Endoglin Null Endothelial Cells Proliferate Faster and Are More Responsive to Transforming Growth Factor β1 with Higher Affinity Receptors and an Activated Alk1 Pathway*

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Endoglin is an accessory receptor for transforming growth factor β (TGFβ) in endothelial cells, essential for vascular development. Its pivotal role in angiogenesis is underscored in Endoglin null (Eng<sup>−/−</sup>) murine embryos, which die at mid-gestation (E10.5) from impaired yolk sac vessel formation. Moreover, mutations in endoglin and the endothelial-specific TGFβ type I receptor, ALK1, are linked to hereditary hemorrhagic telangiectasia. To determine the role of endoglin in TGFβ pathways, we derived murine endothelial cell lines from Eng<sup>+/+</sup> and Eng<sup>−/−</sup> embryos (E9.0). Whereas Eng<sup>+/+</sup> cells were only partially growth inhibited by TGFβ, Eng<sup>−/−</sup> cells displayed a potent anti-proliferative response. TGFβ-dependent Smad2 phosphorylation and Smad2/3 translocation were unchanged in the Eng<sup>−/−</sup> cells. In contrast, TGFβ treatment led to a more rapid activation of the Smad1/5 pathway in Eng<sup>−/−</sup> cells that was apparent at lower TGFβ concentrations. Enhanced activity of the Smad1 pathway in Eng<sup>−/−</sup> cells was reflected in higher expression of ALK1-dependent genes such as Id1, Smad6, and Smad7. Analysis of cell surface receptors revealed that the TGFβ type I receptor, ALK5, which is required for ALK1 function, was increased in Eng<sup>−/−</sup> cells. TGFβ receptor complexes were less numerous but displayed a higher binding affinity. These results suggest that endoglin modulates TGFβ signaling in endothelial cells by regulating surface TGFβ receptors and suppressing Smad1 activation. Thus an altered balance in TGFβ receptors and downstream Smad pathways may underlie defects in vascular development and homeostasis.

Endoglin (CD105) is a homodimeric transmembrane glycoprotein expressed on all types of endothelial cells (1) and increased in cells in culture and during angiogenesis in vivo (2–7). Endoglin expression is also enhanced in vascular smooth muscle cells during injury and inflammation (8–10). Endoglin is critically important in the cardiovascular system as revealed by a lethal phenotype in endoglin null (Eng<sup>−/−</sup>) murine embryos at gestational day E10.5 because of defects in vessel and heart development (11–13). Vasculogenesis in the Eng<sup>−/−</sup> mice is normal, but angiogenesis is impaired along with remodeling of the primary vascular plexus. Mice exhibit poor vascular smooth muscle development that results in dilatation and rupture of the vascular channels. Heart development is arrested in Eng<sup>−/−</sup> mice at E9.0. The atrioventricular canal endocardium fails to undergo mesenchymal transformation and to generate the cushion tissue essential for valve formation and heart separation (11). Transient expression of endoglin is also striking during human development, as it is up-regulated during heart valve formation but subsequently reduced as the valves mature (14). In the adult vasculature, endoglin haploinsufficiency causes the vascular dysplasia hereditary hemorrhagic telangiectasia type 1 (HHT1)<sup>1</sup> associated with dilated vessels and arteriovenous malformations (15, 16).

Endoglin associates with transforming growth factor β (TGFβ) receptors (17). TGFβ is a multifunctional cytokine that controls proliferation, migration, adhesion, and apoptosis of diverse cell types (18, 19). TGFβ signals through a heteromeric complex of type I and type II transmembrane serine/threonine kinase receptors (20). Receptor activation occurs upon binding of ligand to the type II receptor (TβRII), which recruits and phosphorylates type I receptors and then propagates the signal to downstream target receptor-regulated Smads (21, 22). The specificity of cellular responses to TGFβ is mediated by the type I receptors. Most cells utilize the type I receptor ALK5, which phosphorylates Smad2 and Smad3. However, endothelial cells have an additional type I receptor, ALK1, which phosphorylates Smad1 and Smad5 (23, 24). Of note, TGFβ-dependent activation of ALK1 requires ALK5, such that both are present with TβRII in a composite receptor complex that acts via Smad1 and Smad5. Once phosphorylated, one of these receptor-regulated Smads combines with the common Smad4.

<sup>1</sup>The abbreviations used are: HHT, hereditary hemorrhagic telangiectasia; TGFβ, transforming growth factor β; Eng, endoglin gene; E3, ubiquitin-protein isopeptide ligase; MECC, murine embryonic endothelial cells; TβRII, TGFβ type II receptor; ALK, activin receptor-like kinase; ACVR1L, activin receptor-like kinase 1 gene; FBS, fetal bovine serum; pAb, polyclonal antibody; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; PECAM, platelet/endothelial cell adhesion molecule.
and the complex translocates to the nucleus to regulate transcription (22, 25). The inhibitory Smads 6 and 7 are a third class of Smads that down-regulate TGFβ-related responses (26) primarily through recruitment of E3 ligases to active receptor complexes, leading to ubiquitin-mediated degradation by the 26 S proteasome (27, 28).

TGFβ has various roles in development and homeostasis of the vascular system and regulates both vasculogenesis and angiogenesis. It can regulate vascular permeability, endothelial cell proliferation and migration, production of extracellular matrix, and vascular remodeling (29). Recruitment of mesenchymal cells into new vessels is achieved in part by TGFβ. Upon contact of mesenchymal cells with the endothelium, latent TGFβ is activated, inducing differentiation of mesenchymal cells into pericytes and smooth muscle cells (30). Gene ablation of TGFβ, its receptors, and Smads underscores the essential role of TGFβ signaling in vascular development. Gene knock-outs of TGFβ1, TβRII, ALK5, ALK1, and Smad5 all die from improper development of yolk sac vascular networks and/or cardiac malformations. Although TβRII and ALK5 null mice also have yolk sac defects, the most similar phenotypes to endoglin null mice are the ALK1- and Smad5-deficient embryos, which also develop dilated vessels ascribed to poor smooth muscle cell development (31). Moreover, mutations in the ACVR1I gene, which codes for ALK1, cause HHT2, exhibiting vascular lesions similar to HHT1, suggesting that endoglin enhanced the ability of TGFβ to stimulate endothelial cells with antisense oligonucleotides for TGFβ1 ranging from 0 to 100 picomolar in medium containing 1% FBS and 15 μg/ml endothelial mitogen (Biomedical Technologies) and then pulsed for 8 h (1 μCi/well) at various time points. Cells were lysed with water and harvested with a microplate harvester (Inotech Biosystems International); radioactive incorporation was determined by liquid scintillation analysis (Beckman Instruments). Multiple conditions were tested for cell density, growth factor, FBS concentration, and time in culture with both matched sets of lines to obtain maximal inhibition of proliferation by TGFβ1.

Measurement of Smad Phosphorylation by Western Blot—Equivalent numbers of MEEC were seeded in complete medium. After 48 h, cells were starved for 2–3 h in serum-free medium, treated with TGFβ1 for the times and concentrations indicated, and solubilized in lysis solution containing 1% Triton X-100 and a mixture of proteases, gentamicin 50 μg/ml (Wisent), and glutamine 106 (100-mm dish) in complete medium containing 1% FBS and 15 μg/ml endothelial mitogen (Biomedical Technologies) and then pulsed for 8 h (1 μCi/well) at various time points. Cells were lysed with water and harvested with a microplate harvester (Inotech Biosystems International); radioactive incorporation was determined by liquid scintillation analysis (Beckman Instruments). Multiple conditions were tested for cell density, growth factor, FBS concentration, and time in culture with both matched sets of lines to obtain maximal inhibition of proliferation by TGFβ1.

To better understand the role of endoglin in the vascular system, we analyzed TGFβ signaling in Eng−/− and control murine embryonic endothelial cells (MEEC). Our results are consistent with a model in which endoglin modulates TGFβ-dependent activation of ALK1 versus ALK5 to maintain a balance between Smad1/5 and Smad2/3 signaling pathways in endothelial cells.

EXPERIMENTAL PROCEDURES

Derivation of MEEC—Eng−/− and Eng−/− murine embryos and yolk sacs were isolated at E9.0 of gestation (N4 generation, C57BL/6 mice), prior to onset of the lethal phenotype in the Eng null mice as previously described (38). After disaggregation of tissues, endothelial cells were selectively immortalized with polyoma middle T antigen in the retrovirus vector N-TkμT using 8 μg/ml polybrene (Sigma), and lines were established as reported (38). The infected cells were selected by adding the neomycin analogue G418 (Invitrogen) at 800 μg/ml, which also develop dilated vessels ascribed to poor smooth muscle cell development (31). Moreover, mutations in the ACVR1I gene, which codes for ALK1, cause HHT2, exhibiting vascular lesions similar to HHT1, suggesting that endoglin enhanced the ability of TGFβ to suppress growth and migration of these cells (37).

To better understand the role of endoglin in the vascular system, we analyzed TGFβ signaling in Eng−/− and control murine embryonic endothelial cells (MEEC). Our results are consistent with a model in which endoglin modulates TGFβ-dependent activation of ALK1 versus ALK5 to maintain a balance between Smad1/5 and Smad2/3 signaling pathways in endothelial cells.

Two-color Flow Cytometry—MEEC were detached by brief trypsinization, neutralized with 15% FBS, and washed in Ca2+-/Mg2+-free phosphate-buffered saline plus 2% FBS. Cells (1 × 106) were incubated for 1 h with saturating amounts of FITC-conjugated monoclonal antibody (mAb) M57/18 (Biocan Scientific) to murine endoglin (CD105), phosophatidylethanolamine-conjugated mAb MEC 13.3 to PE-CAM (CD31; BD Biosciences) or respective conjugated isotype controls and then washed again. Samples were run on the fluorescence-activated cell sorter Calibur® (BD Biosciences). A gate was set using forward and side scatter and both FL1 (FITC) and FL2 (phosphatidylethanolamine) channels were acquired. Data were analyzed using CellQuest® software where percent positive cells were determined relative to the isotype controls.

Metabolic Labeling and Immunoprecipitation—Equivalent numbers of matched MEEC lines were incubated with 100 μCi/ml [35]S)methionine (Trans-3S-label; ICN Pharmaceuticals) in methionine-free Dulbecco’s modified Eagle’s medium for 3.5 h. Cells were solubilized in lysis solution containing 1% Triton X-100 (Sigma) and a mixture of inhibitors, immunoprecipitated with mAb M7F/18 (Bionca Scientific) to endoglin, and processed according to published procedures (16).
Activated ALK1 Pathway in Endoglin Null Endothelial Cells

PCR program consisted of the initial activation step at 95 °C for 15 min, followed by 50 cycling steps of denaturing for 15 s at 94 °C, annealing at appropriate temperature for 30 s, and extension at 72 °C for 15 s. The PCR data were analyzed using SDS 2.1 software (Applied Biosystems).

Cell Surface Biotinylation and Western Blot—Receptor levels were quantified by Western blot analysis as described for Smad phosphorylation except that cells were not treated with TGFβ1. Antibodies used for detection were pAb C16 to TβRII (Santa Cruz Biotechnology), mAb MJ7/18 (BiocGN Scientific) to murine endoglin, mAb to ALK1 (R&D systems), and pAb V22 to ALK5 (Santa Cruz). For quantification of cell surface TβRII, equivalent numbers of MEEC were seeded in complete medium; after 48 h, intact monolayers were surface labeled with 0.3 mg/ml of sulfo-succinimidyl 6 (biotinamido)-hexanoate (NHS-LC-Biotin; Pierce) as reported previously (16). Labeled cell surface-biotinylated cells were immunoprecipitated with anti-TβRII pAb C16 (Santa Cruz) or anti-endoglin mAb MJ7/18 (BiocGN Scientific). Samples containing equivalent protein content were analyzed by 4–12% SDS-PAGE (Novex) and probed with streptavidin-horseradish peroxidase as reported. Quantification was achieved by chemiluminescence with ECL (Amersham Biosciences) followed by reading with a Fluor-S Max® CCD camera and QuantityOne software (Bio-Rad).

Affinity Labeling of Receptor Complexes, Turnover, and Scatchard Analysis—TGFβ (R&D Systems) was iodinated with 125I using chloramine-T (39). For turnover of active complexes, confluent monolayers were affinity labeled by incubation with 250 pM 125I-TGFβ1 for 2 h at 4 °C, washed, and treated with disuccinimidyl suberate (Pierce). Cells were washed with phosphate-buffered saline-Tris (pH 7.6) and incubated in medium at 37 °C for the times indicated for receptor turnover experiments. Cells were then solubilized in lysis solution containing 1% Triton X-100 and a mixture of inhibitors as reported (40) and solubilized by SDS-PAGE on 4–12% gels (Novex). Receptors were quantified using a Storm Phosphorimager and ImageQuant software (Amersham Biosciences). For Scatchard analysis, confluent monolayers were incubated with increasing concentrations of 125I-TGFβ1 in the presence or absence of 40× competing cold TGFβ1 at 4 °C for 3.5 h and cross-linked as described above. Labeling medium and supernatants were collected and counted in a gamma counter. Lysates were also counted and a phosphatidylethanolamine-conjugated anti-CD31. A representative experiment is shown for two sets of matched MEEC lines, Eng+/−, 150/7 with Eng+/+ 150/9 and Eng−/−, 125/10 with Eng+/− 125/20. 96–98% of Eng+/− cells were in the upper right quadrant, whereas 90–93% of Eng−/− cells were in the upper left quadrant. Markers for each axis were set with FITC-conjugated and phosphatidylethanolamine-conjugated isotype-matched control antibodies shown in lower panels with 99% negative cells in the lower left quadrant. B, endoglin expression was analyzed by metabolic labeling with [35S]methionine and solubilization in Triton X-100. Lysates containing equivalent cpm were immunoprecipitated with anti-CD105 and fractionated using SDS-PAGE under reducing conditions. Monomeric glycosylated endoglin (E) and precursor (P) are observed only in Eng+/+ lysates.

RESULTS

Eng Null Endothelial Cells Show Increased Proliferation and Growth Inhibitory Response to TGFβ—To better understand the role of endoglin in TGFβ-mediated signaling in endothelial cells, we generated endothelial cell lines from murine embryos and yolk sacs (E9.0) prior to onset of the lethal phenotype in cells, we generated endothelial cell lines from murine embryos.

Next we analyzed the proliferation rate of the Eng+/− and control Eng+/+ MEEC lines and found that Eng−/− cells proliferated faster and achieved higher cell densities when compared with wild type cells (Fig. 2, A and B). Subsequently, we examined TGFβ-dependent inhibition of proliferation. Although TGFβ is a potent inhibitor of proliferation in epithelial cells, both wild-type endothelial cell lines examined here displayed poor responses (Fig. 2C), similar to previously published reports in other endothelial cell types (16, 41, 42). In contrast, when we examined the Eng−/− lines, we observed potent anti-proliferative responses to TGFβ, with over 80% inhibition observed at between 25–50 pM (Fig. 2C). Of note, none of our cell lines displayed a TGFβ-dependent proliferative response, contrasting with MEEC cells reported by Lebrin et al. (7) that displayed a “low-dose” TGFβ-induced proliferation dependent on endoglin. Our data suggest that in wild type cells, endoglin functions to block TGFβ-dependent inhibition of proliferation, in agreement with published data of a knockdown of endoglin in endothelial cells using antisense oligonucleotides leading to enhanced growth inhibition by TGFβ1 (37).
Endothenin Does Not Alter TGFβ1 Activation of Smad2 in MECC Lines—To better define a mechanism for the increased TGFβ responsiveness in the Eng−/− lines, we looked at downstream signaling events, namely Smad2 phosphorylation and nuclear accumulation (Fig. 3). We first examined Smad2 phosphorylation in response to increasing doses of TGFβ using a phospho-specific antibody that recognizes activated Smad2. This revealed robust Smad2 activation that was near maximal when comparing Smad6, Smad7, and Id1, which were shown to be specifically up-regulated by constitutive ALK1 activity in endothelial cells (44), using real-time quantitative PCR (Fig. 4D). Consistent with Smad1 pathway activation, we found that when compared with wild type cells, Eng−/− cells displayed elevated levels of Smad2/3 (10-fold), Smad7 (4.5-fold), and Id1 (2–2.5-fold), whereas Smad1, Smad2, and plasminogen activator inhibitor (PAI-1), a TGFβ target gene, were unchanged (Fig. 4C). Our data suggest that loss of Eng has little impact on the temporal kinetics in these cells using a high dose of TGFβ and found a more rapid activation of Smad1/5/8 in the 150/9 Eng−/− cells (Fig. 4B) that was evident within 5 min of stimulation and was quite pronounced when the cells were subjected to a brief pulse with TGFβ1 (Fig. 4C). Similar results were obtained when we analyzed the 152/8 and 152/10 matched set of Eng−/− and Eng−/− cells (data not shown). Our analysis of the Smad1 pathway suggests that it is basally active and highly responsive in the mutant lines. Therefore, we measured the expression of Smad6, Smad7, and Id1, which were shown to be specifically up-regulated by constitutive ALK1 activity in endothelial cells (44), using real-time quantitative PCR (Fig. 4D). Consistent with Smad1 pathway activation, we found that when compared with wild type cells, Eng−/− cells displayed elevated levels of Smad6 (10-fold), Smad7 (4.5-fold), and Id1 (2–2.5-fold), whereas Smad1, Smad2, and plasminogen activator inhibitor (PAI-1), a TGFβ target gene, were unchanged (Fig. 4C). Our data suggest that loss of Eng has little impact on the Smad2/3 pathway but leads to up-regulation of the Smad1 pathway that is likely due to enhanced signaling through ALK1, which specifically regulates Smad1/5/8 (45). Of particular note, we also analyzed TGFβ induction of Smad-independent pathways such as mitogen-activated protein kinase by measuring phosphorylation of p44/p42 as well as p38 and found that these pathways were not strongly regulated by TGFβ in these cells (data not shown).

Eng Null MECC Display Reduced Numbers of Cell Surface TGFβ Receptors but Increased Binding Affinity—Signaling by TGFβ is initiated by binding to TGFβ/RII followed by recruitment of ALK5 or ALK5-ALK1 complexes in endothelial cells. Because loss of Eng leads to enhanced activation of the Smad1/5/8 pathway, we therefore examined the TGFβ receptor complement in
MEEC. Analysis of receptor mRNA levels revealed no difference in ALK1 or TβRII; however, ALK5 levels were elevated 2–2.5-fold in the endoglin null MEEC relative to control (Fig. 5A). Western blot analysis further showed that ALK5 protein was elevated in the endoglin null cells, whereas TβRII protein was somewhat reduced in both endoglin null lines compared with normal, and
ALK1 levels were similar in all the lines (Fig. 5B). Quantitation of protein levels from several experiments showed that ALK5 protein in the mutants was elevated by 2.2-fold and TβRII protein reduced to 70% of control levels. Furthermore, we examined cell surface TβRII using surface biotinylation of all four matched MEEC lines followed by immunoprecipitation with anti-TβRII and detection using streptavidin. This revealed that Eng−/− cells have ~60% of TβRII compared with Eng+/+ cells, similar to the overall 70% reduction observed by Western blot (Fig. 5C). Analysis with anti-endoglin as a control for the same samples confirmed no surface endoglin on the null cells. Because binding of TGFβ to TβRII is required for the assembly of functional heteromeric receptor complexes, we next examined TGFβ binding kinetics to both TβRII and type I receptors on MEEC by Scatchard analysis. As ALK1 co-migrates with ALK5, we could not distinguish between these two receptors. However, previous data have shown that ALK1 binds TGFβ in these cells in a complex with ALK5, so it is reasonable to assume that the affinity and cell surface numbers reflect the binding kinetics of both ALK5 and ALK1. Fig. 5D shows a representative experiment using Eng+/+ 152/8 and Eng−/− 152/10 lines, and Table I gives a summary of the data obtained with these lines. From the Scatchard analysis, we found that the K_d of both TβRII and the type I receptors were 2.5–4-fold lower in the Eng−/− lines, indicating higher affinity binding complexes, whereas the number of sites per cell was reduced by 4–7-fold for both receptors. Although we cannot determine from this data which type I receptor is affected or if there is a shift in binding preferentially to ALK1, it is apparent that in MEEC endoglin expression leads to a larger number of lower affinity receptor complexes. This is the first report providing evidence that endogenous endoglin modulates the affinity and number of TGFβ receptor complexes in endothelial cells. Furthermore, because ALK5 is required for ALK1 activity (46), the increased numbers of ALK5 coupled to the enhanced affinity displayed by TGFβ receptors in Eng−/− MEEC may enhance activation of the Smad1 pathway. Altogether, our results suggest that endoglin alters the balance of Smad1/5/8 versus control Smad−/− line 150/7 for S6 and S7 (n = 9) and for S1, Id-1, and PAI-1 (n = 6) were measured by real-time PCR. The relative amounts of these mRNAs were obtained by normalization to glyceraldehyde-3-phosphate dehydrogenase levels. Error bars represent mean ± S.D. Genes showing a statistical difference between the two groups (p < 0.001) are marked by **.

DISCUSSION

In the mouse embryo, endoglin is essential for angiogenesis. Endoglin has been previously described as a marker of proliferating endothelial cells, as its expression is up-regulated on cycling cells and during angiogenesis (2–7). However, it is not clear whether endoglin mediates an enhanced proliferative rate or may be up-regulated as part of a negative feedback loop. Our observation that Eng−/− MEEC lines display a much
with the antibody.
summarized in Table I.
calculated and plotted quantifying aliquots of labeling medium. A representative experiment is shown along with its corresponding gels. The ratio bound/free was
receptors was quantified by Phosphorimager analysis and corrected with values from cold gels. Pixels were converted to cpm by spotting and
hexonate. Lysates from surface-biotinylated cells were immunoprecipitated with anti-endoglin or anti-T
affinity labeled with increasing concentrations of125I-TGF
protein content were analyzed by SDS-PAGE, probed with streptavidin-horseradish peroxidase, and quantified by chemiluminescence. For total
Supernatants were collected and counted. Lysates were analyzed by SDS-PAGE. The specific amount of125I-TGF
binding sites.

**TABLE I**
Summary of Scatchard analysis data of TGFβ receptor complexes in Eng null and wild type MEEC lines

Confluent monolayers were affinity labeled with increasing concentrations of125I-TGFβ1 in the presence or absence of 40× competing cold TGFβ1 at 4 °C for 3.5 h. Supernatants were collected and counted. Lysates were analyzed by SDS-PAGE. The specific amount of125I-TGFβ1 bound to TβRII and type I receptors was quantified by Phosphorimager analysis and corrected with values from cold gels. Pixels were converted to cpm by spotting and quantifying aliquots of labeling medium. A representative experiment is shown along with its corresponding gels. The ratio bound/free was calculated and plotted versus the amount bound per cell, using the LINEST function of Excel, which draws the best-fit line using the least-squares method of regression analysis. The Kd for each line was estimated from the slope, and the x-axis intercept represents the number of sites/cell, summarized in Table I.

| Cell line | Receptor II | Receptor I |
|-----------|-------------|------------|
|           | Kd (pm)     | No. of Sites/Cel| Kd (pm)     | No. of Sites/Cel|
| Eng+/− 150/7 | 21 pm | 1225 | 56 pm | 1085 |
| Eng−/− 150/9 | 5.7 pm | 300 | 15 pm | 150 |
| Eng+/− 152/8 | 41 pm | 1625 | 41 pm | 770 |
| Eng−/− 152/10 | 17 pm | 270 | 8.7 pm | 180 |

higher proliferative rate than control cells would be consistent with the latter model. This also correlates with our observation that the Smad1/5/8 pathway is activated in the Eng−/− cells, because this pathway has been proposed to promote endothelial cell proliferation (7, 47).

TGFβ is well known for regulating endothelial cell proliferation, extracellular matrix production in the vessel wall, vascular remodeling, and interactions between endothelium and vascular smooth muscle cells, along with their recruitment, and thus it has multiple roles in vascular development and maintenance of vessel integrity. Endothelial cells are unique in that they possess two type I receptor pathways activated by TGFβ, the canonical ALK5 pathway, which activates Smad2/3, and the ALK1 pathway, which stimulates Smad1/5/8, a bone
endothelial cells recruit mesenchymal cell progenitors; upon contact, latent TGFβ is activated, which induces differentiation to pericytes or smooth muscle cells. A recent report by Carvalho et al. (49) indicates that the defect in yolk sac vasculature of Eng null mice may in fact reflect reduced availability of TGFβ protein and impaired recruitment and differentiation of smooth muscle cells. Accordingly, addition of exogenous TGFβ1 to yolk sac cells overcome the defect, confirming that it was the reduced production of TGFβ by the endothelium that was responsible for the apparent inability of mesenchymal cells to differentiate normally. Consistent with this, endothelial cells from HHT1 patients secrete less TGFβ and have lower plasma levels than do controls (50). Thus, defining how the loss of endoglin affects cell responsiveness versus TGFβ1 levels or access in vivo will help define how it impairs vascular development and homeostasis.

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