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Reduced angiogenic responses in adult endoglin heterozygous mice

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Abstract

Objective: To determine if angiogenesis is altered in adult *Endoglin* heterozygous ($Eng^{+/-}$) mice, the animal model for the vascular disorder hereditary hemorrhagic telangiectasia type 1 (HHT1).

Methods: Primary cultures of endothelial cells were generated from $Eng^{+/-}$ and $Eng^{+/+}$ mice and analyzed for proliferation, migration, and ability to form capillary-like tubes. Endothelial cells derived from umbilical veins of newborns (HUVEC) with an HHT1 genotype were also tested for capillary formation. Two in vivo models of angiogenesis were tested in the $Eng^{+/-}$ and $Eng^{+/+}$ mice: Matrigel implant-dependent angiogenesis and reperfusion following hindlimb ischemia.

Results: The $Eng^{+/-}$ endothelial cells displayed significantly reduced proliferation and migration, increased collagen production, and decreased NO synthase expression and vascular endothelial growth factor (VEGF) secretion. They also showed impaired capillary tube formation in vitro, as did the HHT1 HUVEC. These endothelial cell-specific abnormalities were associated with impaired Matrigel-dependent capillary tube formation in vivo and delayed reperfusion following hindlimb ischemia.

Conclusions: Although vascular development is normal in $Eng^{+/-}$ mice, angiogenic abnormalities were observed in the adult mice and their isolated endothelial cells. These results suggest that a normal level of endoglin is required for full angiogenic activity.

Keywords: Angiogenesis; Endoglin; HHT1; Nitric oxide; VEGF

This article is referred to in the Editorial by Maio et al. (pages 781–783) in this issue.

1. Introduction

Hereditary hemorrhagic telangiectasia (HHT) is an autosomal dominant vascular disorder characterized by focal telangiectases and arteriovenous malformations (AVMs). Mutations in the genes encoding endoglin (*ENG*) and activin-like kinase (*ALK1*) are associated with HHT1 and HHT2, respectively. Haploinsufficiency is the disease model for HHT as mutant proteins are generally not expressed or are non-functional (see review by Abdalla and Letarte [1]).

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Endoglin (CD105) is a 180-kDa homodimeric transmembrane glycoprotein expressed mainly in endothelial cells and known to modulate cellular responses to ligands of the transforming growth factor-β (TGF-β) superfamily [2,3]. Endoglin is up-regulated in tissues undergoing angiogenesis and in vitro inhibition of its expression on endothelial cells impairs this process [4]. *Eng*-null mice die at mid-gestation from defective angiogenesis and severe cardiovascular abnormalities, while *Endoglin* heterozygous ($Eng^{+/-}$) mice have normal life spans, but are predisposed to develop HHT-like vascular abnormalities [4–7]. We also demonstrated that endothelial nitric oxide (NO) synthase (eNOS) expression was reduced and that NO synthesis was impaired in these mice [8,9]. Endoglin has been found in endothelial caveolae, where it associates with eNOS and modulates its activation by promoting eNOS/Hsp90 association [9]. Endoglin heterozygous cells (human and murine) not only have reduced eNOS levels, but show uncoupled eNOS activity and generate eNOS-derived superoxide during agonist-induced activation [9]. However, the characterization of endoglin-dependent endothelial mechanisms, necessary to support normal angiogenesis and whether or not these are altered in $Eng^{+/-}$ mice, a physiologically and clinically relevant model of HHT1, remain to be elucidated.

We report that isolated murine $Eng^{+/-}$ endothelial cells display reduced proliferation and migration, impaired capillary tube formation and reduced eNOS activity and VEGF secretion. These changes were associated with decreased blood vessel formation in in vivo models of angiogenesis, indicating that endoglin contributes to normal adult angiogenesis.

2. Materials and methods

2.1. Mice

All procedures conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). $Eng^{+/-}$ and $Eng^{+/+}$ mice were sex- and age-matched 8- to 12-week-old N3–N5 C57BL/6 backcrosses.

2.2. Isolation and characterization of endothelial cells

Mouse aortic endothelial cells (MAECs) from $Eng^{+/+}$ and $Eng^{+/-}$ mice were isolated and cultured up to 4 passages as previously described [8]. The identity and purity of the cells were determined by indirect immunofluorescence using monoclonal antibodies (mAb) to CD31 (Santa Cruz), von Willebrand factor (Sigma) and endoglin (MJ178), followed by Cy3 or Alexa Fluor 488-conjugated secondary antibodies (Jackson Immunoresearch) [10]. Cells were visualized under a Zeiss fluorescence microscope equipped with a digital camera and analyzed using Openlab (Improvision, Inc.). Human umbilical vein endothelial cells (HUVECs) were derived from newborns with an *ENG* mutation, or from control babies. These cells were characterized and maintained in complete M199 medium supplemented with 10% FBS and 30 μg/ml endothelial mitogen. Samples from HHT1 families were tested by protein and mutation analysis as described.

2.3. Proliferation and migration assays

MAECs were plated at 5×10^3 cells/well and proliferation assessed by counting the number of viable cells, determined by crystal violet nuclei staining, over 8 days. Cell cycle analysis was evaluated by flow cytometry.

Confluent monolayers of $Eng^{+/+}$ and $Eng^{+/-}$ MAECs were wounded using a sterile pipette and the extent of wound closure was determined over 24 h by calculating migrated distance/total wound distance using digital microscopy. Endothelial cell migration was also assessed by the Boyden Chamber assay, used in the bottom chamber medium with 2% (negative control) or 10% serum. After 12 h, inserts were washed with PBS and migrated cells fixed with 10% glutaraldehyde, stained with 2% crystal violet, treated with 10% acetic acid and quantified at 595 nm.

2.4. Collagen synthesis and VEGF secretion

MAECs were grown to 70–80% confluence and serum-starved for 24 h. Cells were grown in the presence and absence of TGF-β1 (1 ng/ml) or in the presence of anti-TGF-β1 antibody (100 ng/mL; R&D Systems, Minneapolis, MN, USA). The collagen content in the medium was quantified by measuring [^{3}H]-proline incorporation over a period of 24 h [10]. Media VEGF accumulation over a 24-h period was determined by ELISA (Quantikine™, R&D Systems) and normalized for DNA content.

2.5. Western blot

Tissue or cell protein extracts were prepared in 10 mM Tris buffer containing 1% Triton X-100 supplemented with protease inhibitors (Roche). Protein extracts were quantified by the Bradford method and 20 μg were fractionated by SDS–PAGE, transferred onto nitrocellulose and probed with a rabbit polyclonal antibody to eNOS (1:1000, Santa Cruz Biotechnology) or rat monoclonal anti-endoglin (MJ7/18). Protein levels were normalized to α-tubulin levels.

2.6. In vitro and in vivo Matrigel assays

$Eng^{+/+}$ and $Eng^{+/-}$ MAECs or HUVECs derived from newborns with different *ENG* mutations or from control newborns ($n = 6$/group) were plated at 5×10^4 cells/well in 6-well plates coated with 0.5 mL of growth factor free Matrigel™ (Becton Dickinson). After 6 h, images were taken at 20× magnification using an inverted microscope and a digital camera; capillary tube number, area and wall width were quantified using Openlab software (Improvision Inc.).

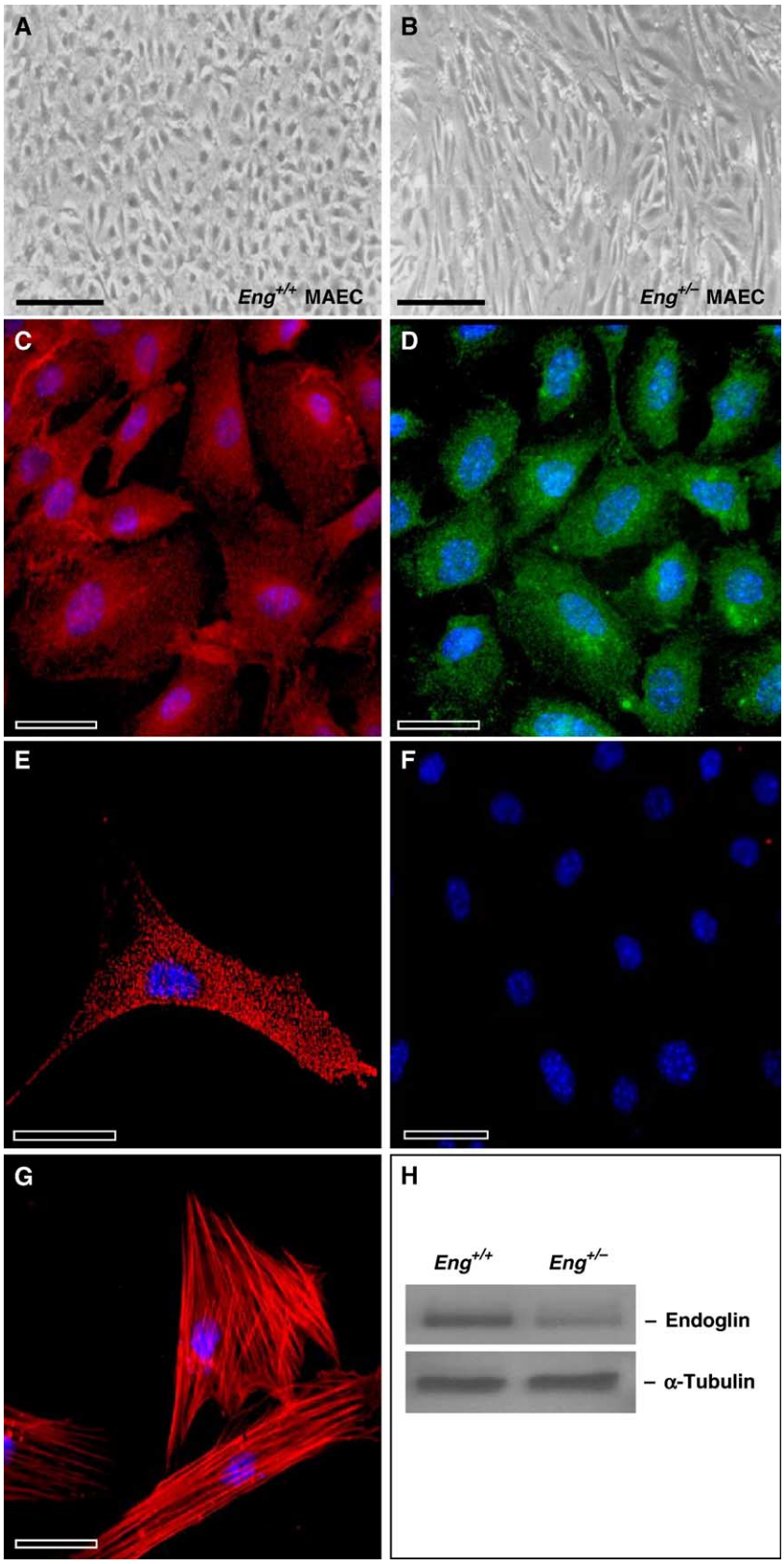

Fig. 1. Characterization of primary mouse aortic endothelial cells (MAEC) cultures: (A) confluent monolayers of cells from $Eng^{+/+}$ mice show a cobblestone appearance while (B) those from $Eng^{+/-}$ mice display an elongated morphology. Scale bar=50 μm. Immunofluorescence analyses of $Eng^{+/+}$ cells indicate the presence of endothelial markers: (C) von Willebrand factor, (D) PECAM-1 and (E) endoglin. (F) αSM-actin was not detectable in $Eng^{+/+}$ cells but (G) was observed in rat smooth muscle cells (scale bar=5 μm). (H) Western blot analysis shows reduced endoglin levels in $Eng^{+/-}$ compared to $Eng^{+/+}$ MAECs; α-tubulin was used as a loading control.

Separately, $Eng^{+/-}$ mice and $Eng^{+/+}$ littermates ($n=4$/group) were anaesthetized with ketamine/xylazine (100/10 mg/kg i.p.) and 0.4 mL of Matrigel™ (with growth factors) was injected into the subcutaneous space along the dorsal midline. Mice were sacrificed 12 days later and Matrigel plugs were carefully removed. Samples were fixed in 4% paraformaldehyde, paraffin embedded, cross-sectioned and stained with haematoxylin/eosin. Sections were analyzed

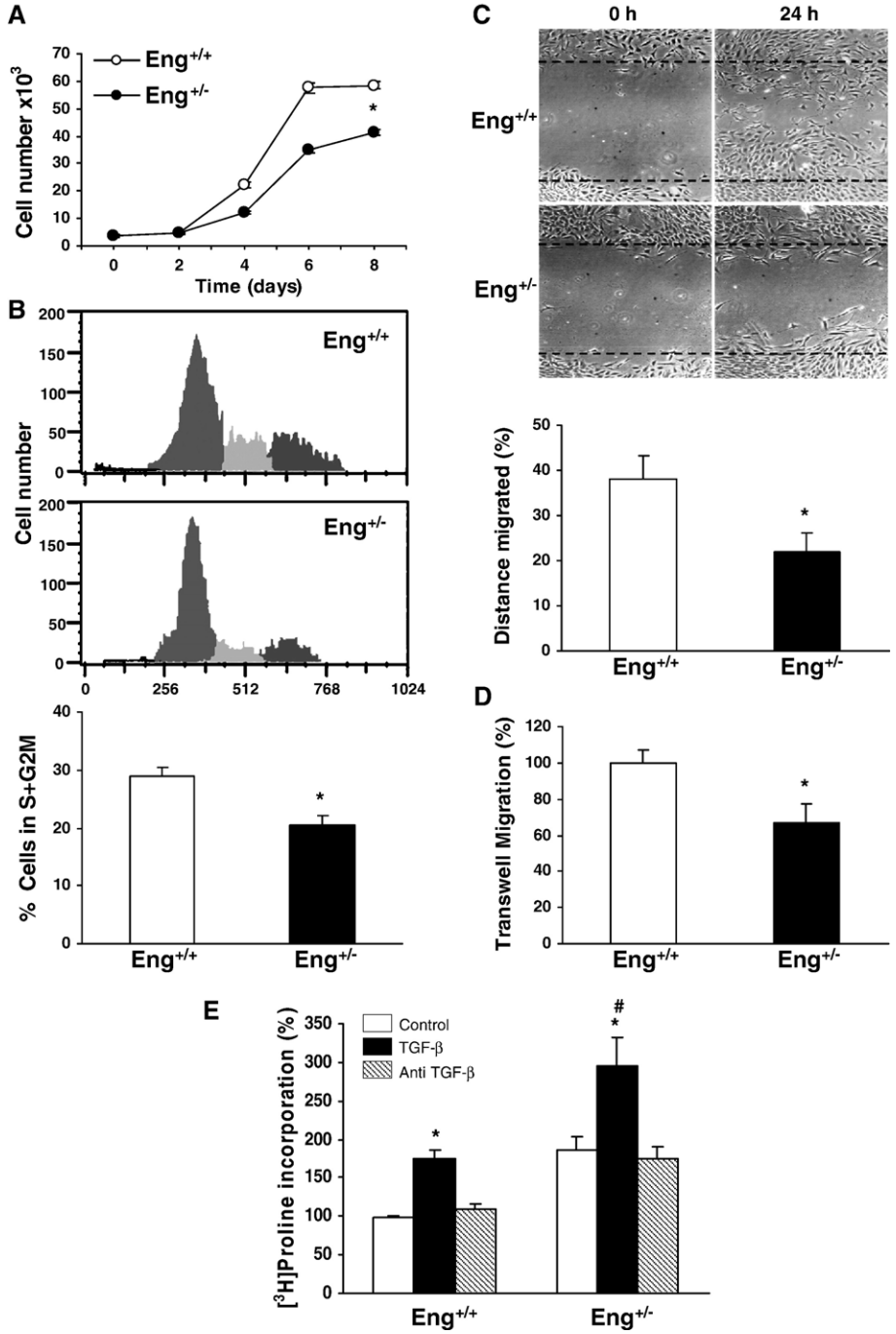

Fig. 2. Decreased proliferation and migration in $Eng^{+/-}$ cells. (A) Cell number estimated over a period of 8 days was reduced in $Eng^{+/-}$ vs. $Eng^{+/+}$ MAECs (*$P<0.01$). (B) Representative flow cytometry profiles and graph showing a smaller proportion of $Eng^{+/-}$ MAECs in S+G2M phases of the cell cycle (*$P<0.01$, vs. $Eng^{+/+}$). (C) Representative images and graph showing the decreased migration from a wound edge of $Eng^{+/-}$ MAECs (*$P<0.01$ vs. $Eng^{+/+}$; $n=6$/group; scale bar=200 μm). (D) Reduced transwell migration of $Eng^{+/-}$ MAECs, measured in a Boyden chamber (*$P<0.01$ vs. $Eng^{+/+}$; $n=6$/group). (E) Basal (†) and TGF-β1 (50 pM for 24 h) – stimulated collagen synthesis (#), quantified by [^{3}H]-proline incorporation, were increased in $Eng^{+/-}$ MAECs ($n=8$/group; $P<0.01$ vs. $Eng^{+/+}$). In both groups, collagen synthesis was stimulated by TGF-β1 (*$P<0.01$ vs. untreated), but basal levels were not affected by incubation of the cells for 24 h with an anti-TGF-β antibody.

using Openlab software and quantified for the number of structures having circular or ellipsoid shapes with a visible lumen with or without blood.

2.7. Hindlimb ischemia and reperfusion

Eng$^{+/+}$ and *Eng*$^{+/-}$ mice were anaesthetized with isoflurane and the left femoral artery was ligated 2–3 mm distal to the inguinal ligament. In some animals, NO synthesis was inhibited with 1 mM N^G-nitro-L-arginine methyl ester (L-NAME; Sigma) in the drinking water. Hindlimb perfusion was measured every other day up to 28 days by laser Doppler (Periflux 2B, Perimed) using a 0.45-mm probe, and the results are expressed as the ratio of perfusion in the ischemic versus non-ischemic limb. Perfusion studies have been completed in 10 untreated animals per group and in 6 animals per group in mice treated with L-NAME. Four days after femoral ligation, groups of animals (6 per group) were anaesthetized with isoflurane and the adductor muscle rapidly excised from both ischemic and non-ischemic limbs, rinsed in PBS and snap-frozen in liquid nitrogen for total protein and RNA extractions.

2.8. Northern blot

Total RNA was isolated from tissues by phenol/chloroform extraction. About 20 μg was fractionated on a 2% formaldehyde agarose gel, transferred onto a nylon membrane, probed with random ^{32}P-labeled fragments to murine endoglin (938 bp, GenBank Accession No.: X77952). The 28S ribosomal RNA (700 bp) served as loading control and specific bands were visualized by radioautography.

2.9. Statistical analysis

Between-group comparisons were performed by ANOVA and corrected for repeated measures when appropriate. If ANOVA revealed overall significant differences, individual means were evaluated post hoc using Bonferroni's procedure. Results are expressed as the mean ± S.E.M.

3. Results

3.1. Characterization of *Eng*$^{+/+}$ and *Eng*$^{+/-}$ MAECs

Endothelial sprouts from 4 to 6 aortic rings per mouse were first visible after 9 days and grew into a homogeneous population as observed by flow cytometry analyses of size and granularity (data not shown). In culture, *Eng*$^{+/+}$ MAECs formed typical cobblestone monolayers (Fig. 1A), whereas *Eng*$^{+/-}$ MAEC showed a more elongated morphology (Fig. 1B). Immunofluorescence staining of *Eng*$^{+/+}$ MAEC revealed von Willebrand factor, CD31 and endoglin expression, confirming their endothelial phenotype (Fig. 1C, D and E). α-smooth muscle actin was absent in MAEC cultures (Fig. 1F) while readily detectable in rat smooth muscle cells (Fig. 1G). MAECs from *Eng*$^{+/-}$ mice showed the same positive CD31 and von Willebrand factor staining patterns than those from Eng$^{+/+}$ mice and no α-smooth muscle actin staining (data not shown). However, Western blot analysis showed reduced endoglin levels corresponding to 50% of those found in *Eng*$^{+/+}$ cells (Fig. 1H).

3.2. *Eng*$^{+/-}$ MAECs display reduced proliferation and migration and increased collagen synthesis

Endothelial cell proliferation and migration are key early events in angiogenesis. The effects of endoglin heterozygosity on cellular proliferation were tested by determining the increase in cell number during 8 days of growth in complete medium. *Eng*$^{+/-}$ MAECs proliferated significantly slower than *Eng*$^{+/+}$ MAECs reaching a 9-fold increase in total cell count by day 6, compared to a 16-fold increase for *Eng*$^{+/+}$ cells (Fig. 2A). Flow cytometry analysis revealed a

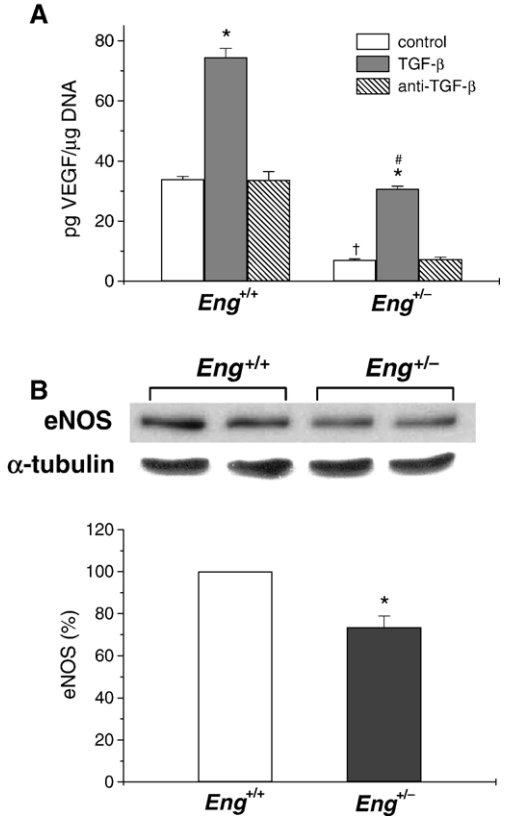

Fig. 3. Decreased VEGF and eNOS in *Eng*$^{+/-}$ cells. (A) When quantified by ELISA and normalized for DNA content, the basal (†) and TGF-β1 stimulated VEGF secretion (#) were lower in *Eng*$^{+/-}$ MAECs ($n=6$/group; $P<0.01$ vs. *Eng*$^{+/+}$). In both groups VEGF secretion was increased by TGF-β1 (*$P<0.01$), whereas basal levels were not affected by pre-treatment of cells for 24 h with an anti-TGF-β antibody. (B) Representative Western blot and summary of data showing reduced eNOS levels in *Eng*$^{+/-}$ MAECs relative to *Eng*$^{+/+}$ MAECs (*$P<0.01$; $n=6$/group). eNOS levels were corrected using α-tubulin levels.

greater percentage of $Eng^{+/-}$ cells arrested in G0/G1 (76±2%) compared to $Eng^{+/+}$(68±2%) cells. The proportion of cells in S+G2M phases was significantly lower in $Eng^{+/-}$ (20%) than in $Eng^{+/+}$ cells (29%) (Fig. 2B).

Endothelial cell migration was analysed by two independent techniques, which yielded similar results. $Eng^{+/-}$ MAEC confluent monolayers when damaged by a scratch wound assay migrated a significantly shorter

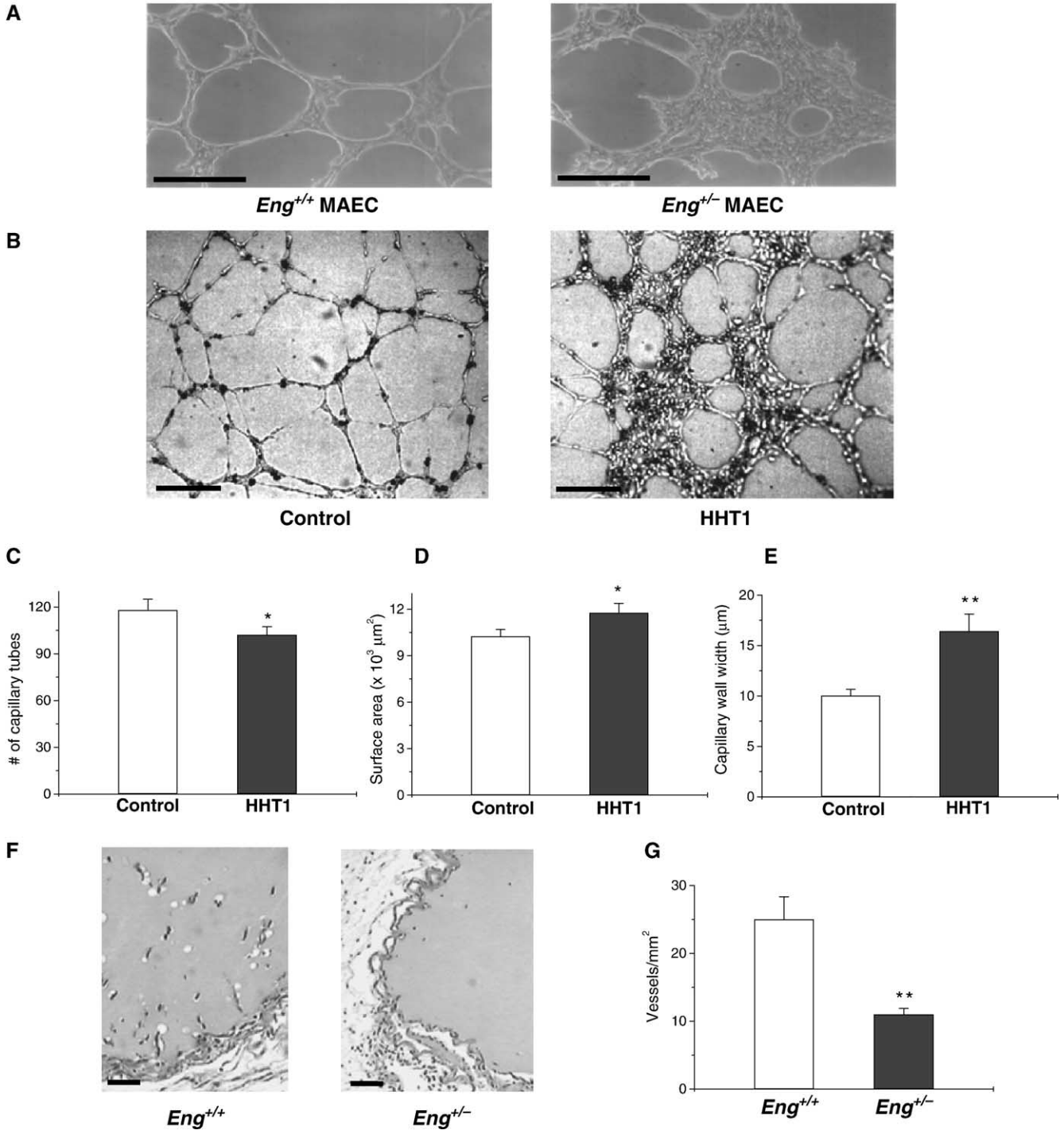

Fig. 4. Impaired capillary formation in endothelial cells with reduced endoglin expression. (A) Representative images showing impaired capillary network formation on Matrigel™ by $Eng^{+/-}$ MAECs and (B) HUVEC with an *ENG* mutation ($n=6$) and control HUVECs. (C) These HHT1 HUVEC formed fewer capillary tubes (*$P<0.05$) with (D) greater luminal areas (*$P<0.05$) and (E) wall widths (**$P<0.01$) compared to control HUVECs. (F) Matrigel plugs implanted dorsally into mice were removed 12 days later, sectioned and stained with hematoxylin/eosin. Representative images reveal decreased cellular infiltration and blood vessel formation in $Eng^{+/-}$ mice. (G) Analysis of sections showed a lower number of blood vessels in Matrigel plugs from $Eng^{+/-}$ mice relative to $Eng^{+/+}$(**$P<0.01$). Scale bar=50 µm for all panels.

distance, covering 21.9±4.1% of the total wound versus 38.2±5.1% for $Eng^{+/+}$ cells (Fig. 2C). Similarly, the number of migrated cells assayed using a Boyden chamber was significantly lower in the $Eng^{+/-}$ than in $Eng^{+/+}$ MAECs (Fig. 2D).

Several in vitro studies have demonstrated that endoglin expression regulates extracellular matrix (ECM) production. Increased collagen secretion is an important step in the resolution phase of angiogenesis, but may have inhibitory effects on endothelial migratory function during the

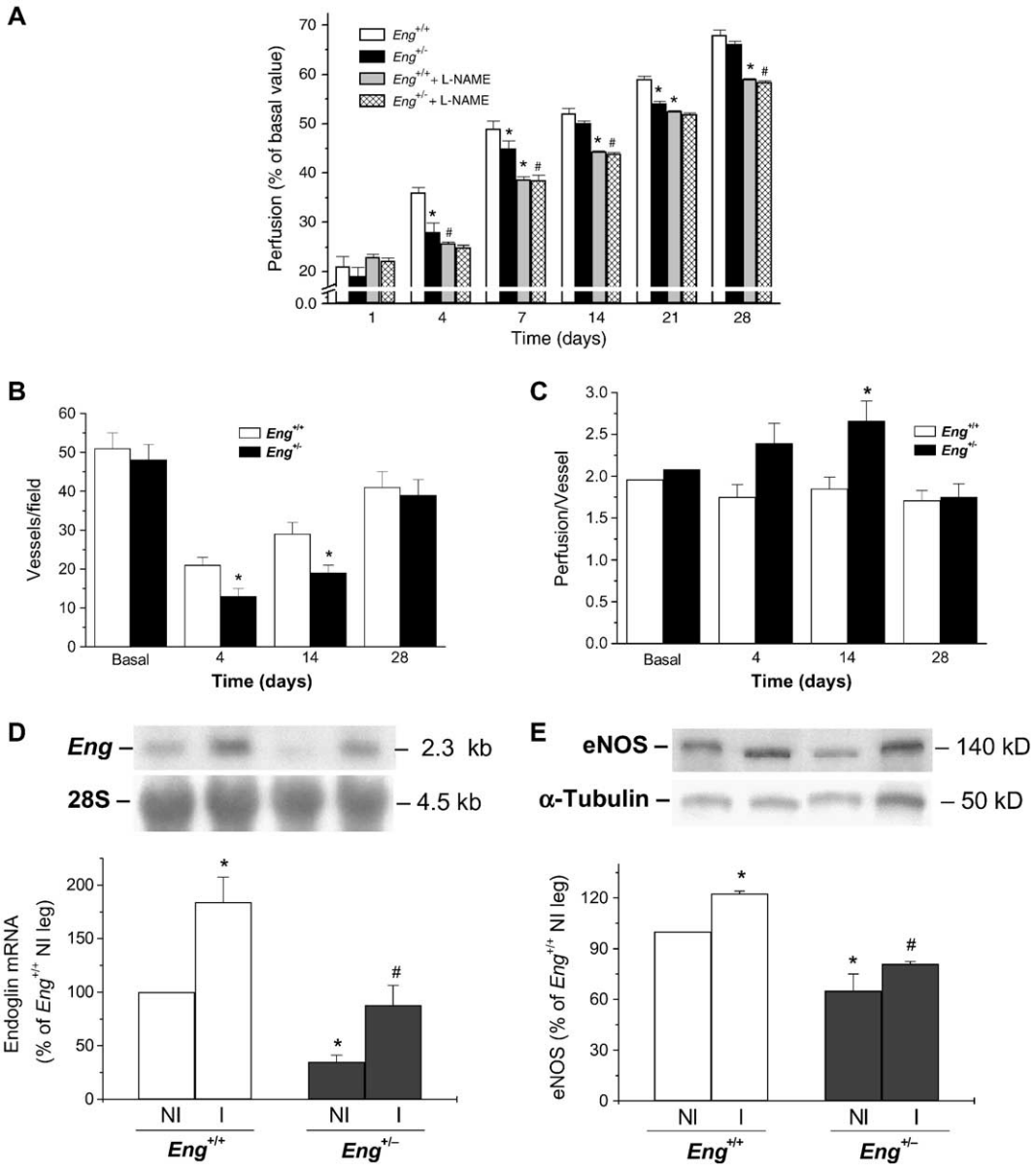

Fig. 5. The ratio of ischemic to non-ischemic limb reperfusion following femoral artery ligation was measured by laser Doppler flow analysis in $Eng^{+/+}$ and $Eng^{+/-}$ mice, treated with or without L-NAME. (A) Untreated $Eng^{+/-}$ mice display lower reperfusion compared to untreated $Eng^{+/+}$ littermates (*$P<0.05$). L-NAME impaired reperfusion in the $Eng^{+/+}$ mice (*$P<0.05$ vs. untreated group) and in the $Eng^{+/-}$ mice ($^{\#}P<0.05$ vs. untreated $Eng^{+/-}$ group). No differences were observed between the L-NAME-treated groups. Each data point is a mean obtained from 10 animals per group in the untreated $Eng^{+/+}$ and $Eng^{+/-}$ mice and 6 animals per group in mice treated with L-NAME. (B) Adductor muscle vascularity, estimated from the number of $CD31^{+}$ vessels/mm^2, was reduced in $Eng^{+/-}$ mice after femoral artery ligation (*$P<0.05$ vs. $Eng^{+/+}$ group; $n=5$ mice with 5 sections each). (C) The calculated rate of perfusion/vessel showed an increased trend in $Eng^{+/-}$ mice 4 days after ligation and was significantly higher at 14 days (*$P<0.05$ vs. $Eng^{+/+}$; $n=5$ mice/group). (D) Representative Northern blot and summary of endoglin mRNA levels in non-ischemic (NI) and ischemic (I) adductor muscles 4 days post femoral artery ligation. Levels were reduced in $Eng^{+/-}$ NI muscles (*$P<0.05$ vs. NI $Eng^{+/+}$) and proportionally increased following ischemia, maintaining the difference between groups ($^{\#}P<0.05$ for I $Eng^{+/-}$ vs. I $Eng^{+/+}$; $n=6$/group). (E) Western blot analysis revealed increased eNOS levels in the I $Eng^{+/+}$(*$P<0.05$ vs. NI $Eng^{+/+}$) and reduced eNOS levels in NI $Eng^{+/-}$ muscles (*$P<0.05$ vs. NI $Eng^{+/+}$), and in I $Eng^{+/-}$ muscles ($^{\#}P<0.05$ vs. I $Eng^{+/+}$; $n=8$ mice/group).

activation phase. Basal [^{3}H]-proline incorporation was significantly greater in $Eng^{+/-}$ compared to $Eng^{+/+}$ MAECs (Fig. 2E). TGF-β1 treatment caused a 1.7-fold increase in collagen production in both groups, resulting in significantly higher total levels in $Eng^{+/-}$ cells. Incubation with a neutralizing antibody to TGF-β1 had no effect on collagen accumulation in either cell type, suggesting that the elevated basal collagen synthesis in the $Eng^{+/-}$ cells was not caused by higher endogenous levels of TGF-β1.

3.3. Decreased VEGF secretion by $Eng^{+/-}$ MAECs

Given that VEGF is a central mediator of endothelial cell proliferation and migration, its basal levels were quantified in the culture medium. They were significantly reduced in $Eng^{+/-}$ MAECs versus control $Eng^{+/+}$ cells (Fig. 3A). TGF-β1 significantly increased VEGF levels in both groups, resulting in higher total levels in $Eng^{+/+}$ cells. A neutralizing antibody to TGFβ1 did not affect baseline VEGF secretion, suggesting that the lower levels observed in $Eng^{+/-}$ cells are not due to a lower concentration of endogenous TGF-β1.

3.4. Reduced eNOS expression in $Eng^{+/-}$ MAECs

The mitogenic and chemotactic effects of VEGF on endothelial cells are NO-dependent and necessitate normal eNOS expression and activity. Consistent with previous observations [8,9], $Eng^{+/-}$ MAECs displayed significantly reduced eNOS levels relative to $Eng^{+/+}$ cells (Fig. 3B).

3.5. Abnormal capillary-tube formation in vitro and in vivo

When plated on Matrigel, endothelial cells rapidly organize and form capillary-like structures. $Eng^{+/-}$ MAEC generated a less extensive capillary network than the $Eng^{+/+}$ cells (Fig. 4A). HUVECs derived from six newborns with a distinct *ENG* mutation, and therefore an HHT1 genotype, expressed reduced levels of endoglin (ranging from 37% to 64%) relative to the control group, as assessed by metabolic labelling [11]. A median of 45% was estimated for 30 newborns with HHT1 while unaffected newborns had a median of 98% of control HUVEC, as reviewed recently and in keeping with the haploinsufficiency model [1]. The HHT1 HUVECs generated an altered capillary network when plated on Matrigel (Fig. 4B). A lower number of capillary tubes (101 ± 4.5 vs. 118 ± 5, $P < 0.05$) was observed (Fig. 4C), with greater luminal areas reaching 11,765 ± 611 μm^2 vs. 10,252 ± 434 μm^2 in the control group ($P < 0.05$) (Fig. 4D). Moreover, the walls of the capillary tubes formed by HUVECs with an *ENG* mutation were significantly thicker than those of control HUVEC (16.4 ± 1.7 μm versus 10.0 ± 0.6 μm, $P < 0.01$) (Fig. 4E).

Matrigel plugs were implanted dorsally in $Eng^{+/-}$ and $Eng^{+/+}$ mice as a way to monitor endothelial cell outgrowth and invasion of extracellular matrix in vivo. Representative sections of the implanted Matrigel plugs show much reduced numbers of invading cells in the $Eng^{+/-}$ mice (Fig. 4F). Quantitative analysis revealed a significantly lower number of blood vessels (11 ± 0.9 vessels/mm^2) in $Eng^{+/-}$ mice, relative to $Eng^{+/+}$ mice (25 ± 3.3 vessels/mm^2, $P < 0.01$) (Fig. 4G).

3.6. Lower post-ischemic capillary generation and reperfusion in $Eng^{+/-}$ mice

The importance of NO and the capacity for ischemia-induced angiogenesis in $Eng^{+/-}$ mice were evaluated by measuring reperfusion recovery in femoral artery-ligated hindlimb following chronic treatment with or without L-NAME. $Eng^{+/-}$ mice had significantly lower overall hindlimb reperfusion compared to $Eng^{+/+}$ mice and L-NAME significantly reduced this parameter to similar levels in both groups of mice (Fig. 5A).

Non-ischemic and ischemic adductor muscle vascularity was quantified in tissue sections by counting the number of CD31 positive vessels. Non-ischemic muscles showed similar vessel density in $Eng^{+/+}$ and $Eng^{+/-}$ mice (Fig. 5B; basal). Following ischemia, $Eng^{+/-}$ mice had a significantly lower overall adductor muscle vessel density; most noticeable were the differences at 4 and 14 days (60% of control values). By day 28, capillary density was similar in both groups. When the rate of perfusion was calculated per vessel, a trend at 4 days and a significant increase at 14 days were observed in the $Eng^{+/-}$ muscles, suggesting that increased flow was compensating for reduced vascularity (Fig. 5C).

3.7. Endoglin and eNOS expression in ischemic adductor muscles

Endoglin mRNA was significantly reduced in $Eng^{+/-}$ non-ischemic muscles, due to expression of a single *Eng* allele. Following ischemia, muscle endoglin mRNA levels were increased in both groups, maintaining a higher level in $Eng^{+/+}$ mice (Fig. 5D). eNOS protein levels were significantly lower in non-ischemic $Eng^{+/-}$ than in $Eng^{+/+}$ muscles and remained so despite a significant ischemia-induced increase in both groups (Fig. 5E).

4. Discussion

Our findings demonstrate that endothelial cell proliferation, migration and capillary tube formation are impaired in an animal model of HHT1, the $Eng^{+/-}$ mouse. In addition to in vitro findings, two different in vivo angiogenic assays showed reduced vessel formation. However, a higher rate of perfusion per vessel was observed in the hind limb ischemia model. Increases in local blood flow, associated with HHT, may compensate for the angiogenic defects and account for normal perfusion 28 days after injury-induced ischemia.

VEGF regulates both vasculogenesis and angiogenesis. It was demonstrated that VEGF induced proliferation of HUVECs and their ability to form vessel-like structures in

vitro was NO-dependent [12]. Long-term VEGF exposure stimulates eNOS protein levels while short-term stimulation of NO production was mediated by PI-3 Kinase signaling. It was subsequently shown that the AKT serine threonine kinase, known to phosphorylate eNOS and cause its activation and NO production, was the downstream effector of VEGF stimulated actin reorganization and endothelial cell migration [13,14]. NO promotes endothelial cell proliferation and migration, while NOS inhibitors suppress these responses. Endothelial cell growth and angiogenic sprouting are impaired in *eNOS*$^{-/-}$ mice. Association with heat shock protein 90 (Hsp90) is required for eNOS activation and NO production [15]. Impaired eNOS/Hsp90 association causes uncoupling of the enzyme and production of eNOS-derived superoxide (O_2^-) [16]. Agents that perturb eNOS/Hsp90 association or uncouple eNOS activity were shown to attenuate endothelial cell proliferation, migration and capillary tube formation [17,18]. Endoglin was shown to associate with eNOS and modulate its activation by promoting eNOS/Hsp90 association. *Eng*$^{+/-}$ endothelial cells have uncoupled eNOS activity, evidenced by less NO and more eNOS-derived superoxide (O_2^-) production during agonist-induced activation [9]. Therefore, the abnormalities in proliferation, migration and capillary formation observed in *Eng*$^{+/-}$ cells may be due to uncoupled eNOS activity and reduced NO production.

Our data also revealed a decrease in basal VEGF production in *Eng*$^{+/-}$ versus *Eng*$^{+/+}$ endothelial cells, which may contribute to the observed reduction in proliferation, migration and capillary tube formation. Although NO was shown to stimulate VEGF expression in human and rat vascular smooth muscle cells [19], our findings suggest a potential endothelial autocrine loop whereby eNOS activity and NO levels may regulate VEGF production. TGF-β-stimulated increase in VEGF secretion was unchanged in both groups of cells and treatment with a neutralizing antibody to TGF-β did not alter basal VEGF secretion suggesting that the reduced basal VEGF production in *Eng*$^{+/-}$ cells is independent of TGF-β.

Basal collagen synthesis was elevated in *Eng*$^{+/-}$ cells, consistent with a reduced migratory activity. Fibronectin synthesis was also increased (data not shown), indicating that extracellular matrix deposition is higher in *Eng*$^{+/-}$ cells. This likely leads to increased cell adhesion and hindered locomotion. The stimulation of collagen synthesis by TGF-β1 still occurred in *Eng*$^{+/-}$ endothelial cells, indicating that reduced endoglin levels did not affect this response. Furthermore, treatment with a neutralization antibody to TGF-β did not affect basal collagen production suggesting that the increased collagen synthesis in *Eng*$^{+/-}$ endothelial cells is independent of TGF-β.

The finding that *Eng*$^{+/-}$ endothelial cells display impaired proliferation and migration is consistent with reports demonstrating that endoglin: i) promotes cell proliferation and TGF-β/ALK-1 signaling [20]; ii) is up-regulated in angiogenesis [21]; iii) down-regulation impairs proliferation and increases cell apoptosis [4]; and iv) normal cellular levels are required for the formation of new blood vessels [22].

In addition to the impaired in vitro angiogenic activity of isolated *Eng*$^{+/-}$ endothelial cells, *Eng*$^{+/-}$ mice showed impaired angiogenesis in vivo. The migration of adjacent cells into Matrigel implants and their ability to form vessels was markedly inhibited in the *Eng*$^{+/-}$ mice. The lesser migration of *Eng*$^{+/-}$ cells may be due to their lower production of NO, and the consequent reduction in metalloproteinase activity as reported in our recent study [23]. We demonstrated that NO elicits migration in murine and bovine aortic endothelial cells, by increasing extracellular MMP-13 expression, which leads to higher collagen breakdown.

Hindlimb revascularization following femoral artery ligation, was significantly lower in *Eng*$^{+/-}$ mice during the first 2-week recovery period, but reached normal levels after 28 days suggesting a delay in neo-vascularization. Interestingly, there were relatively large differences in hindlimb vascularity between *Eng*$^{+/+}$ and *Eng*$^{+/-}$ mice, which did not correlate with the much smaller differences in perfusion. This suggests that despite a reduction in local vascularity in *Eng*$^{+/-}$ mice, there is increased collateral blood flow as evidenced by an elevated perfusion per vessel. These observations are consistent with the impaired myogenic reactivity and enhanced endothelium-dependent dilatation observed in *Eng*$^{+/-}$ resistance or feeding vessels, due to uncoupled eNOS activity [9]. Indeed, chronic treatment of mice with L-NAME reduced hindlimb perfusion to a similar level in both groups of mice suggesting that the increased perfusion per vessel in *Eng*$^{+/-}$ mice is entirely NOS-dependent. This endothelial abnormality in *Eng*$^{+/-}$ resistance vessels leads to increased local blood flow and maintains adequate oxygen delivery in response to tissue metabolic demand, despite reduced vascularity during the early phase of recovery. As recently proposed [9], consequent excessive increases in hemodynamic stress may abnormally dilate or damage downstream fragile venular and capillary structures and represent an early event in the pathogenesis of HHT-type vascular lesions.

Angiogenesis is crucial for tumor growth and progression. Without an increased blood supply from newly formed vessels, neoplastic cell aggregates remain dependent on diffusion for nutrients, oxygenation and the removal of waste metabolites. Under these conditions, their growth is restricted to a maximum of 1–2 mm^3 and most remain clinically quiescent. The increased expression of endoglin in proliferating endothelial cells and in tumor vasculature suggests that it may be highly relevant in the diagnostic, prognostic and potential treatment of solid malignancies [21]. We have found that endothelial cell proliferation, migration and capillary tube formation are impaired in cultured *Eng*$^{+/-}$ endothelial cells and that these abnormalities may be related to reduced NO bioavailability and VEGF production, and to an increase in collagen deposition. Reduced vessel formation was observed in two distinct in vivo angiogenic assays. Interestingly, hindlimb reperfusion per vessel following ischemia

was in fact elevated in $Eng^{+/-}$ mice as would be expected from impaired arterial myogenic reactivity and enhanced endothelium-dependent dilation in collateral $Eng^{+/-}$ resistance vessels [9]. Therefore, such inherent tendencies for increased local blood flow in $Eng^{+/-}$ mice can serve to maintain adequate tissue oxygenation during early reduction in neo-vascularization. However, such a seemingly beneficial vascular response would cause excessive hemodynamic stress leading to abnormal venular dilatation or capillary damage, and subsequent HHT-type vascular lesions.

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