Figure 4B through 4E) but were completely absent in controls (Figure 4A). In addition, ~20% of Eng-iKO^e retinas had bleeding AVMs (Figure 4D and 4E). The Eng-iKO^e retinas also showed an increase in venous caliber and enlargement of AVMs as the plexus developed. To determine whether the increase in AVM diameter was attributable to increased EC number we stained retinal vessels with VE-cadherin, a marker of EC junctions. This clearly revealed that the numbers of ECs lining the AVMs (Figure 5A and 5B) increased in larger AVMs, and that there was a significant increase in AVM diameter during the first week of postnatal development (Online Figure V, A), pointing to excess EC proliferation in these vessels. This was tested using BrdUrd labeling. In control retinas at P7, veins and peripheral capillaries show proliferating cells, whereas arteries are almost exclusively composed of nonproliferating cells (Figure 5C). In contrast, all AVMs, veins, and arteries and the entire capillary plexus show increased cell proliferation in the Eng-iKO^e retinas (Figure 5D and 5E), even in the central region of the plexus where the majority of vessels appeared normal (Online Figure V, B). To determine whether any of the proliferating vascular cells were endothelial, we performed detailed confocal analysis of retinas stained for CD31 and BrdUrd and confirmed that proliferating endothelial cells were present (Figure 5F; Online Figure VI). In light of the role of endoglin in regulating TGFβ family signaling, we anticipated ECs in Eng-iKO^e retinas might show altered Smad phosphorylation. However, we were unable to detect any changes in phospho-Smad1/5/8 or phospho-Smad2 staining in retinal ECs of Eng-iKO^e mice compared with controls. Similar results were obtained using both immunofluorescent staining of whole mount preparations and immunohistochemistry of retinal sections (Online Figure VII, A and B).

It has previously been proposed that AVMs may occur in response to loss of arterial and/or venous identity. To test arterial and venous identities in Eng-iKO^e retinas we first examined vessels for expression of Jagged-1 and ephrinB2 (known to be associated with arterial identity) and for EphB4 and the apelin receptor Aplnr (associated with venous identity). Jagged-1 and ephrinB2 expression were restricted to arteries in control and Eng-iKO^e retinas suggesting that arterial identity is maintained (Figure 6A through 6E). Also, veins in control and Eng-iKO^e retinas expressed EphB4 and Aplnr indicating that veins retained their molecular identity in the absence of Endoglin (Figure 6F through 6I).
hand AVMs appeared to be venous in nature as they expressed EphB4 and Aplnr, but not Jagged-1 or ephrinB2 (Figure 6D through 6F, 6H, and 6I).

Another widely used marker to identify neonatal retinal arteries is vascular smooth muscle cell coverage, recognized by immunostaining for aSMA, and which is absent from veins at this stage of development (Figure 7A and 7C). In the Eng-iKO\textsuperscript{e}, the smooth muscle organization is no longer arterial-specific and aSMA expression follows the pattern of predicted blood flow across the AVM and into veins on the “downstream” side of the shunt (Figure 7B and 7D). This pattern is seen from an early stage of AVM formation (eg, P6, Figure 7B), is maintained as the AVM progresses, and indicates that muscularization is a secondary response to increased blood flow.

**Discussion**

This study has revealed the importance of endoglin in endothelial cells in the remodeling of blood vessels during angiogenesis. Either exogenous or endogenous angiogenic stimuli promote vessel abnormalities in Eng-iKO\textsuperscript{e} mice, with the common feature of an abnormal increase in EC proliferation. In adult skin this effect was observed in the veins, whereas in the neonatal retina all vessels show a higher frequency of proliferating vascular cells, including endothelial cells, compared with controls. These findings are consistent with the role of endoglin and ACVRL1 in cytostasis in response to angiogenesis triggers and with data showing that endoglin null endothelial cells proliferate faster than control cells in culture. Our data are also consistent with a recent report showing that angiogenesis is required for AVM formation. AVMs in Eng-iKO\textsuperscript{e} retinas show delayed progression of the vascular plexus and abnormal interconnections between arteries and veins generating multiple AVMs (red arrows). Note also the increased caliber of major vessels compared with controls. One AVM in the boxed area is shown at higher power (C). Scale bars, 500 μm. D, Hemorrhage in a freshly dissected retina; boxed area corresponds to the AVM that can be seen in higher power after isolectin staining (E).

Figure 5. Increased vascular cell proliferation in Eng-iKO\textsuperscript{e} retinas. A and B, AVMs in Eng-iKO\textsuperscript{e} retinas immunostained for VE-Cadherin (red) reveals that the cellular footprint of individual ECs is similar in all AVMs, but a large AVM (B) has increased numbers of ECs compared with a small AVM at P5 (A). Topro3 is used to visualize nuclei (pseudocolored blue). C through E, BrdUrd staining reveals proliferating vascular cells (red nuclei) and collagen IV staining (green) is used to indicate the vascular basement membrane. Eng-iKO\textsuperscript{e} retinas at P7 (D and E) show more extensive vascular cell proliferation in arteries and capillaries than age-matched controls (C). AVMs are also associated with large numbers of proliferating cells (red arrows) (D and E). F, High-power confocal image of an AVM from a mutant retina stained for CD31 and BrdUrd reveals proliferating endothelial cells (yellow arrows) and proliferating perivascular cells (white arrows), as indicated by optical sections (see Online Figure VI). Scale bars, 10 μm (A and B); 100 μm (C through E); 20 μm (F).
development in the absence of Acvrl1.30 Similarly, increased EC proliferation has been reported in cranial vessels of zebrafish Acvrl1 mutants, and form lethal arteriovenous shunts in a process that is exacerbated by blood flow.31,32 Because the clinical phenotypes of HHT1 (endoglin) and HHT2 (ACVRL1) patients are closely related, it is likely that endoglin and ACVRL1 play a similar role in regulating vessel caliber during angiogenesis. Our evidence suggests endoglin plays a part in restricting endothelial proliferation during angiogenesis, consistent with a role in BMP9 signaling in vivo and that blocking this function results in an increased frequency of AVM formation. In addition, the importance of endoglin in regulating the vascular architecture appears to be angiogenesis-specific because we observed no effects in unstimulated Eng-iKOe adult skin vessels (Figure 1).

The role of endoglin is likely to be most important where it is more strongly expressed. In the retina this corresponds to veins and remodeling capillaries behind the tip cells. In the absence of endoglin we observed delayed progression of the retinal capillary plexus that did not appear to result from defects in branching per se. Capillary remodeling and extension is a process that is currently poorly understood,33 but in the absence of endoglin, a thickened capillary plexus formed that was slow to extend to the retinal periphery. One possible explanation for this is a defect in intercalation, a process in which endothelial cells “slide” past each other while maintaining cell–cell contact to extend the vascular tube with a lumen. A possible role for endoglin in intercalation was previously suggested because the cytoplasmic domain of endoglin is homologous to that of Piopio, a protein required for intercalation movements in the developing Drosophila tracheal network.34 If, in the absence of endoglin, a newly formed capillary fails to extend longitudinally by intercalation before the onset of blood flow, this leaves it vulnerable to formation of small opportunistic shunts once blood flow commences.

Figure 6. Arterial and venous identity in Eng-iKOe retinal vessels. Retinal blood vessels are identified by isoelectin staining (green, lower images) (C and D and G through I). A through D, Jagged-1 (red) is expressed strongly in endothelial cells of arteries but not veins of control (A and C) or Eng-iKOe mice (B). Jagged-1 is not upregulated in AVMs (D) at P7. E, In situ hybridization shows ephrin-B2 expression (black) in artery of Eng-iKOe retina but not in vein or AVM at P5. F, In situ hybridization shows Apnrl expression (black) in vein and AVM but not artery of Eng-iKOe retina at P5. G through I, EphB4 (red) is expressed by venous endothelial cells in control (G) and Eng-iKOe mice (H and I), and is also expressed in small and large AVMs (H and I) at P7.

Figure 7. Abnormal organization of vascular smooth muscle cells in Eng-iKOe retinal vessels. In control retinas, vascular smooth muscle cells, recognized by expression of aSMA (anti–aSMA-cy3, red), are associated with arteries (a) but not veins (v) at P6 (A) and P8 (C). Vascular smooth muscle cells appear at an early stage in AVM formation in Eng-iKOe retinas, seen here at P6 (B) and maintained at P8 (D); vascular smooth muscle cells support AVMs (large arrows) that are predicted to have increased blood flow (D). Scale bar, 100 μm (A and B) and 500 μm (C and D).
Previous work has shown that AVMs can be associated with abnormal Notch signaling which is critical for arterial and venous identity.\textsuperscript{35-37} Moreover, it has previously been proposed that disruption of arterial and venous identity also underlies AVM formation in HHT.\textsuperscript{26} However, expression of key molecular regulators of arterial and venous identity (Jagged-1, ephrin-B2, Apmlr and EphB4) is maintained in the arteries and veins of the retina in the absence of endoglin, indicating loss of arterial and venous molecular identity is not the main cause of AVMs in HHT.

Our data also indicate that increased blood flow promotes muscularization of the AVM, and this occurs from the earliest stages, suggesting muscularization is a sensitive response to increased shear stress and/or wall pressure. This is a well-recognized phenomenon used to generate arteriovenous fistula for kidney dialysis patients. In the context of HHT, the abnormal muscularization of AVMs and collecting veins seen in Eng\textsuperscript{-iKO}\textsuperscript{e} retinas parallels the increased number of smooth muscle cells associated with AVMs and postcapillary venules first reported in skin biopsies of HHT patients.\textsuperscript{38}

We were surprised that there was no detectable change in either pSmad2 or pSmad1/5/8 activity in Eng\textsuperscript{-iKO}\textsuperscript{e} retinal vessels. Both groups of Smads have previously been shown to be endoglin dependent using in vitro studies.\textsuperscript{9,39} However, the situation is more complex in vivo, as the effect of endoglin loss on pSmad1/5/8 activity in the pulmonary vasculature is seen in quiescent, but not angiogenic blood vessels.\textsuperscript{40} Although it is possible that the increased cell proliferation and angiogenesis defects observed in Eng\textsuperscript{-iKO}\textsuperscript{e} retinas are caused by abnormal Smad-independent responses, this becomes less likely when considered in the context of SMAD4 mutations causing HHT in a combined syndrome involving juvenile polyposis.\textsuperscript{41} Such events may parallel those occurring following overexpression of Smad7, which stimulates AVMs during angiogenesis in a chick model.\textsuperscript{42} We are currently investigating the in vivo signaling defects that occur in the absence of endoglin and are likely to underlie development of AVMs.

The results of our study raise the question of why AVM formation is frequent in HHT1 (ENG\textsuperscript{1+/+}) patients and occur in various tissue locations, including the retina,\textsuperscript{43,44} whereas AVMs are extremely rare in heterozygous Eng\textsuperscript{1/2} mice\textsuperscript{17} and become frequent only when endoglin is depleted during angiogenesis (Figure 4).\textsuperscript{17} There are (at least) 2 possible explanations. It has been proposed that AVMs may develop following a somatic mutation in the normal endoglin allele and that such mutations have sufficient time to accumulate during the lifetime of HHT1 patients, in contrast to the shorter lifespan of mice. Alternatively, inflammatory cytokines such as tumor necrosis factor-\alpha have been shown to trigger events leading to release of approximately 50% endoglin protein from the endothelial cell surface.\textsuperscript{45-47} Because endoglin levels are already reduced in HHT1 patients, loss of a similar amount of endoglin protein during inflammation may generate a transient and local endoglin-null phenotype (Figure 8). Our evidence supports the idea that local absence of endoglin, either attributable to somatic mutation or to a transient release of endoglin protein, would lead to abnormal vessel remodeling, but only in the presence of angiogenic stimuli (Figure 8).

In support of this hypothesis, we have previously shown that inflammation was associated with bleeding vascular lesions in Eng\textsuperscript{1/-} mice.\textsuperscript{17} As yet, there is no evidence for somatic mutations in HHT1 patient AVM lesions,	extsuperscript{49} but further screening is probably required before this possibility can be completely excluded.

In addition, our findings may be relevant to the occurrence of sporadic AVMs because, recently, these have been causally associated with local increases in soluble endoglin.\textsuperscript{49} Reduced endoglin activity in endothelial cells would result if soluble endoglin depletes the amount of circulating ligand locally available. However, our data suggest that sporadic AVMs resulting from reduced endoglin activity in this way would only form in the context of physiological or pathological angiogenesis and that antiangiogenic therapy may be protective in patients at high risk of developing AVMs.
Acknowledgments
We thank Dr P. Avery for statistical advice and N. Hamilton, L. Hodgson, R. Hussain, and S. Smith for technical support.

Sources of Funding
This research was supported by the Wellcome Trust, British Heart Foundation, Cookson Trust, and Borwick Trust. Z.Z. was supported by an Overseas Research Student Award, and H.M.A is supported by a senior basic science research fellowship from the British Heart Foundation.

Disclosures
None.

References

Novelty and Significance

What Is Known?

- Arteriovenous malformations (AVMs) cause a wide range of clinical pathologies.
- Causes and pathophysiology of AVMs are not yet understood.
- High AVM frequency in patients carrying endoglin mutations suggests that endoglin is important in preventing AVMs.

What New Information Does This Article Contribute?

- AVMs develop in an endoglin-deficient mouse model in an angiogenesis-dependent manner.
- Loss of arterial and venous identity of major vessels is not a prerequisite for AVM formation.
- Increased endothelial cell proliferation underlies AVM development in this model.

The aim of this study was to gain an improved understanding of how AVMs occur when endoglin levels are reduced in endothelial cells. Endoglin is an auxiliary receptor for the TGFβ family of cytokines, which have an important, but poorly understood, role in vascular development. Patients with the inherited disorder, hereditary hemorrhagic telangiectasia type I (HHT1), carry endoglin mutations, resulting in haploinsufficient endoglin levels that predispose them to a high burden of AVMs. Furthermore, spontaneous AVMs (in non-HHT patients) may be associated with increased levels of soluble endoglin, which is known to be released during inflammation. To better understand AVM formation, we used an inducible endothelial-specific endoglin knockout mouse to follow AVM development in the neonatal retinal plexus. Our observations help to elucidate the abnormal cellular changes that occur during AVM development. In the absence of endoglin, extension of the vascular plexus is delayed, in combination with an increase in endothelial cell proliferation. This generates small arteriovenous shunts that enlarge over time. As this work may be relevant to sporadic as well as familial (HHT1) AVMs, a better understanding of how AVMs form in vivo brings forward the likelihood of effective therapies for a broad group of AVM patients.
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Circ Res. 2010;106:1425-1433; originally published online March 11, 2010; doi: 10.1161/CIRCRESAHA.109.211037

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**Online Figure I**

**A**, Summary diagram showing how tamoxifen treatment of *Cdhl(PAC)-Cre^{ERT2} Eng^{2fl/2fl}* mice leads to deletion of endoglin exons 5 and 6.

**B**, X-Gal staining shows efficient Cre^{ERT2} activation in adult dermal blood vessels following tamoxifen treatment of *Cdhl(PAC)-Cre^{ERT2} Rosa26R* mice. Blue color represents endothelial expression of lacZ resulting from Cre activation of the Rosa26R allele^{20}. Abbreviations: a, artery; v, vein; c, capillaries.
Online Figure II

3D reconstructions of veins (blue) and arteries (red) from serial sections of adult mouse skin. Images show how the reconstructions are based on individual sections and the lowest section has been kept for illustration purposes. The position of the epidermis (e) is marked for orientation.

A shows a parallel vein and artery close to the Panniculus Carnosus (pc) in normal skin from a control mouse.

B shows an enlarged vein running parallel to an artery from an equivalent region of skin but close to matrigel (m) in an Eng-iKOe mouse. Slight imperfections in the serial sectioning lead to the small ‘gap’ seen here in the artery.

Approximately 60 serial 5µm sections were reconstructed in each case using Amira 4.0 software.
Online Figure III Relative progression of the capillary plexus was significantly reduced in Eng-iKOe retinas at P6, P7 and P8 compared with controls without change in retinal size. The relative distance covered by the vascular plexus was calculated as a ratio of the vascular radius, indicated by the blue arrows (A,B), and the retinal radius, indicated by the red arrows (A,B) in 4 separate regions for each retina. The size of each retina was calculated as the mean of the retinal radii and individual values are plotted together with mean values +/- standard error (** P<0.001). Numbers of neonates analysed are P6 controls (n=3); P6 Eng-iKOe (n=8); P7 controls (n=4); P7 Eng-iKOe (n=11); P8 controls (n=4); P8 Eng-iKOe (n=12).
**Online Figure IV** Endoglin (CD105) is expressed in tip cells and in tip cell filopodia (A, arrows), that are also seen with isolectin staining (B, C, arrows). When endoglin protein is depleted in Eng-iKO e retinas (D), the tip cells appeared to have normal filopodia (E, F, arrows). The numbers of filipodia in tip and stalk cells appears normal in Eng-iKO e retinas compared with controls and representative images of P7 retinas are shown (G,H).
Online Figure V

A, Enlargement of AVMs during development of the retinal plexus. The diameter of AVMs in the retinal vasculature of Eng-iKO<sup>e</sup> pups at P4, P6, P7 and P9 show a statistically significant increase between P4 and P9.

B, Increased vascular cell proliferation in Eng-iKO<sup>e</sup> retinal blood vessels compared with controls. Vascular BrdUrd-positive nuclei were counted in 5 fields of view (using a 10X objective) in the central region of the retina where vascular remodelling appeared similar to controls. Counts were normalised to isolectin positive area per field of view and plotted as mean values plus SEM. An unpaired students t test showed that vascular cell proliferation is significantly increased in P6 Eng-iKOe retinas (n=4) compared with controls (n=3).
Online Figure VI

Proliferating endothelial cells are seen in confocal images of an AVM using serial Z slices of 0.5 µm thickness. P7 retinal whole mount preparations were stained for endothelial cells with CD31 and for proliferating nuclei with BrdU. Proliferating endothelial cells are indicated by yellow arrows. Proliferating cell nuclei on the periphery of the vessel that have no surrounding cellular CD31 staining are likely supporting muscle cells (white arrows). The vessel lumen (lu) is visible in the final 3 optical slices.
No differences in pSmad2 or pSmad1/5/8 levels were observed in vascular endothelial cells of Eng-iKOe retinas compared with controls. Confocal images of isolectin and pSmad1/5/8 or pSmad2 stained retinal vessels are shown with nuclear stain To-pro3 to indicate endothelial nuclear location (A-D).
No differences in pSmad2 or pSmad1/5/8 levels were observed in vascular endothelial cells of Eng-iKOe retinas compared with controls. Immunohistochemistry of pSmad1/5/8 and pSmad2 in retinal sections confirm the nuclear location (E-H). High power images of vessels (red box) are shown as inset images. Retinal neuronal cells strongly express pSmad2 and pSmad1/5/8 (asterisks).