

Endoglin Mediates Vascular Maturation by Promoting Vascular Smooth Muscle Cell Migration and Spreading

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Objective—Endoglin, a transforming growth factor- β superfamily coreceptor, is predominantly expressed in endothelial cells and has essential roles in vascular development. However, whether endoglin is also expressed in vascular smooth muscle cells (VSMCs), especially in vivo, remains controversial. Furthermore, the roles of endoglin in VSMC biology remain largely unknown. Our objective was to examine the expression and determine the function of endoglin in VSMCs during angiogenesis.

Approach and Results—Here, we determine that endoglin is robustly expressed in VSMCs. Using CRISPR/CAS9 knockout and short hairpin RNA knockdown in the VSMC/endothelial coculture model system, we determine that endoglin in VSMCs, but not in endothelial cells, promotes VSMCs recruitment by the endothelial cells both in vitro and in vivo. Using an unbiased bioinformatics analysis of RNA sequencing data and further study, we determine that, mechanistically, endoglin mediates VSMC recruitment by promoting VSMC migration and spreading on endothelial cells via increasing integrin/FAK pathway signaling, whereas endoglin has minimal effects on VSMC adhesion to endothelial cells. In addition, we further determine that loss of endoglin in VSMCs inhibits VSMC recruitment in vivo.

Conclusions—These studies demonstrate that endoglin has an important role in VSMC recruitment and blood vessel maturation during angiogenesis and also provide novel insights into how discordant endoglin function in endothelial and VSMCs may regulate vascular maturation and angiogenesis.

Visual Overview—An online [visual overview](#) is available for this article. (*Arterioscler Thromb Vasc Biol.* 2017;37:1115-1126. DOI: 10.1161/ATVBAHA.116.308859.)

Key Words: endoglin ■ endothelial cells ■ integrins ■ muscle, smooth, vascular ■ signal transduction



Angiogenesis is the process through which new blood vessels form from pre-existing blood vessels. The process of angiogenesis begins with vasodilation, increased vascular permeability, and extracellular matrix (ECM) degradation in response to an angiogenic stimulus such as VEGF (vascular endothelial growth factor). Activated endothelial cells (ECs) proliferate and migrate toward the angiogenic stimulus, assemble into solid cords, and subsequently acquire a lumen. Once the new vessel has been assembled, pericytes and vascular smooth muscle cells (VSMCs) are recruited and migrate to the newly formed vessel to stabilize the nascent vessel in a quiescent state.¹ Hence, proper cell–cell communication between ECs, pericytes, and VSMCs is critical for vascular remodeling and maturation.

Endoglin is a 95-kDa glycoprotein that directly binds TGF- β 1 (transforming growth factor-beta 1)² and BMP-9 (bone morphogenetic protein-9)³ and can bind TGF- β 3, Activin A and BMP-2/7 in conjunction with their respective ligand binding receptors.⁴ Endoglin has been reported to

either increase^{4–8} or decrease^{5,9,10} TGF- β superfamily signaling in ECs in vitro. In addition, endoglin also regulates non-Smad or non-TGF- β pathways to regulate endothelial biology via interaction with other proteins, including β -arrestin2,¹¹ zyxin¹² or zyxin-related protein-1,¹³ and α 5 β 1 integrin.¹⁴

Endoglin has an important role in angiogenesis, as mice lacking endoglin die at embryonic days 10.5 to 11.5 because of defects in angiogenesis.¹⁵ Mutation of *Eng* results in type I hereditary hemorrhagic telangiectasia 1, a vascular disease, characterized by dilated vessels and arteriovenous malformations that lead to recurrent hemorrhage and shunting in the lung, brain, and the gastrointestinal tract.^{16,17} In addition, endoglin is overexpressed in neoangiogenic vessels, during inflammation,^{18,19} and in solid tumors.^{20,21} Although previous studies have focused largely on the roles of endoglin as a TGF- β superfamily coreceptor in ECs, these findings cannot fully explain the roles of endoglin in developmental and pathological angiogenesis. For example, a defect of VSMCs recruitment was found in endoglin knockout embryos.¹⁵

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Nonstandard Abbreviations and Acronyms	
ALK1	activin receptor-like kinase-1
BMP	bone morphogenetic protein
ECM	extracellular matrix
EC	endothelial cell
ERK	extracellular signal-regulated kinase
FAK	focal adhesion kinase
HMEC-1	human microvascular endothelial cell-1
MEEC	mouse embryonic endothelial cells
PASMC	pulmonary artery smooth muscle cells
RNA-SEQ	RNA-sequencing
TGF-β	transforming growth factor-beta
VSMC	vascular smooth muscle cells

However, in a neonatal retinal vascular development model, endoglin-specific knockout in ECs did not decrease VSMC recruitment by arteries, but increased VSMC recruitment by veins.²² In patients with hereditary hemorrhagic telangiectasia, in addition to EC defects, mural cell defects, including increased layers of smooth muscle cells and an increase in stress fibers in pericytes, have been reported.²³ Furthermore, endoglin deletion mediated by SM22 α -Cre (smooth muscle protein 22 α -Cre), which results in recombination in \approx 100% of arterial SMCs, including in the aorta²⁴ but is exceptionally rare in ECs,²⁵ caused arteriovenous malformations in the postnatal brain, spinal cord, and intestines.²⁶ However, conditionally overexpressing endoglin in VSMCs in endoglin null embryos partially rescues VSMCs recruitment.²⁷ In addition, endoglin is expressed in other nonECs, including neural crest stem cells²⁸ and smooth muscle cells in human atherosclerotic plaques²⁹ or after arterial injury.³⁰ Previous reports also demonstrated that endoglin is expressed in normal primary VSMCs in vitro,^{29,31,32} in freshly isolated VSMCs from human aorta,³¹ and in rare VSMCs in vivo,³⁰ whereas another report detected no endoglin in normal VSMCs in vivo.²⁹ Taken together, these studies suggest that endoglin in the vascular support cells, including VSMCs, may be important for angiogenesis and the pathogenesis of endoglin-associated diseases, including hereditary hemorrhagic telangiectasia. Here, we investigate the expression and roles of endoglin in VSMCs.

Materials and Methods

Materials and Methods are available in the [online-only Data Supplement](#).

Results

Endoglin Promotes VSMC Recruitment During Angiogenesis In Vitro

To investigate endoglin's roles in nonECs during angiogenesis, we investigated whether endoglin is expressed in VSMCs. Consistent with a recent report,³² we determined that endoglin is robustly expressed in a panel of human primary VSMCs, including umbilical artery smooth muscle cells, pulmonary artery smooth muscle cells (PASMC), and aortic smooth muscle cells at both the mRNA (Figure 1A in the [online-only Data Supplement](#)) and cell surface protein

levels (Figure 1B in the [online-only Data Supplement](#)). To obtain more direct evidence that endoglin is also expressed in VSMCs in vivo, we costained murine lung tissue using endoglin antibody, endothelial marker, CD31, and smooth muscle cells' marker, α -smooth muscle actin. Both endothelial and VSMCs expressed endoglin (Figure 1A; Figure 1C in the [online-only Data Supplement](#)). However, smooth muscle cells in the airway expressed low levels of endoglin (Figure 1A; right panel of Figure 1C in the [online-only Data Supplement](#)). Furthermore, as aorta has multiple layers of VSMCs, we stained endoglin in mouse aorta using immunohistochemistry with peroxidase and counterstain. Endoglin is expressed in the multilayered media of both abdominal and ascending aorta (Figure 1B; Figure 1D in the [online-only Data Supplement](#)). In addition, consistent with previous report,³¹ we also detected endoglin expression at mRNA level (Figure 1C) and cell surface protein level (Figure 1D) in the primary VSMCs and ECs (ECs) isolated from the murine aorta. These data indicate that VSMCs also express endoglin.

To investigate endoglin's role in VSMC recruitment, we performed endothelial/VSMC cells coculture experiments in transwells (Figure 1E). CRISPR-induced endoglin knockout (*ENG*^{-/-}; Figure 1E in the [online-only Data Supplement](#)) or short hairpin RNA (shRNA)-mediated silencing of endoglin expression (sh*ENG*; Figure 1E and 1F in the [online-only Data Supplement](#)) in PASMC cells dramatically decreased PASMC recruitment by ECs (Figure 1F and 1G). However, murine EC lines generated from endoglin knockout mice (MEEC^{-/-})¹⁰ either had no effect or slightly increased PASMCs recruitment compared with wild-type MEEC cells (MEEC^{+/+}; Figure 1F and 1G). These effects were specifically because of loss of endoglin, as restoring endoglin expression in *ENG*^{-/-} PASMC cells (Figure 1G in the [online-only Data Supplement](#)) rescued recruitment (Figure 1H in the [online-only Data Supplement](#)). In addition, shRNA-mediated silencing of endoglin in PASMC cells also inhibited PASMC cells being recruited by (Figure 1H and 1I) and spreading on (Figure 1H and 1J) HMEC-1 (human microvascular endothelial cell-1) endothelial tubules formed on Matrigel. These data support a specific role for endoglin in VSMCs in promoting VSMC recruitment and spreading on ECs during angiogenesis.

Endoglin Promotes VSMC Recruitment via Increasing Migration

A recent study demonstrated that endoglin promotes VSMC adhesion to the ECs during vascular development.³² As there are multiple steps involved in VSMC recruitment, including migration, adhesion, and spreading, to obtain unbiased insight into how endoglin may regulate VSMC biology, we performed RNA sequencing (RNA-SEQ; GSE78271) with control and CRISPR-induced endoglin knockout PASMC cells. Among 26440 genes detected, there were 1623 differential expressed genes (Figure 2A; Data set I in the [online-only Data Supplement](#)). We analyzed the potential molecular and cellular functions implicated by the 1623 differential expressed genes using ingenuity pathway analysis software. Based on this unbiased analysis, the molecular and cellular functions most affected by loss of endoglin expression in VSMCs was

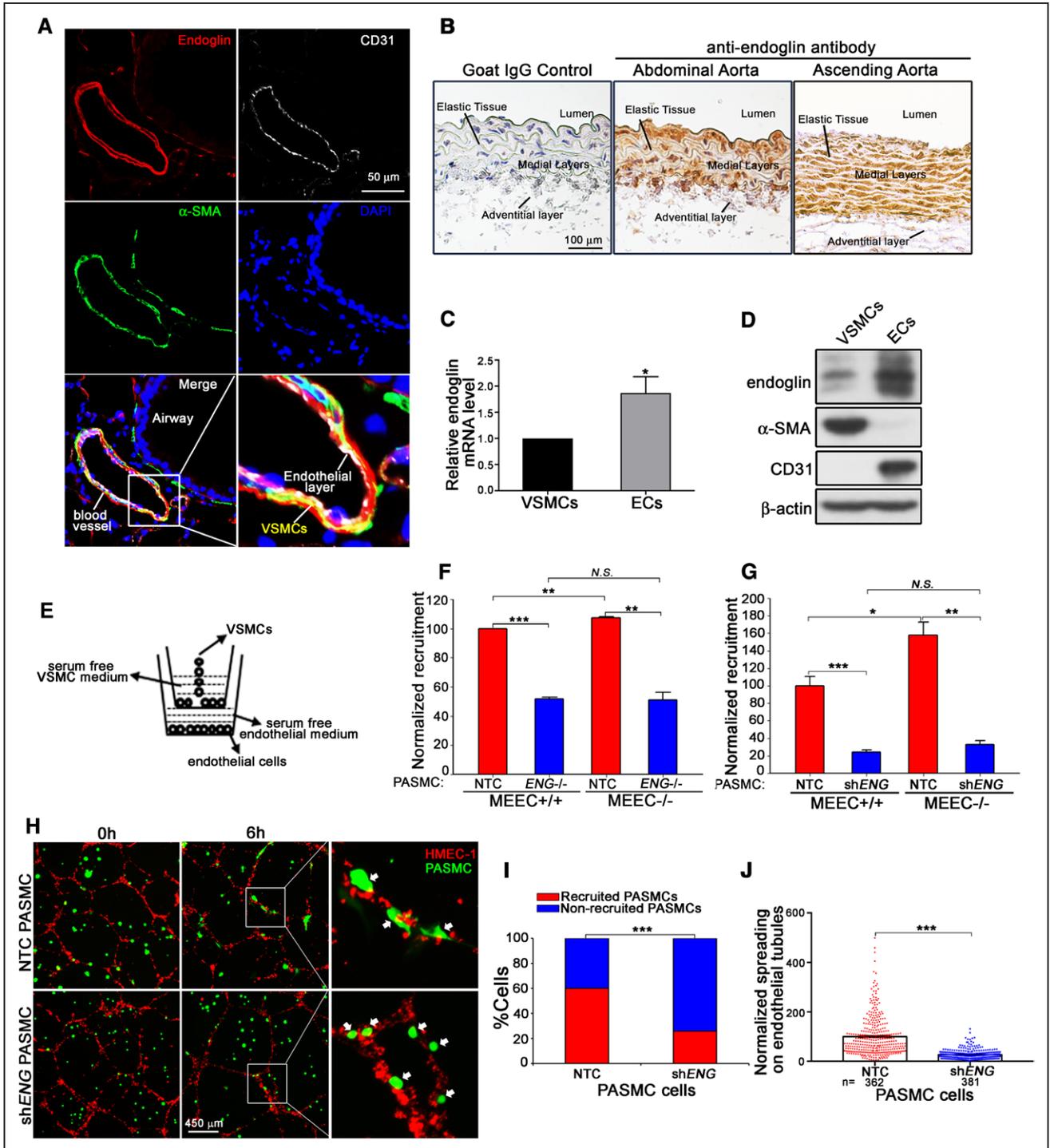


Figure 1. Endoglin promotes vascular smooth muscle cells (VSMC) recruitment. **A**, Fluorescent staining of endoglin (red), α -smooth muscle actin (SMA; green), CD31 (white), and DAPI (4',6-diamidino-2-phenylindole; blue) in murine lung tissue. **B**, Immunohistochemistry staining of endoglin in murine abdominal aorta and ascending aorta, goat IgG was used as a negative control for anti-endoglin staining. **C** and **D**, mRNA (**C**) and cell surface protein (**D**) levels in primary VSMC cells and endothelial cells (ECs) isolated from murine aorta detected using quantitative real-time polymerase chain reaction assay (**C**) and cell surface protein biotinylation assay (**D**). **E**, Schematics for VSMC/endothelial recruitment coculture transwell model. **F** and **G**, Confluent mouse embryonic endothelial cell (MEEC)^{+/+} or MEEC^{-/-} cells were cultured in the bottom transwell in blank medium for 24 h. Nontargeted control (NTC) pulmonary artery smooth muscle cell (PASC) cells, *ENG*^{-/-} (**F**), or *shENG* (**G**) PASC cells were plated in the upper chamber and allowed to migrate for 24 h toward ECs. Recruited PASC were quantified from 3 independent experiments with triplicates \pm SEM. **H–J**, HMEC-1 cells were stained with red fluorescence and plated on Matrigel for 12 h to form tubules. *shNTC* or *shENG* PASC cells (green) were plated and cultured for 6 h. The percentage of recruited PASC cells (**I**) and normalized PASC spreading on the endothelial tubules (**J**) were quantified from 3 independent experiments \pm SEM.

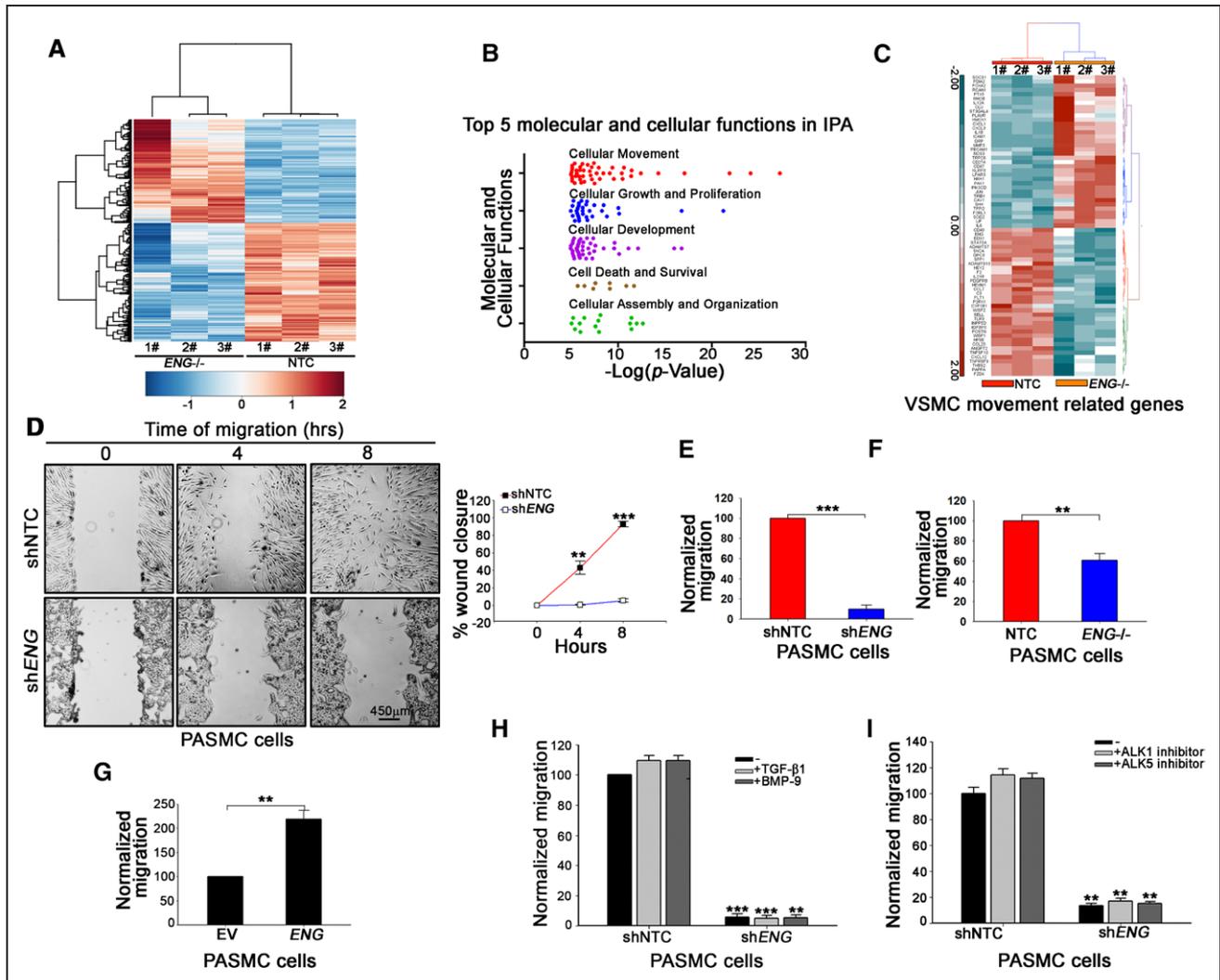


Figure 2. Endoglin promotes vascular smooth muscle cells (VSMC) migration. **A**, Heat map of the differentially expressed genes between nontargeted control (NTC) and *ENG*^{-/-} pulmonary artery smooth muscle cell (PASMC) cells (3 independent RNA samples) identified by RNA sequencing analysis. **B**, The top 5 categories of molecular and cellular functions affected by knockout of endoglin analyzed using ingenuity pathway analysis software. **C**, Heat map of differentially expressed genes involved in VSMC movement and recruitment. **D**, Confluent shNTC or shENG PASMC cells were wounded. The wound closure was measured after 4 and 8 h. Quantitative data are from 3 independent experiments \pm SEM. **E** and **F**, Nontargeting control (shNTC or NTC), shENG (**E**), or *ENG*^{-/-} (**F**) PASMC cells were plated on the transwell and assessed for migration after 8 h. Quantitative data are from 3 independent experiments \pm SEM. **G**, Empty vector (EV) or endoglin overexpressing PASMC cells were plated on the transwell and assessed for migration after 8 h. Quantitative data are from 3 independent experiments \pm SEM. **H** and **I**, shNTC and shENG PASMC cells were treated with 100 pmol/L TGF- β 1 (transforming growth factor-beta 1) or 5 ng/mL BMP-9 (**H**), or 200 ng/mL activin receptor-like kinase-1 (ALK1) inhibitor, ALK1-Fc, or 10 μ mol/L ALK5 inhibitor, SB-431542 (**I**), and were plated on the transwell and assessed for migration after 8 h. Quantitative data are from 3 independent experiments \pm SEM.

cellular movement (Figure 2B), including VSMC movement and cell recruitment (Figure 2C).

As cell movement plays a pivotal role in VSMC recruitment during angiogenesis, we investigated whether endoglin promotes VSMC recruitment via promoting VSMC migration. shRNA-mediated silencing of endoglin expression (Figure 2D and 2E) or CRISPR-induced endoglin knockout (Figure 2F) potentially inhibited PASMC migration as assessed using wound healing (Figure 2D) or transwell migration assay (Figure 2E and 2F). In addition, CRISPR-induced endoglin knockout also inhibited PASMC migration in transwells coated with collagen (Figure IIA in the online-only Data Supplement). In a reciprocal manner,

overexpression of endoglin (Figure IIB in the online-only Data Supplement) increased PASMC migration (Figure 2G). The ability of endoglin to promote VSMC cell migration was specific, as consistent with our previous reports,^{11,14,33} loss of endoglin in MEEC cells (*MEEC*^{-/-}) increased EC migration in noncoated transwells (Figure IIC in the online-only Data Supplement) or collagen-coated transwells (Figure IID in the online-only Data Supplement). *MEEC*^{-/-} cells also migrated more than *MEEC*^{+/+} cells in a wound healing model (Figure IIE in the online-only Data Supplement). Endoglin's effect on VSMC migration occurred in a TGF- β superfamily signaling-independent manner, as the TGF- β superfamily ligands for endoglin, TGF- β 1, and BMP-9 (Figure 2H),

and activin receptor-like kinase (ALK) 1 and ALK5 inhibitors (ALK1-FC and SB-431542, Figure 2I) did not significantly regulate VSMC migration in the presence or absence of endoglin. These studies support a cell-type-dependent effect of endoglin in regulating cell migration, with endoglin in VSMC promoting VSMC migration, and endoglin in ECs inhibiting endothelial migration.

Endoglin Promotes VSMC Spreading

We then turned our attention to endoglin-mediated VSMC adhesion and spreading, which are important in the later stages of VSMC recruitment and blood vessel maturation. shRNA-mediated silencing of endoglin in PASMCs slightly promoted PASMC cell adhesion to HMEC-1 ECs (Figure 3A and 3B) and decreased PASMC cell spreading on HMEC-1 cells (Figure 3A and 3C). Fibronectin,³⁴ collagen,³⁵ and laminin³⁶ have prominent roles in regulating angiogenesis. To determine which ECM components are involved in endoglin-regulated VSMC adhesion and spreading, we assessed adhesion and spreading on the ECM components in both ECs and VSMCs. Loss of endoglin expression increased MEEC endothelial adhesion to all of these ECM components (Figure IIIA in the [online-only Data Supplement](#)). However, shRNA-directed knockdown of endoglin had modest effects on increasing PASMC adhesion to these ECM components (Figure IIIB in the [online-only Data Supplement](#)), whereas CRISPR-directed knockout endoglin had no effect (Figure IIIC in the [online-only Data Supplement](#)). This is consistent with Kyoto Encyclopedia of Genes and Genomes analysis of the RNA-SEQ data, in which cell adhesion pathways

were not significantly affected ($P=0.06$) by CRISPR-induced endoglin knockout (Table I and Figure IIID in the [online-only Data Supplement](#)). However, CRISPR-induced endoglin knockout (Figure 3D and 3E) or shRNA-mediated silencing of endoglin expression (Figure 3F) in PASMCs significantly decreased PASMC spreading on these 3 different ECM components, whereas MEEC^{-/-} endothelial spreading was modestly decreased on fibronectin, not affected on laminin and slightly increased on collagen (Figure IIIE and IIIF in the [online-only Data Supplement](#)). Furthermore, overexpression endoglin in *ENG*^{-/-} PASMC cell using adenovirus rescued spreading on the dishes coated with fibronectin and collagen (Figure IIIG in the [online-only Data Supplement](#)). These data suggest that endoglin in VSMCs promotes blood vessel maturation by promoting VSMC spreading on ECs via different ECM components.

Endoglin Promotes VSMC Recruitment and Spreading Via the Focal Adhesion Kinase Signaling

To further investigate the mechanism by which endoglin promotes VSMC recruitment and spreading, we analyzed the differentially expressed genes regulated by endoglin in VSMCs using the Kyoto Encyclopedia of Genes and Genomes database and DAVID software (<https://david.ncicrf.gov/>). Many of these endoglin-regulated genes belonged to the ECM-receptor interaction and focal adhesion categories (Figure 4A; Table I in the [online-only Data Supplement](#)), both of which have a central role in cell migration and spreading. Accordingly, we investigated whether endoglin regulates VSMC recruitment and spreading via the ECM-integrin-focal

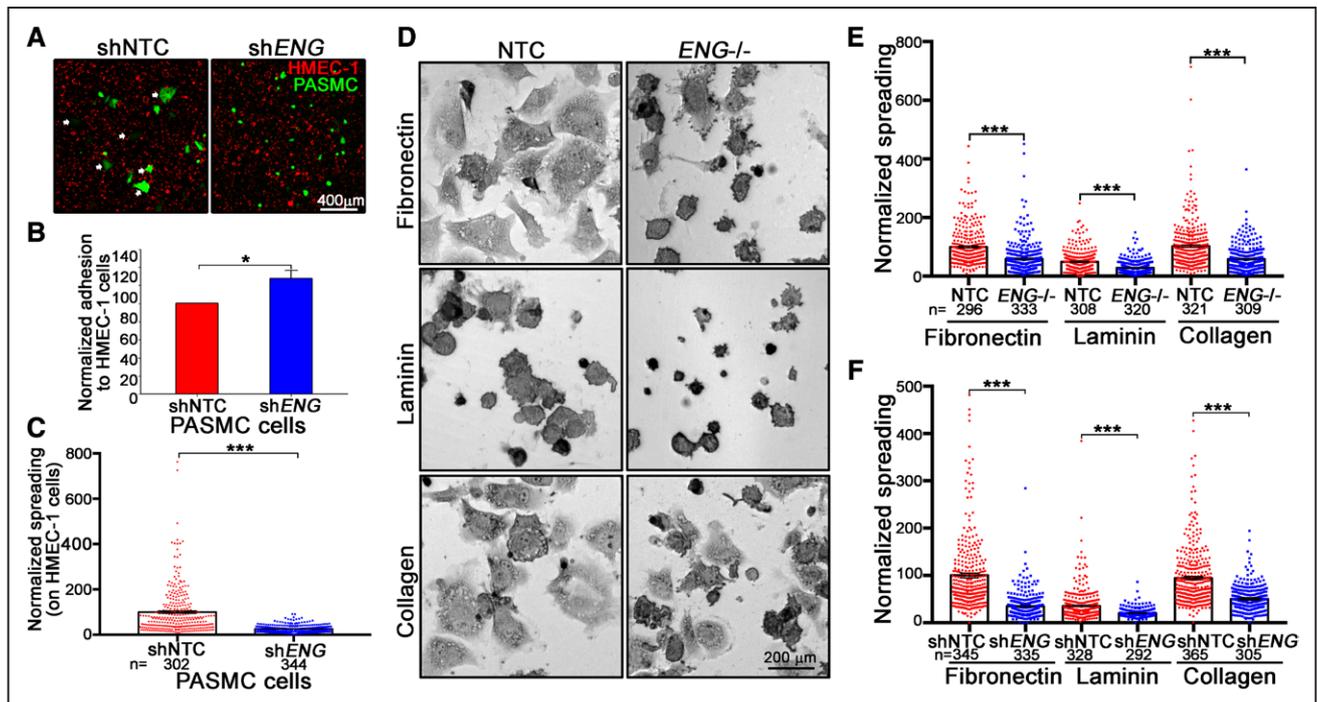


Figure 3. Endoglin promotes vascular smooth muscle cell (VSMC) spreading. **A–C**, sh-nontargeted control (NTC) or shENG pulmonary artery smooth muscle cell (PASMC) cells (green) were plated on confluent HMEC-1 cells (red) and cultured for 1 h. Cell adhesion (**B**) and spreading (**C**) were normalized and quantified from 3 independent experiments \pm SEM. **D–F**, NTC (or shNTC), *ENG*^{-/-} (**D** and **E**), or shENG (**F**) PASMC cells were plated on dishes coated with fibronectin, laminin, or collagen for 30 min, fixed, and stained with violet crystal. Cell spreading was normalized and quantified from 3 independent experiments \pm SEM.

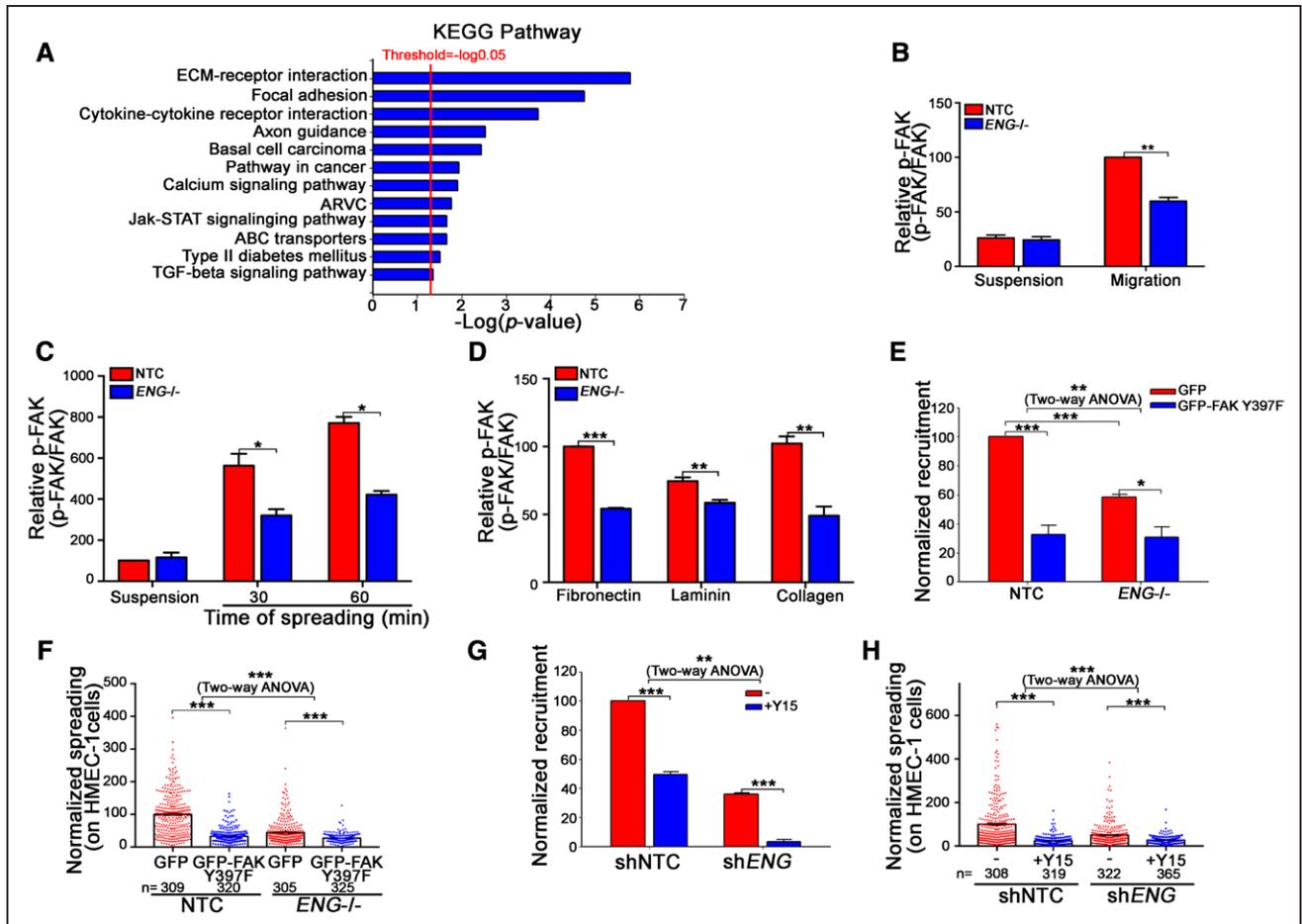


Figure 4. Endoglin promotes vascular smooth muscle cells (VSMC) recruitment and spreading via FAK signaling. **A**, The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways significantly affected by knockdown of endoglin, as analyzed using DAVID software. **B**, Nontargeted control (NTC) and *ENG*^{-/-} pulmonary artery smooth muscle cells (PASMC) were plated in transwells and allowed to migrate for 3 h, the resulting cell lysates were analyzed with the indicated antibodies. Quantitative data are representative of 3 independent experiments \pm SEM. *P* values were obtained by 2-tailed Student *t* test. **C**, NTC and *ENG*^{-/-} PASMC cells were plated in non-extracellular matrix-coated dishes and allowed to spread for the indicated times, the resulting cell lysates were analyzed with the indicated antibodies. Quantitative data are from 3 independent experiments \pm SEM. *P* values were obtained by 2-tailed Student *t* test. **D**, NTC and *ENG*^{-/-} PASMC cells were plated in fibronectin, laminin, or collagen-coated dishes for 1 h, and the cell lysates were analyzed with the indicated antibodies. Quantitative data are from 3 independent experiments \pm SEM. *P* values were obtained by 2-tailed Student *t* test. **E** and **F**, NTC and *ENG*^{-/-} PASMC cells were transfected with GFP (green fluorescent protein) or GFP-FAK Y397F mutant for 24 h. PASMC recruitment by **(E)** and spreading on **(F)** HMEC-1 endothelial cells was measured. Data were normalized and quantified from 3 independent experiments \pm SEM. *P* values were obtained by 2-tailed Student *t* test, and the statistical interaction between endoglin expression and GFP-FAK Y397F transfection was obtained by 2-way ANOVA analysis. **G** and **H**, shNTC and sh*ENG* PASMC cells were pretreated with 10 μ mol/L Y15 for 1 h. PASMC recruitment by **(G)** and spreading on **(H)** HMEC-1 endothelial cells were measured. Data were normalized and quantified from 3 independent experiments \pm SEM. *P* values were obtained by 2-tailed Student *t* test, and the statistical interaction between endoglin expression and Y15 treatment was obtained by 2-way ANOVA analysis.

adhesion kinase (FAK) pathway. FAK phosphorylation at Y397 was decreased in CRISPR-induced endoglin knockout PASMC cells during migration (Figure 4B; Figure IVA in the [online-only Data Supplement](#)) and spreading (Figure 4C; Figure IVB in the [online-only Data Supplement](#)). As loss of endoglin decreased PASMC cells spreading on different ECM components (Figure 3D through 3F), we investigated FAK signaling in these contexts. Consistently, loss of endoglin decreased FAK phosphorylation in the context of fibronectin, laminin, or collagen (Figure 4D; Figure IVC and IVD in the [online-only Data Supplement](#)). These effects were specifically because of loss of endoglin, as restoring endoglin expression rescued FAK phosphorylation in CRISPR-induced endoglin knockout PASMC cells (Figure IVE in the

[online-only Data Supplement](#)). As extracellular signal-regulated kinase (ERK)³⁷ and p38³⁸ are downstream of FAK, we investigated the effect of loss of endoglin on the activation of ERK and p38. ERK and p38 phosphorylation were also decreased in the PASMC cells with CRISPR-induced endoglin knockout (Figure IVF through IVH in the [online-only Data Supplement](#)) or shRNA-mediated silencing of endoglin expression (Figure IVI through IVK in the [online-only Data Supplement](#)). These data indicate that endoglin promotes FAK pathway signaling in VSMCs.

We then investigated whether regulation of the FAK pathway is the mechanism by which endoglin regulates VSMCs recruitment and spreading. Dominant negative FAK (Y397F; Figure IVL in the [online-only Data Supplement](#)) dramatically

decreased control PASC recruitment by (Figure 4E) and spreading on (Figure 4F) HMEC-1 ECs. However, in the PASCs with loss of endoglin that already exhibited decreased FAK activation (Figure 4B through 4D; Figure IVA through IVD in the [online-only Data Supplement](#)), dominant negative FAK had attenuated effects on VSMCs recruitment and spreading (Figure 4E and 4F). Furthermore, 10 $\mu\text{mol/L}$ of the FAK Y397 phosphorylation inhibitor, Y15, partially inhibited FAK phosphorylation at the Y397 site in sh-nontargeted control PASC cells to the basal level observed in sh*ENG* PASCs (Figure IVM in the [online-only Data Supplement](#)) without causing dramatic cell apoptosis.³⁹ Consistently, Y15 decreased recruitment (Figure 4G) and spreading (Figure 4H) in the sh-nontargeted control PASC cells to the basal level of sh*ENG* PASCs, while further inhibiting sh*ENG* PASCs recruitment (Figure 4G) and spreading (Figure 4H). The more dramatic effects of Y15 on recruitment in sh*ENG* PASC cells may reflect the total FAK inhibition here (Figure IVM in the [online-only Data Supplement](#)) and the longer time scale of these studies (hours) relative to the spreading studies (minutes). In addition, consistent with a role for FAK and downstream ERK and p38 mediating the effects of loss of endoglin expression, the ERK inhibitor, U0126, and p38 inhibitor, SB203580, also inhibited PASC migration (Figure IVN in the [online-only Data Supplement](#)). These data suggest that endoglin promotes VSMC recruitment and spreading at least partially via the FAK pathway.

Endoglin Promotes Different Integrin Subunits Expression

As the kinase activity of FAK is regulated through Y397 autophosphorylation on integrin α and β subunits clustering and further activation of β integrins,⁴⁰ we examined the effect of endoglin expression on integrin subunit expression. The mRNA of most integrin subunits were decreased in PASC cells on loss of endoglin expression (Figure VA in the [online-only Data Supplement](#)). Consistently, the protein levels of some integrin subunits whose mRNA levels were downregulated were also decreased in PASC cells on loss of endoglin expression, including $\alpha 5$, αv , $\beta 1$, and $\beta 3$ (Figure 5A; Figure VB in the [online-only Data Supplement](#)). Furthermore, the cell surface integrin $\beta 1$ and $\alpha 5$ subunits were also decreased in *ENG*^{-/-} cells (Figure VC and VD in the [online-only Data Supplement](#)). However, for integrin $\alpha 6$ and $\alpha 11$, whose mRNA were increased in PASCs lacking endoglin, the protein level of $\alpha 11$ was slightly increased (Figure VE in the [online-only Data Supplement](#)), whereas $\alpha 6$ was decreased (Figure VF in the [online-only Data Supplement](#)), suggesting other post-transcriptional regulation.

Our data have demonstrated that loss of endoglin decreased FAK signaling and VSMC spreading in the context of different ECM components. As the integrin $\beta 1$ subunit forms complexes with different integrin α subunits to serve as receptors for different ECM components, we investigated whether endoglin's function is through integrins, especially through the integrin $\beta 1$ subunit. shRNA-directed knockdown of integrin $\beta 1$ (Figure 5B) decreased FAK phosphorylation (Figure 5C and 5D) and recruitment (Figure 5E) in control PASC cells. However, in

ENG^{-/-} PASC cells, which have lower $\beta 1$ integrin levels, loss of $\beta 1$ integrin had attenuated effects (Figure 5C through 5E). In a reciprocal manner, overexpressing integrin $\beta 1$ increased phosphorylation of FAK (Figure 5F) and recruitment (Figure 5G) in control PASCs. However, overexpression of integrin $\beta 1$ only slightly rescued FAK signaling and recruitment in *ENG*^{-/-} PASC cells (Figure 5F and 5G), suggesting that besides $\beta 1$ integrin, other integrin subunits regulated by endoglin are involved in endoglin regulated VSMC recruitment. In addition, endoglin interacted with endogenous $\beta 1$ integrin in *ENG*^{-/-} PASC cells overexpressing hemagglutinin-endoglin (Figure 5H). This interaction was not dependent on the RGD (arginine-glycine-aspartic acid) domain on human endoglin, as mutation of RGD to TAD (threonine-alanine-aspartic acid) had no effect on the endoglin and $\beta 1$ integrin interaction (Figure 5H), consistent with the lack of conservation of the RGD domain across species (Figure VG in the [online-only Data Supplement](#)). Furthermore, endogenous endoglin interacted with endogenous $\beta 1$ integrin as determined using proximity ligation assays (Figure 5I and 5J). Loss of endoglin also decreased the interaction between endogenous $\beta 1$ integrin and FAK (Figure 5K). Collectively, these data support that endoglin promotes VSMC recruitment and spreading at least in part through regulation of the integrin-FAK pathway.

Endoglin Promotes VSMC Recruitment and Blood Vessels Maturation In Vivo

To investigate whether endoglin promotes VSMC recruitment to and spreading on ECs in vivo, we injected sh-nontargeted control or sh*ENG* PASCs intravenously into day 10 chicken embryos and observed their recruitment to chorioallantoic membrane arterioles within 24 hours. Compared with the sh-nontargeted control PASC, shRNA-mediated silencing of endoglin expression dramatically decreased PASC recruitment to the blood vessels (Figure 6A and 6B). Furthermore, although the recruited control PASC cells spread and covered the blood vessels, shRNA-mediated silencing of endoglin expression decreased PASC spreading on the blood vessels (Figure 6A and 6C, red arrows). These data indicate that endoglin in VSMCs promotes VSMC recruitment and blood vessel maturation in vivo.

Discussion

Collectively, we determined here that endoglin is robustly expressed in VSMCs, which promotes VSMC recruitment and spreading on endothelial tubules during angiogenesis (Figure 6D). As we have determined previously that in ECs, endoglin cross talks with fibronectin/integrin $\alpha 5\beta 1$ to regulate endothelial migration and stabilize the newly formed tubules before VSMC and other pericytes recruitment,¹⁴ these studies provide strong support for endoglin's roles in the maturation of blood vessels.

Although a few prior studies identified endoglin's roles in VSMCs,^{27,32} including a role in VSMC adhesion to ECs,³² and VSMC recruitment,²⁷ the functions and mechanism of action of endoglin in VSMCs during angiogenesis are still largely unknown. To obtain unbiased insight into how endoglin regulates VSMCs function, we utilized an unbiased bioinformatics

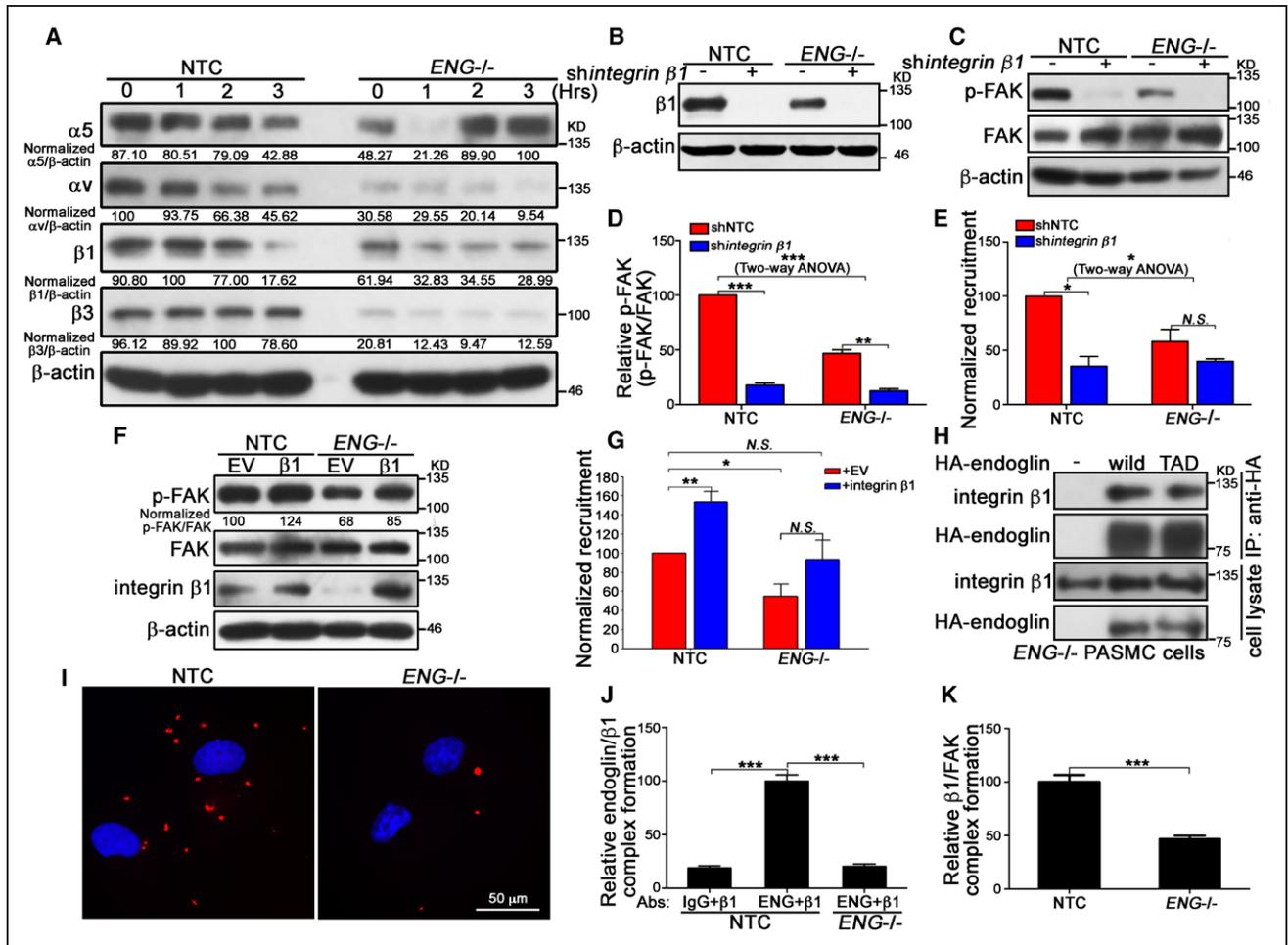


Figure 5. Endoglin promotes integrin expression. **A**, Nontargeted control (NTC) and *ENG*^{-/-} pulmonary artery smooth muscle cell (PASC) were plated in dishes and cultured for the indicated times, and the resulting cell lysates analyzed with the indicated antibodies. **B**, Stable knockdown of integrin β 1 using lentivirus in NTC and *ENG*^{-/-} PASC cells, knockdown efficiency determined using Western blot. The results are representative of 3 dependent experiments. **C** and **D**, shNTC and *shintegrin* β 1 PASC cells were plated on dishes and cultured for 1 h, and the cell lysates were analyzed with the indicated antibodies. Quantitative data are from 3 independent experiments \pm SEM (**D**). *P* values were obtained by 2-tailed Student *t* test, and the statistical interaction between endoglin and integrin β 1 expression was obtained by 2-way ANOVA analysis. **E**, shNTC and *shintegrin* β 1 PASC cells recruitment by HMEC-1 endothelial cells was normalized and quantified from 3 independent experiments \pm SEM. The statistical interaction between endoglin and integrin β 1 expression was obtained by 2-way ANOVA analysis. **F**, NTC and *ENG*^{-/-} PASC cells transfected with integrin β 1 for 24 h were plated on dishes and cultured for 1 h, and the cell lysates were analyzed with the indicated antibodies. The results are representative of 3 independent experiments. **G**, NTC and *ENG*^{-/-} PASC cells were transfected with integrin β 1, and PASC recruitment by HMEC-1 endothelial cells was normalized and quantified from 3 independent experiments \pm SEM. **H**, Immunoprecipitates were prepared from *ENG*^{-/-} cells overexpressing hemagglutinin (HA)-tagged wild-type endoglin or endoglin RGD (arginine-glycine-aspartic acid) to TAD (threonine-alanine-aspartic acid) mutant with anti-HA antibody, HA-endoglin, and β 1 integrin were detected in immunoprecipitates (IP) and cell lysates by Western blot analysis. **I** and **J**, Interaction between endogenous endoglin and β 1 integrin was assessed in NTC and *ENG*^{-/-} PASC cells by Duolink proximity ligation assay. Nuclei were stained using DAPI (4',6-diamidino-2-phenylindole). *P* values were obtained by 2-tailed Student *t* test from 3 independent experiments. **K**, Interaction between endogenous FAK and β 1 integrin was assessed in NTC and *ENG*^{-/-} PASC cells by Duolink proximity ligation assay. *P* values were obtained by 2-tailed Student *t* test from 3 independent experiments.

analysis of RNA-SEQ data comparing wild-type and endoglin loss of function VSMCs. In this analysis, cell movement was the function most affected by loss of endoglin expression, suggesting a role in inhibiting migration. This is consistent with the key role of cell migration plays in VSMC recruitment during angiogenesis. In the Kyoto Encyclopedia of Genes and Genomes pathway analysis, there were 12 pathways significantly affected by loss of endoglin (Figure 4A). Some of these pathways have been reported previously to be regulated by endoglin. For example, regulation of the TGF- β signaling was expected as endoglin is a TGF- β superfamily coreceptor, mediating TGF- β 1 and BMP-9/10 signaling.^{7,8} In addition,

cytokine signaling was supported by the prior observation that endoglin is involved in BMP9 regulated SDF1/CXCR4 chemokine signaling,⁴¹ promoting CXCL12-mediated leukocyte adhesion and transmigration through ECs.⁴² Furthermore, regulation of cancer pathways is supported by endoglin's involvement in cancer progression.^{43,44} Interestingly, ECM-receptor interaction and focal adhesion pathways were the top 2 pathways affected by loss of endoglin. These 2 pathways play a central role in cell migration,⁴⁵⁻⁴⁷ adhesion,^{48,49} and spreading,⁵⁰ 3 key steps during VSMCs recruitment during angiogenesis that are all regulated by integrin-FAK, suggesting regulation of the integrin-FAK pathway as the mechanism by which

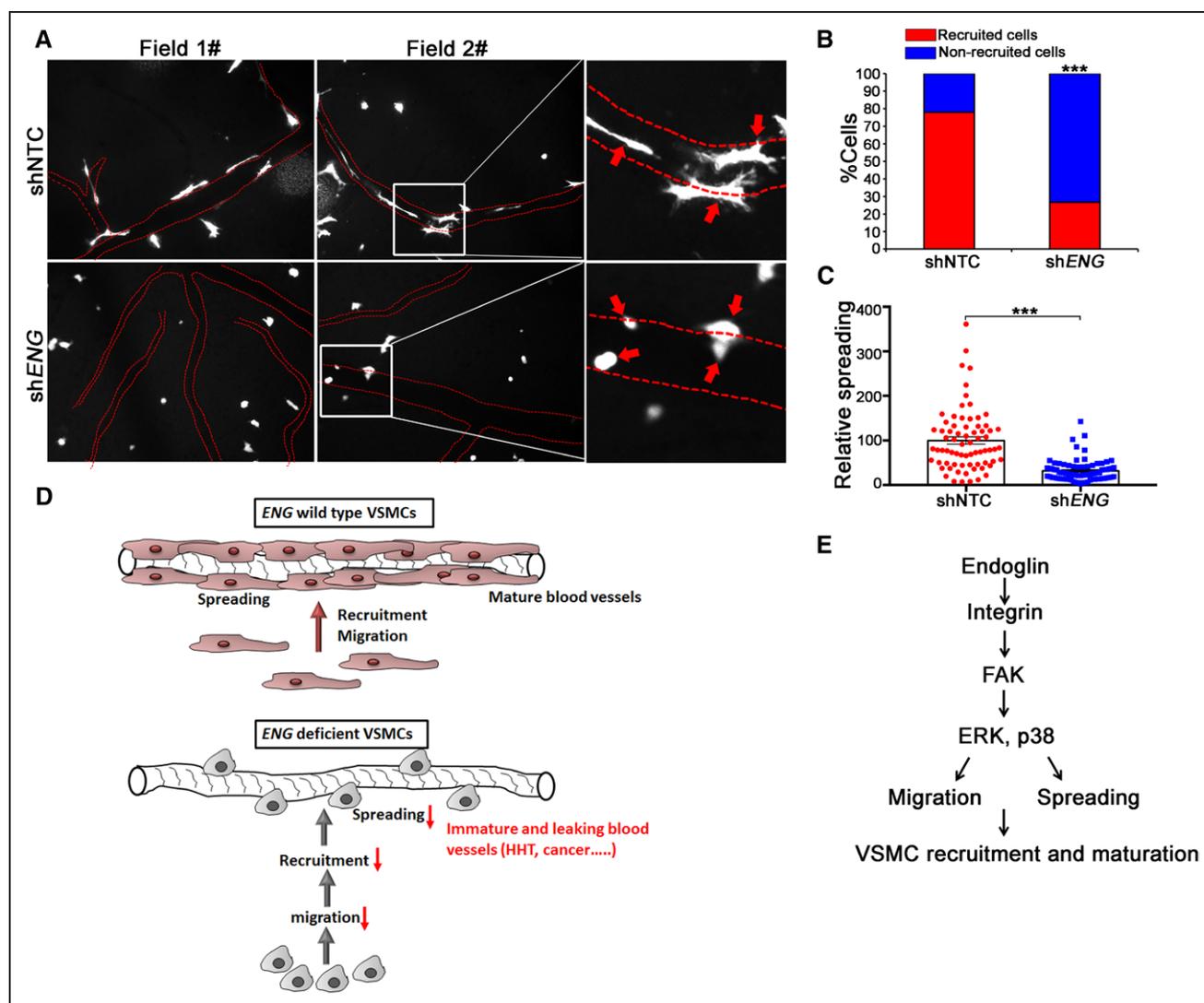


Figure 6. Endoglin promotes blood vessel maturation in vivo. **A–C**, sh-nontargeted control (NTC) and shENG pulmonary artery smooth muscle cells (PASMC) were injected intravenously into the allantoic vein of day 10 chicken embryos and their recruitment to the arterioles of the chorioallantoic membrane was monitored 24 h later. Percentage of PASMC recruitment (**A** and **B**) and spreading (**A** and **C**) were quantified from 3 independent experiments \pm SEM. **D**, Schematic of endoglin-regulated vascular smooth muscle cells (VSMC) biology during angiogenesis. **E**, Schematic of the proposed model by which endoglin regulates VSMC function during angiogenesis.

endoglin regulates VSMCs recruitment. Indeed, we determined that loss of endoglin inhibits VSMCs migration and spreading. However, loss of endoglin only slightly increased VSMCs adhesion to ECs (Figure 3A and 3B). Consistent with this observation, our unbiased bioinformatics analysis demonstrated that CRISPR-induced endoglin knockout did not significantly ($P>0.05$) affect cell adhesion molecule pathways (Table I and Figure IIID in the [online-only Data Supplement](#)), with the expression of some adhesion molecules mediating adhesion to ECs actually increasing, including ICAM1 (intercellular adhesion molecule 1), LICAM (L1 cell adhesion molecule), and PECAM1 (platelet and endothelial cell adhesion molecule 1; Figure IIID in the [online-only Data Supplement](#)).

In terms of the molecular mechanism by which endoglin regulates VSMCs recruitment, based on our RNA-SEQ data, we determined that the integrin–FAK pathway plays a central role in endoglin-regulated VSMC recruitment, migration, and spreading (Figure 6E). Endoglin has been reported

to regulate the FAK pathway by different mechanisms in different cell contexts. In ECs, endoglin inhibits cell migration via regulating the composition of focal adhesions.¹² We have demonstrated previously that TGF- β promotes internalization of endoglin/integrin $\alpha 5\beta 1$ complex to enhance FAK signaling.¹⁴ In Ewing sarcoma and melanoma, endoglin enhances $\beta 3$ integrin expression and promotes FAK pathway to maintain tumor cell plasticity and promotes tumor invasion.⁵¹ In the context of VSMCs, we have observed here that multiple integrin subunits were transcriptionally silenced by loss of endoglin expression in VSMCs. Among them, $\beta 1$ integrin, which can form complexes with multiple α subunits to serve as receptors for several ECM components, was dramatically decreased. This may explain why endoglin can regulate FAK signaling (Figure 4D; Figure IVC and IVD in the [online-only Data Supplement](#)) and VSMC biology (Figure 3D through 3F) in the context of different ECM components. In line with the effects on FAK signaling, signaling to ERK and p38,^{37,38,52} 2

direct downstream targets of FAK involved in cell migration and spreading, was decreased in endoglin-silenced VSMCs, with inhibition of ERK and p38 decreasing VSMC migration. Although transcriptional regulation of integrin subunits may be the primary mechanism by which endoglin regulates FAK signaling in VSMCs, endoglin may also regulate FAK signaling through other mechanisms. We have established here that endoglin interacts β 1 integrin in VSMCs (Figure 5H through 5J). Furthermore, loss of endoglin inhibits integrin β 1/FAK complex formation (Figure 5K). In addition, the mRNA levels of α 6 and α 11 integrin were increased in VSMCs lack of endoglin, whereas their protein levels were only decreased or slightly increased, suggesting other post-transcriptional regulation, such as micro-RNA. Collectively, these data indicate that endoglin promotes FAK signaling via regulation of integrin subunits to promote VSMCs recruitment and spreading during angiogenesis.

The cell context-dependent functions of endoglin in VSMCs relative to ECs is of significant interest. For example, although endoglin promotes migration, spreading, and has minimal effect on adhesion in VSMC cells, endoglin inhibits migration and adhesion while having minimal effect on spreading in ECs. These differences are likely to be because of context-dependent mechanisms by which endoglin regulates biology. For example, endoglin inhibits EC migration via interaction with zyxin to alter the composition of focal adhesions.¹² However, we determined here that in VSMCs, endoglin promotes VSMC migration and spreading via promoting integrin expression and focal adhesion pathway signaling. In addition, we have determined previously that endoglin antagonized ERK signaling to inhibit endothelial migration via β -arrestin-2 and TGF- β pathways,¹¹ whereas here we determine that endoglin promotes ERK signaling potentially downstream of integrin/FAK in VSMCs. One of the contextual differences could be the presence of different interacting receptors in VSMCs and ECs. For example, in ECs, endoglin is a coreceptor for ALK1 and many functions of endoglin in ECs are mediated through ALK1, including effects on migration and proliferation.^{7,8,14} Endoglin and ALK1 both bind the TGF- β superfamily ligand, BMP-9, a quiescent signaling factor that inhibits cell migration in ECs.^{3,53} However, in VSMCs, BMP-9 (Figure 2H) or an ALK1 inhibitor (Figure 2I) had no significant effects on migration, suggesting that VSMCs do not express or express very low levels of ALK1. The cell context-dependent functions of endoglin in VSMCs relative to ECs is important for blood vessels maturation during angiogenesis. In the later stages of angiogenesis (resolution phase), ECs decrease migration and proliferation to finish tubulogenesis, whereas VSMCs increase migration and spreading to facilitate recruitment by immature blood vessels. These distinct cellular behaviors, especially cell movement, between ECs and VSMCs cooperate to mature the newly formed blood vessels. The distinct roles of endoglin in endothelial and VSMC biology defined here may provide a novel mechanism for coordinating these functions.

Mutations in endoglin and ALK1 result in hereditary hemorrhagic telangiectasia (HHT).^{54–57} The important role of endoglin in VSMC recruitment and spreading described here may explain why blood vessels in HHT patients are fragile and how

recurrent bleeding occurs (Figure 6D). However, endoglin regulates VSMC recruitment in a TGF- β and ALK1-independent manner (Figure 2H and 2I). Furthermore, endoglin and ALK1 heterozygous mice have a distinct pulmonary and hepatic angiogenic profiles and response to anti-VEGF treatment.⁵⁸ Thus, besides functioning as a coreceptor for ALK1, endoglin may regulate angiogenesis and HHT at least partially in an ALK1-independent manner. Indeed, ALK1 is also involved in VSMC recruitment, as the ALK1 inhibitor, ALK-FC, increases vessel pericyte coverage,⁵⁹ suggesting that ALK1 inhibits VSMC recruitment. The distinct roles and mechanism of endoglin and ALK1 in HHT could be used as the basis for developing therapies for different molecular causes of HHT. For example, the current studies suggest that targeting FAK may be a rationale approach for type I HHT patients, who have endoglin mutations. The roles and mechanism by which ALK1 regulates VSMC recruitment remain to be explored.

Collectively, these studies provide novel insights into how endoglin regulates angiogenesis in the context of VSMCs, and how its disruption contributes to the pathophysiology of endoglin associated vascular diseases, including hereditary hemorrhagic telangiectasia and cancer-associated angiogenesis.

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Disclosures

None.

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Highlights

- Endoglin is not expressed only in endothelial cells, but also in VSMCs.
- Endoglin promotes VSMCs recruitment during angiogenesis in a cell autonomous manner.
- Endoglin promotes VSMCs recruitment via promoting VSMCs migration and spreading.
- Endoglin promotes integrin/FAK signaling.